Springer Protocols

Antibody Engineering

Volume 1

Edited by
Roland Kontermann
and Stefan Dübel

Second Edition

Springer

Antibody Engineering

Springer Protocols

Roland Kontermann • Stefan Dübel
Editors

Antibody Engineering Volume 1

Second Edition

Springer

Editors
Prof. Dr. Roland Kontermann
(Biomedical Engineering)
Institut für Zellbiologie und Immunologie
Universität Stuttgart
Allmandring 31
70569 Stuttgart
Germany
roland.kontermann@izi.uni-stuttgart.de

Professor Dr. Stefan Dübel
Technische Universität Braunschweig
Institut für Biochemie und Biotechnologie
Spielmannstraße 7
38106 Braunschweig
Germany
s.duebel@tu-bs.de

ISBN 978-3-642-01143-6 e-ISBN 978-3-642-01144-3
DOI 10.1007/978-3-642-01144-3
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2010922366

Originally published in one volume within the series Springer Lab Manuals

Cover design: deblik Berlin, Germany

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Foreword

Antibodies, naturally produced for protection by a variety of organisms, are also extremely powerful tools for research, diagnosis, and therapy. Since publication of the first edition of *Antibody Engineering* in 2001, the field of antibody research and development (R&D) has continued to grow at a remarkable pace. The research arena has seen advances in understanding structure-function relationships, antibody engineering techniques, and production of various antibody fragments. Clinical development has expanded, with novel monoclonal antibodies directed toward an array of targets entering the study at a rapid pace and the study of more than 200 monoclonal antibodies as treatments for a wide variety of diseases on-going.

A key feature of the global surge in antibody R&D activity is the need for updated information by both novice and experienced researchers. The publication of this second edition of *Antibody Engineering* is thus timely. In this manual, Roland Kontermann and Stefan Dübel provide comprehensive coverage of both new and well-established techniques. Volume 1 reviews techniques that serve as the foundation for antibody research (e.g. humanization, antibody production in eukaryotic expression systems), key information on the measurement of antibody structure and function, and current thinking on preclinical development practices. Volume 2 focuses on antibody fragment or derivative research. This area has greatly increased in importance, as the limitations of full-size antibodies have become more apparent. Up-to-date information on techniques to generate single-chain variable fragments, bispecific antibodies, and single domain antibodies are included.

The manual provides topic overviews that place information in context, and materials and methods that are described in clear, concise language. Newcomers to the field will benefit from the practical advice included, and experts will appreciate both the wealth of information collected and the extensive reference lists provided for each section. *Antibody Engineering* 2nd edition will, thus, be an invaluable resource to anyone engaged in antibody R&D.

Janice M. Reichert, Ph.D.
Editor-in-Chief, mAbs
Senior Research Fellow
Tufts Center for the Study of Drug Development

Preface

More than a century after the first Nobel Prize was awarded for an antibody-based therapy, these molecules continue to fascinate researchers and inspire novel therapeutic approaches. More than ever, antibodies are used for a very broad and still steadily expanding spectrum of applications – from proteomics to cancer therapy, from microarrays to in vivo diagnostics. Responsible for the renaissance of this class of molecules are recombinant approaches that allow the modification and improvement of almost all properties. Today, affinity, valency, specificity, stability, serum half-life, effector functions, and even the species origin and thus the immunogenicity, just to name a few aspects, can be engineered at will. More than 20 antibodies are approved for clinical use, and almost all are genetically engineered, recombinant molecules. The next generations of these antibodies are already in the pipeline, and a plethora of alternative antibody formats are under development for various applications.

We look back on exciting 25 years of development from humble beginnings in the early 1980s, when the mere production of an antibody chain in *Escherichia coli* was a goal hard to achieve, to today's impressive list of protein engineering tools. Among them, in particular, the methods that allow us to make human antibodies outside the human body, such as transgenic human Ig mice and phage display, have shaped and driven the developments during the past decade.

Ten years ago, in the preface of the first edition of *Antibody Engineering* – which was comprehensive at its time with less than half of the pages – we predicted that "...it can be expected that recombinant antibody based therapies will be a widespread and acknowledged tool in the hands of the physicians of the year 2010." This vision has become true within the past decade, and even was exceeded, since we also see that these technologies have broadly entered basic research, allowing us to bring to reality the vision of generating sets of antibodies to entire proteomes – in high throughput robots without a single animal involved.

Antibody Engineering aims to provide the toolbox for many exciting developments, and it will help the reader to stay up-to-date with the newest developments in this still fast moving field. It is designed to lead the beginners in this technology in their first steps by supplying the most detailed and proven protocols, and also by supplying professional antibody engineers with new ideas and approaches.

Stuttgart and Braunschweig — Roland Kontermann and Stefan Dübel

Contents

Part IV Engineering and Production of Immunoglobulins

Part V Antibody Characterization

Part I
Cloning of the Antigen-binding Site from Hybridoma

Chapter 1
Cloning of Variable Domains from Mouse Hybridoma by PCR

Nina Strebe, Frank Breitling, Dieter Moosmayer, Bodo Brocks, and Stefan Dübel

1.1 Introduction

Despite a growing number of recombinant antibodies being isolated from phage display libraries, most known antibody specificities are available from hybridoma cell lines. Here, a method is presented to obtain the genetic information for the antigen-binding part of the antibody from hybridoma cells, and to assemble it into a functional bacterially produced fusion protein (scFv fragment). To achieve this, vectors have been constructed, which combine the two variable regions (Vh and Vl) with a peptide linker. The genetic information for Vh and Vl is amplified from hybridoma cells using the polymerase chain reaction (PCR) with antibody-specific primers.

What are the reasons for cloning an scFv from a hybridoma? First, some hybridoma cell lines are very low producers, or antibody production is lost upon prolonged culture. In this case, a recombinant "hybridoma immortalization" can rescue a valuable antibody specificity. Second, the recombinant format can be

N. Strebe
Institut für Biochemie und Biotechnologie, Technische Universität Braunschweig, Spielmannstr. 7, Braunschweig 38106, Germany
Sanofi-Aventis Deutschland GmbH, Industriepark Hoechst, Bldg. H790, Frankfurt am Main 65926, Germany
F. Breitling
Karlsruhe Institute of Technology, Helmholtzplatz 1, 76344 Eggenstein-Leopoldshafen, Germany
D. Moosmayer
Bayer Schering Pharma AG, Müllerstr. 178, Berlin 13342, Germany
B. Brocks
Bodo Brocks, MorphoSys AG, Lena-Christ-Str. 48, Martinsried 82152, Germany
S. Dübel (✉)
Institut für Biochemie und Biotechnologie, Technische Universität Braunschweig, Spielmannstr. 7, Braunschweig 38106, Germany
e-mail: s.duebel@tu-bs.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_1,

required for the desired application. Examples are complex fusion proteins such as immunotoxins and antibody-enzyme fusions, or bispecific antibodies, which cannot be prepared with defined stoichiometry and coupling points by conventional chemical modification. Another example is the application as an intrabody (Strebe et al. 2009; Breitling and Dübel 1999). It has also been shown that the scFv format itself can be beneficial for the desired function. In one example, a monoclonal antibody against TNFα-Receptor (TNFR1), with limited TNF-agonistic activity was converted into a potent TNFα antagonist by producing it as a monomeric scFv fragment, thus preventing ligand binding and receptor cross linking (Moosmayer et al. 1995). In another example, the scFv fragment, but not the original antibody, was able to act as an enzyme inhibitor, probably because of its smaller size, avoiding sterical hindrance (Liu et al. 1999).

A problem frequently obstructing the functional cloning of V region DNA from hybridoma cell lines is their sequence heterogenicity. Point mutations and insertions as well as entirely different V regions may be found in the PCR products. Even if an antibody sequence has already been determined from the hybridoma, e.g., by PCR sequencing, it is not necessarily the sequence coding for the functional V regions, as shown, e.g., for the Myc1-9E10 (anti c-myc) hybridoma cell line (Fuchs et al. 1997; Schiweck et al. 1997). Various explanations for this sequence heterogenicity can be considered. Mutations can accumulate in the hybridoma cell population upon prolonged culture, which are not evident from functional analysis of the supernatant as long as a sufficient fraction of the cells still produce the correct antibody chains. These types of mutations can be minimized by preparing the cDNA from a freshly produced hybridoma subclone. Entirely different V sequences might derive from traces of expressed mRNA from the myeloma fusion partner, or even from the second allele of the B cell partner, since hybridoma cells are not controlled anymore by the rigid regulation mechanisms of the immune system. Even antibody pseudogene transcripts with stop codons inside the V region have been found in PCR products. Further, point mutations at both ends of the sequence can result from base pair mismatches during PCR priming.

In conclusion, an assay for antigen-binding function should be done as early as possible in the process of cloning. Creating a small phage display library from the PCR products, and screening for function is recommended where possible, e.g., when sufficient amounts of soluble antigen are available to perform a panning (see "troubleshooting"). This process is described in detail in Sect. 1.1.3. In this chapter, we present a direct cloning approach for hybridoma antibodies, recognizing antigens that are not available in significant amounts, such as cell surface antigens.

1.2 Outline

The method comprises hybridoma subcloning, RNA isolation, cDNA synthesis, PCR, stepwise cloning into a bacterial expression vector, and initial characterization steps for structure, production, and function of the antibody. The key to the successful

cloning is the PCR primer set. Two different choices for PCR primers are given. The first is a minimal set, which was empirically tested and evolved over more than 7 years (Dübel et al. 1994). To date, it has allowed successful amplification of V region DNA from over 40 hybridoma lines, including several rat hybridomas, and we have not observed a case so far where no PCR amplification is possible. However, this primer design strategy resulted in quite long oligonucleotides, thus introducing primer mutations at mismatch nucleotides, which may interfere with antigen binding of folding. The second primer set has been designed based on more recent and extended knowledge of antibody sequences and also includes IgM and lambda primers. It has not been tested to a similar extent, but proved to amplify V region DNA from several hybridoma antibodies, and it also has been successfully applied for cloning highly diverse repertoires from immunized mice (Brocks et al. 2001). In general, primers designed for the generation of murine V region libraries (see Sect. 1.1.2) might be used as well for cloning of V regions from hybridoma.

The entire procedure is outlined in Fig. 1.1. Bacterial culture, DNA manipulations, transformation, and gel electrophoresis methods are performed according to standard protocols (Sambrook et al. 1989).

1.3 Materials

1.3.1 Equipment

- ELISA reader
- PCR thermocycler.

1.3.2 Reagents

- RNA extraction Kit (RNeasy mini kit, Qiagen)
- Reverse transcriptase (SuperScript II, Invitrogen)
- Oligonucleotide primers, e.g., as described in Tab.1
- DNA polymerase (CombiZyme polymerase, Invitek)
- PCR-reaction buffer (supplied with the enzyme)
- Nucleotide stock solution containing 10 mM of each dNTP
- Bacterial culture, gel electrophoresis and agarose gel extraction equipment
- *Escherichia coli* K12, JM109, TG1 or XL1-blue competent cells
- Bacterial growth medium (LB) agar plates containing 100 μg/mL glucose and 100 μg/mL ampicillin
- Bacterial growth medium (LB) agar plates containing 50 μM isopropyl β-D-1-thiogalactopyranoside (IPTG)
- Media and agar plates are prepared according to standard protocols as previously described (Sambrook et al. 1989)

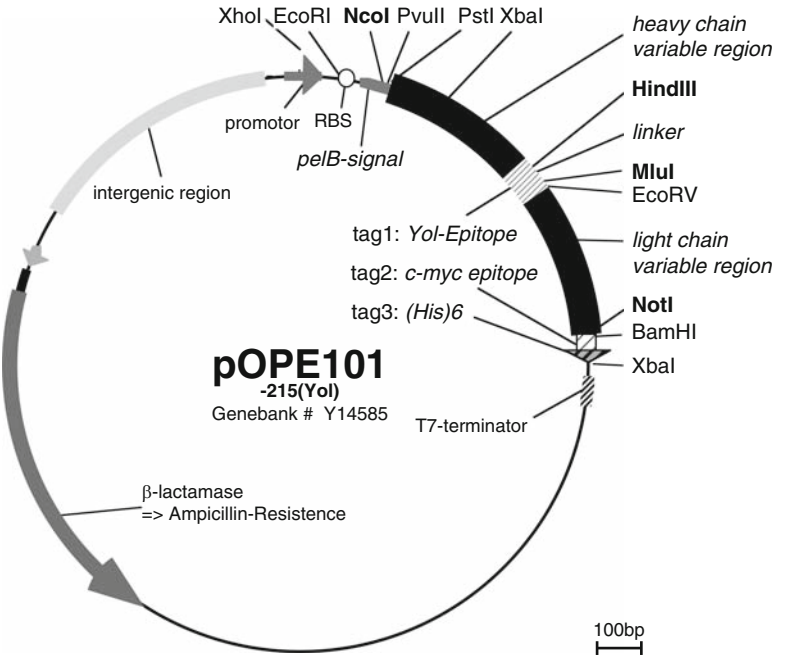


Fig. 1.1 The *E. coli* scFv expression vector pOPE101

- Tris–NaCl–Tween (TNT): 20 mM Tris–HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.4
- Tris-buffered-saline (TBS): 20 mM Tris–HCl, 0.15 M NaCl, pH 7.4
- Nitrocellulose membrane filters fitting the petri dish used for plating of the transformed bacteria, e.g., BA85 0.45 μM Ø82 mm, Schleicher & Schuell
- Indian Ink (Pelikan, Braunschweig)
- Chloroform
- Sodium azide powder
- 1% skimmed milk powder in TNT
- Antibody recognizing a tag peptide coded by the expected fusion protein
- Enzyme-labeled antiserum recognizing the first antibody
- Precipitating substrate system for the enzyme labeled antiserum
- Autoclaved glycerol.

1.4 Procedure

1.4.1 Isolation of Antibody DNA

1. Prepare a freshly subcloned culture of the hybridoma cells and check the supernatant for antigen binding. Collect at least 10^6 hybridoma cells by centrifugation.

2. Isolate the total RNA using a kit.
3. 9 µL total RNA, 2.5 µL oligo(dT)12–18 primer (10 µM), and 5 µL dNTPs were mixed. They were incubated for 5 min at 70°C and for 5 min on ice.
4. Afterward, 5 µL RT buffer (5×), 2.5 µL DTT (0.1 M) and 1 µL reverse transcriptase were added and incubated for 60 min at 42°C.
5. The reaction was stopped by incubation for 15 min at 70°C. The generated cDNA was stored at −20°C.
6. Mix the first strand cDNA with the PCR constituents on ice. Each 50 µL reaction contains 25 pmol of each primer, polymerase buffer as described by the supplier and 250 µM dNTPs. Use 1 µL of cDNA for each 50 µL PCR reaction.
7. Preheat the thermocycler to 95°. Add 0.5 U per 50 µL reaction volume of DNA polymerase on ice and mix. Avoid warming to room temperature, put the tubes quickly into the preheated thermocycler. Denature for max. 60 s. **Note**: *Taq*-DNA polymerase may be used, but there is a higher risk of introducing mutations during amplification. Some enzyme products consist of a mixture of proofreading/nonproofreading enzymes; they may be used as well. Longer denaturation than 60 s at the start of the program is not necessary, and may even result in loss of yield.
8. Perform 25–30 cycles of 30 s denaturation at 95°C, 1 min hybridization at the appropriate primer hybridization temperature, and 1 min polymerization at 72°C. Use the primers described in Tables 1.1 and 1.2 in individual reactions for each appropriate primer pair. After the end of the cycles, immediately cool down to 4°C. **Note:** A hybridization temperature of 55°C should be tried initially. If no products are amplified, perform a set of 4 PCRs, differing only by their hybridization temperatures of 42, 45, 48, and 51°C. Buffer, nucleotides, and primers may be stored as a premix at −20°C. Overcycling with *vent*-DNA polymerase may lead to product degradation.
9. Purify the PCR products. In case you have used the primer set of Table 1.1, you can directly proceed to Step 10. In case you have used the primer set of Table 1.2, you have to perform a second PCR to introduce the restriction cited necessary for cloning. To do this, use 1 µL of the purified first PCR reaction as a template in a reaction similar to step 7 and 8, but do only nine cycles, employing a hybridization temperature of 57°C.
10. Collect 1/5 volume of the reaction for analysis on a 1.5% agarose gel, containing ethidium bromide. Phenol extract the PCR product or freeze the PCR tubes immediately, until you have time to extract it. **Note**: The remaining activity of DNA polymerase needs to be removed after completion of the PCR reaction by phenol extraction to prevent digestion of 3′ overhanging ends by the intrinsic 3′-5′-exonuclease activity of the Polymerases. This step is essential for efficient cloning after the subsequent restriction digest. Omission of this step may result in drastically reduced ligation efficiency. Be aware that gel purification or spin columns do not remove this polymerase activity completely.

Table 1.1 Minimal Oligonucleotide set for the amplification of mouse and rat immunoglobulin variable region DNA. According to Dübel et al. (1994)

κ chain FR1 region:	(EcoRV)
Bi6	5′-GGTGATATCGTGAT(A/G)AC(C/A)CA(G/A) GATGAACTCTC
Bi7	5′-GGTGATATC(A/T)TG(A/C)TGACCCAA(A/T) CTCCACTCTC
Bi8	5′-GGTGATATCGT(G/T)CTCAC(C/T)CA(A/G) TCTCCAGCAAT
κ chain constant domain:	(BamHI)
Bi5	5′-GGGAAGATGGATCCAGTTGGTGCAGCATCAGC
Heavy chain FR1 region: (PstI, PvuII)	
Bi3	5′-GAGGTGAAGCTGCAGGAGTCAGGACCTAGCCTGGTG
Bi3b	5′-AGGT(C/G)(A/C)AACTGCAG(C/G)AGTC(A/T)GG
Bi3c	5′-AGGT(C/G)(A/C)AGCTGCAG(C/G)AGTC(A/T)GG
Bi3d	5′-AGGT(C/G)CAGCTGCAG(C/G)AGTC(A/T)GG
γ chain CH1 domain: (HindIII)	
Bi4	5′-CCAGGGGCCAGTGGATAGACAAGCTTGGGTGTCGTTTT
Reamplification primers for the introduction of other restriction sites	
Heavy chain FR1 region: Bi3f	5′- CAGCCGGCCATGGCGCAGGT(C/G)CAGCTGCAG(C/G) AG NcoI PvuII,PstI
κ chain constant domain: Bi5c	5′- GAAGATGGATCCAGCGGCCGCAGCATCAGC BamHI NotI
κ chain FR1 region: Bi8b	5′- AATTTTCAGAAGCACGCGTAGATATC(G/T)TG(A/C)T(G/C)ACCCAA(T/A)CTCCA MluI EcoRV

As a standard set, the combination Bi3f + Bi4 should be used for Vh and Bi8b + Bi5c for Vl. If no product is found in this first approach, other combinations can be tried. Preferentially, Bi7 should be tried instead of Bi8b, and Bi3b/3c instead of 3f. In addition, in case that restriction sites required for cloning are present internally in the amplification products, they should be reamplified (not more than 5–8 PCR cycles) to introduce alternative cloning sites. In case internal restriction sites of enzymes essential for cloning are present in the amplification products, they can be reamplified (not more than 5–8 PCR cycles) with the primers containing alternative sites. Please note that overcycling with *vent*-DNA polymerase may lead to a degradation of the correct product

11. Double digest the purified PCR product with the appropriate restriction endonucleases. **Note:** Calculate the amount of required enzyme carefully. Over digestion may reduce the ligation efficiency .
12. Purify the digested PCR fragment. We recommend spin column kit systems.

1.4.2 Cloning and Colony Screening

1. Ligate the appropriate dephosphorylated vector fragment (see Fig. 1.2) with the digested PCR product.

Table 1.2 Extended Oligonucleotide set for the two step amplification of mouse and rat immunoglobulin variable region DNA. According to Brocks et al. (2001)

First PCR	
Heavy chain	
Gamma chain CH1 domain:(IgG)	
Bi4	CCA GGG GCC AGT GGA TAG ACA AGC TTG GGT GTC GTT TT
Mu chain CH1 domain:(IgM)	
Bi4m	GGA GAC GAG GGG GAA AAG CTT TGG GAA GGA CTG ACT CTC
Heavy chain FR1:	
MHV.B1	GAT GTG AAG CTT CAG GAG TC
MHV.B2	CAG GTG CAG CTG AAG GAG TC
MHV.B3	CAG GTG CAG CTG AAG CAG TC
MHV.B4	CAG GTT ACT CTG AAA GAG TC
MHV.B5	GAG GTC CAG CTG CAA CAA TCT
MHV.B6	GAG GTC CAG CTG CAG CAG C
MHV.B7	CAG GTC CAA CTG CAG CAG CCT
MHV.B8	GAG GTG AAG CTG GTG GAG TC
MHV.B9	GAG GTG AAG CTG GTG GAA TC
MHV.B10	GAT GTG AAC TTG GAA GTG TC
MHV.B11	GAG GTC CAG CTG CAA CAG TC
MHV.B12	GAG GTG CAG CTG GAG GAG TC
Light chain	
Kappa chains	
kappa chain constant domain	
MKC. F	GGA TAC AGT TGG TGC AGC ATC
Kappa chain FR1	
MKV.B1	GAT GTT TTG ATG ACC CAA ACT
MKV.B1	GAT GTT TTG ATG ACC CAA ACT
MKV.B2	GAT ATT GTG ATG ACG CAG GCT
MKV.B3	GAT ATT GTG ATA ACC CAG
MKV.B4	GAC ATT GTG CTG ACC CAA TCT
MKV.B5	GAC ATT GTG ATG ACC CAG TCT
MKV.B6	GAT ATT GTG CTA ACT CAG TCT
MKV.B7	GAT ATC CAG ATG ACA CAG ACT
MKV.B8	GAC ATC CAG CTG ACT CAG TCT
MKV.B9	CAA ATT GTT CTC ACC CAG TCT
MKV.B10	GAC ATT CTG ATG ACC CAG TCT
Lambda chains	
Lambda chain constant domain	
MLC.F	GGT GAG TGT GGG AGT GGA CTT GGG CTG
Lambda chain FR1 region	
MLV.B	CAG GCT GTT GTG ACT CAG GAA
Second PCR	
Heavy chain	
Gamma chain CH1 domain:(IgG), Hind III site	
Bi4	(identical to 1st PCR)
Mu chain CH1 domain:(IgM), Hind III site	
Bi4m	(identical to 1st PCR)

(*continued*)

Table 1.2 (continued)

Heavy chain FR1 with NcoI site:	
MHV.B1.Nco	GAA TAG GCC ATG GCG GAT GTG AAG CTG CAG GAG TC
MHV.B2.Nco	GAA TAG GCC ATG GCG CAG GTG CAG CTG AAG GAG TC
MHV.B3.Nco	GAA TAG GCC ATG GCG CAG GTG CAG CTG AAG CAG TC
MHV.B4.Nco	GAA TAG GCC ATG GCG CAG GTT ACT CTG AAA GAG TC
MHV.B5.Nco	GAA TAG GCC ATG GCG GAG GTC CAG CTG CAA CAA TCT
MHV.B6.Nco	GAA TAG GCC ATG GCG GAG GTC CAG CTG CAG CAG TC
MHV.B7.Nco	GAA TAG GCC ATG GCG CAG GTC CAA CTG CAG CAG CCT
MHV.B8.Nco	GAA TAG GCC ATG GCG GAG GTG AAG CTG GTG GAG TC
MHV.B9.Nco	GAA TAG GCC ATG GCG GAG GTG AAG CTG GTG GAA TC
MHV.B10.Nco	GAA TAG GCC ATG GCG GAT GTG AAC TTG GAA GTG TC
MHV.B11.Nco	GAA TAG GCC ATG GCG GAG GTC CAG CTG CAA CAG TC
MHV.B12.Nco	GAA TAG GCC ATG GCG GAG GTG CAG CTG GAG GAG TC
Light chain	
Kappa chains	
Kappa chain constant domain with NotI site	
MKC. F.Not	TGA CAA GCT TGC GGC CGC GGA TAC AGT TGG TGC AGC ATC
Kappa chain FR1 with MluI site	
MKV.B1.Mlu	TA CAG GAT CCA CGC GTA GAT GTT TTG ATG ACC CAA ACT
MKV.B2.Mlu	TA CAG GAT CCA CGC GTA GATATT GTG ATG ACG CAG GCT
MKV.B3.Mlu	TA CAG GAT CCA CGC GTA GAT ATT GTG ATA ACC CAG
MKV.B4.Mlu	TA CAG GAT CCA CGC GTA GAC ATT GTG CTG ACC CAA TCT
MKV.B5.Mlu	TA CAG GAT CCA CGC GTA GAC ATT GTG ATG ACC CAG TCT
MKV.B6.Mlu	TA CAG GAT CCA CGC GTA GAT ATT GTG CTA ACT CAG TCT
MKV.B7.Mlu	TA CAG GAT CCA CGC GTA GAT ATC CAG ATG ACA CAG ACT
MKV.B8.Mlu	TA CAG GAT CCA CGC GTA GAC ATC CAG CTG ACT CAG TCT
MKV.B9.Mlu	TA CAG GAT CCA CGC GTA CAA ATT GTT CTC ACC CAG TCT
MKV.B10.Mlu	TA CAG GAT CCA CGC GTA GAC ATT CTG ATG ACC CAG TCT

(*continued*)

Table 1.2 (continued)

Lambda chains	
lambda chain constant domain with NotI site	
MLC.F.Not	GA CAA GCT TGC GGC CGC GGT GAG TGT GGG AGT GGA CTT GGG CTG
lambda chain FR1 region with MluI site	
MLV.B.Mlu	TA CAG GAT CCA CGC GTA CAG GCT GTT GTG ACT CAG GAA

2. Transform *E.coli* cells and plate on LB agar plates containing 100 μg/mL glucose and 100 μg/mL ampicillin. Incubate overnight at 28–32°C to obtain small colonies. **Note**: The glucose should not be omitted since it is necessary for the tight suppression of the synthetic promoter of pOPE vectors, and thus, for maintaining the stability of the insert.
3. When colonies with a diameter of about 0.5 mm have formed, put a nitrocellulose filter on the plate, wait a few seconds until it is entirely moistured.
4. Label the orientation of the filter on the agar plate by piercing a syringe needle dipped into Indian ink through the filter into the agar.
5. Use a scalpel or razor blade to cut out a section of about one fifth of the filter for the negative control.
6. Carefully remove both pieces of the filter with forceps, put them on new plates with the attached bacteria pointing upward. Put the negative control onto selection medium with glucose, the major section onto selection medium containing 100 μM IPTG. **Note**: With pOPE-vectors in *E.coli* JM109, we achieved optimal protein secretion with 20 μM IPTG at 25°C. This optimal IPTG concentration can vary between different Fv-sequences by a factor of about two. Higher IPTG concentrations lead to higher amounts of total protein, but in this case, most of the scFv fragments still carry the bacterial leader sequence (Dübel et al. 1992) and form aggregates. However, for the immunoblot analysis of total cellular SDS extracts, it is not necessary to discriminate between unprocessed and processed protein. Therefore, a higher IPTG concentration is used to increase the intensity of the protein band on the blot.
7. Incubate for 3 h at 37°C.
8. Expose the filters for 15 min to chloroform vapor (in a glass chamber containing an open chloroform vessel) **Note**: This step increases the staining intensity at the margin of a colony, thus improving the signal. It might be omitted in the case of strong reactions.
9. Wash the filters 2× in an excess of TNT (50 mL for a filter with a diameter of 10 cm) with the colonies pointing downwards. Remove the bacteria by gently rubbing the filters on the bottom of the washing vessel.
10. Wash the filters in TNT containing 0.01% NaN_3 (Caution: sodium azide is very toxic) with the colonies pointing downward, to kill residual bacteria. **Note**: NaN_3 should not be stored as a stock solution since it degrades rapidly in water.
11. Wash the filters 3× in an excess of TNT with the colonies pointing downwards.

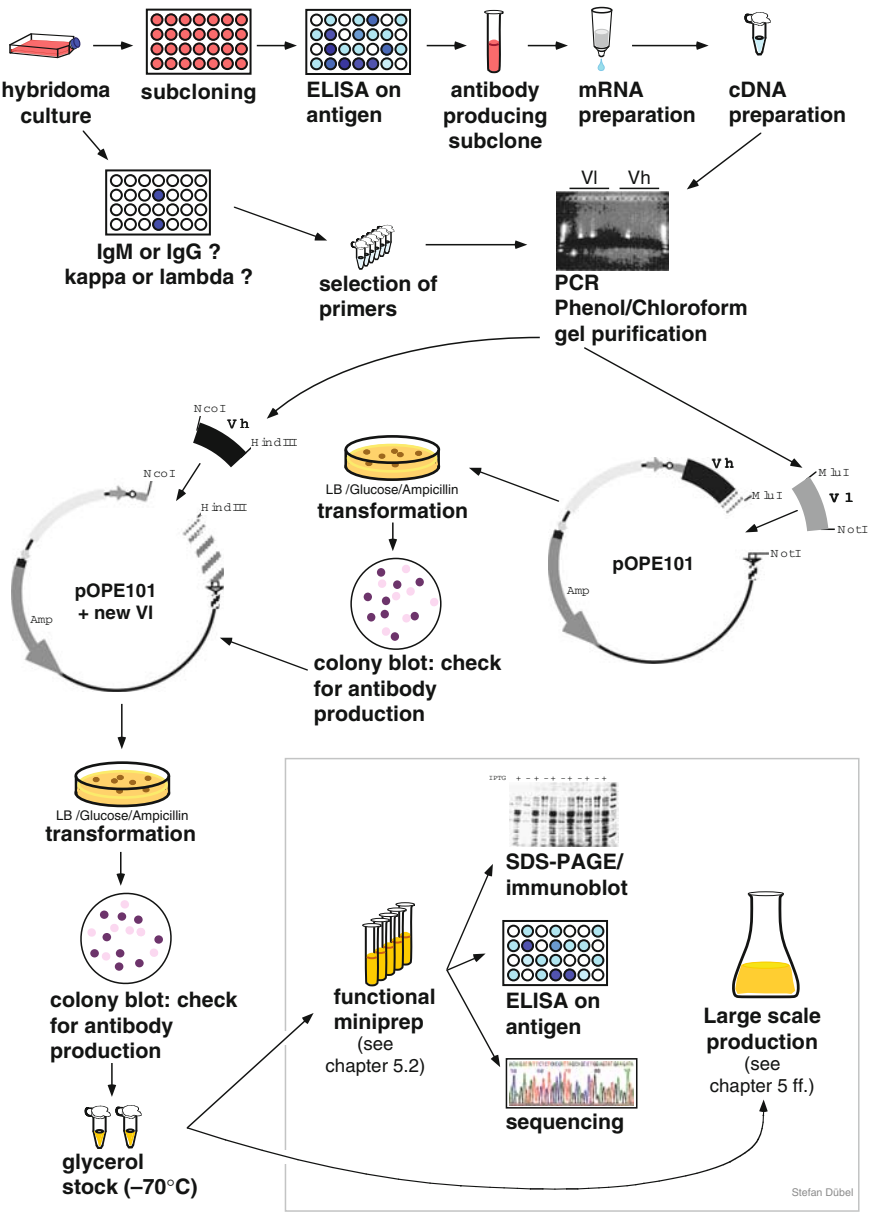


Fig. 1.2 Flow chart of scFv cloning from hybridoma cell lines ("hybridoma immortalization")

12. Block unspecific binding sites by shaking for 30 min in 1% milk powder in TNT. **Note**: Store 1% milk powder in TNT at 4°C for a maximum of 1 day; for longer storage, freeze aliquots. For a filter of 10 cm diameter, 4–5 mL of blocking or antibody solution are usually enough to obtain an even staining.

The best results are obtained with a platform shaker, which has a tilting motion in only one direction. The use of platform shakers with a tumbling movement requires larger incubation volumes to obtain an even staining. Only one filter should be used per incubation vessel.

13. Incubate in 1% milk powder/TNT containing the first antibody. **Note:** For scFv fragments cloned into pOPE51 or pOPE101, the monoclonal antibody Myc1-9E10 that binds to the internal epitope EQKLISEEDLN (Evan et al. 1985, commercially available from Cambridge Research Biochemicals) can be used. The recommended dilution is 1/10,000. The most specific result is obtained after incubation overnight at 4°C. For most applications, however, 1–2 h incubation at room temperature is sufficient. Alternatively, the His-tag can be utilized for detection with Ni-NTA HRP conjugate (Qiagen, Hilden, Germany). In this case, a second antibody is not necessary; proceed directly with Step 17 after incubation.
14. Wash 3× for 5 min in TNT.
15. Incubate in 1% milk powder/TNT containing the second antibody. **Note**: Commercially available antimouse IgG antisera, labeled with horseradish peroxidase (HRP), usually require a dilution of 1:1,000–1:5,000 in a 1–2 h incubation.
16. Wash 3× for 5 min in TNT, and briefly in TBS to remove detergent
17. Place the filters in substrate solution until the desired noise/signal ratio between the induced and the non-induced piece of the filter is reached. **Note**: We use cobalt enhanced diaminobenzidine/H_2O_2 – substrate system (Caution: diaminobenzidine is a cancerogene): Dilute 200 µL of diaminobenzidine stock solution (25 mg/mL in water, stock solution should be stored at −20°C in aliquots for max. 6 months) in 10 mL TBS, containing 0.02% (w/v) $CoCl_2$ and add 1 µL of 30% (v/v) H_2O_2, use immediately. The addition of $CoCl_2$ enhances sensitivity about 30 fold. Commercially available premixed TMB substrate solutions may be used instead.
18. After sufficient substrate reaction (usually not more than 10 min), wash 3× for 5 min in tap water. Air dry for documentation. **Note**: Scan or photograph within 1 day since bleaching may occur.
19. Pick a few positive colonies. Inoculate 1 mL of LB_{GA} (LB medium containing 100 mM glucose and 50 µg/mL ampicillin). Grow overnight at 37°C. Add 250 µL glycerol, mix and freeze at −30°. **Note**: The master plates should not be stored for longer than 1 day at 4°C. Cultures freshly inoculated directly from the frozen glycerol stock should be used for all subsequent experiments. Never thaw the glycerol stock; it is sufficient to scrape a bit of ice from the surface for inoculation.

1.4.3 Troubleshooting

Frequently, more than one primer pair amplifies a PCR product of the correct size. The reasons are discussed in the introduction. In this case, it is recommended to

pick, clone, and sequence 5–10 clones of each product. In case different sequences are found, all have to be tested for function. A set of clones has to be generated containing all possible combinations of Vh and Vl regions. Alternatively, a phage display screening (panning) can be employed after cloning the PCR products directly into a phagemid surface expression vector (e.g., pHAL, see chapter 5) and screened for functional antibody fragments. The latter procedure is always recommended if soluble and purified antigen is available in amounts above a few micrograms. Only if the antigen is not available, e.g., in case of cell surface antigens, the first approach has to be used.

References

Breitling F, Dübel S (1999) Recombinant antibodies. Wiley, New York, ISBN:0-471-17847-0

Brocks B, Garin-Chesa P, Behrle E, Park JE, Rettig WJ, Pfizenmaier K, Moosmayer D (2001) Species-crossreactive scFv against the tumor stroma marker "fibroblast activation protein" selected by phage display from an immunized FAP-/- knock-out mouse. Mol Med 7:461–469

Dübel S, Breitling F, Klewinghaus I, Little M (1992) Regulated secretion and purification of recombinant antibodies in *E.coli*. Cell Biophys 21:69–79

Dübel S, Breitling F, Fuchs P, Zewe M, Gotter S, Moldenhauer G, Little M (1994) Isolation of IgG antibody Fv-DNA from various mouse and rat hybridoma cell lines using the polymerase chain reaction with a simple set of primers. J Immunol Methods 175:89–95

Evan GI, Lewis GK, Ramsay G, Bishop M (1985) Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. Mol Cell Biol 5:3610–3616

Fuchs P, Breitling F, Little M, Dübel S (1997) Primary structure and functional scFv antibody expression of an antibody against the human protooncogen c-myc. Hybridoma 16:227–233

Liu Z, Song D, Kramer A, Martin AC, Dandekar T, Schneider-Mergener J, Bautz EK, Dübel S (1999) Fine mapping of the antigen-antibody interaction of scFv215, a recombinant antibody inhibiting RNA polymerase II from Drosophila melanogaster. J Mol Recognit 12:103–111

Moosmayer D, Dübel S, Brocke B, Watzka H, Hampp C, Scheurich P, Little M, Pfizenmaier K (1995) A single chain TNF receptor antagonist is an effective inhibitor of TNF mediated cytotoxicity. Ther Immunol 2:31–40

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schiweck W, Buxbaum B, Schatzlein C, Neiss HG, Skerra A (1997) Sequence analysis and bacterial production of the anti-c-myc antibody 9E10: the V(H) domain has an extended CDR-H3 and exhibits unusual solubility. FEBS Lett 414:33–38

Strebe N, Guse A, Schüngel M, Schirrmann T, Hafner M, Jostock T, Hust M, Müller W, Dübel S (2009) Functional knockdown of VCAM-1 at the posttranslational level with ER retained antibodies. J Immunol Methods 341:30–40

Chapter 2
Coning Hybridoma cDNA by RACE

Andrew Bradbury

2.1 Introduction

V region primer PCR is usually successful in the amplification of hybridoma V genes, especially if using diverse primer sets (Wang et al. 2000; Krebber et al. 1997; Coloma et al. 1991; Gavilondo-Cowley et al. 1990; Rohatgi et al. 2008). However, there are a number of potential pitfalls in using V region PCR. Mutations within the 5′ or 3′ ends of the V genes may inhibit primer annealing, and so, prevent amplification. In some cases, the use of universal V region primers can introduce mutations that can reduce stability, production yield, and antigen affinity (Honegger and Pluckthun 2001; Jung et al. 2001). Another problem is the presence of other V genes within the hybridoma that are preferentially amplified. These arise for two reasons. The first is nonproductive rearrangments, which, not being mutated, are very good PCR templates (Carroll et al. 1988; Storb et al. 1980), while the second is probably caused by the fusion of more than one spleen cell to the myeloma cell line, resulting in multiple functional (as well as nonfunctional) V genes (Zack et al. 1995). In this situation, an alternative to V gene PCR is to use either traditional cDNA cloning or rapid amplification of cDNA ends (RACE) (Frohman et al. 1988). This technique relies on knowledge of a small part of gene sequence to amplify from that gene sequence to either end of the cDNA. For both cases, an oligo-dT primer containing a specific tag is used to amplify the cDNA end. In the case of the 3′ end, the sequence to which it anneals is the naturally occurring poly-A tail, while in the case of the 5′ end (which is that used when RACE is used to clone hybridoma V genes), a poly-A tail is added using terminal transferase. PCR specificity can be

A. Bradbury
B division, MS-M888, Los Alamos National Laboratory, Los Alamos, NM 87545, USA
e-mail: amb@lanl.gov

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_2,

subsequently improved by using the specific tag primer and a nested sequence specific primer (Pescatori et al. 1995).

When applied to immunoglobulins (Ruberti et al. 1994; Doenecke et al. 1997), the isotype of the monoclonal to be cloned provides the sequence knowledge, which can be used for the internal primer. This is used to create the cDNA. A poly-A tail is added to the 5′ end of the cDNA using terminal transferase, and the complete V gene, including 5′ untranslated region, leader sequence, and a small part of the constant region, can then be amplified. Once amplified, the V genes can then be cloned into standard cloning vectors such as pUC, from which they may be sequenced or reamplified using V region primers for cloning into specific phage display (McCafferty et al. 1990; Sblattero and Bradbury 2000) or eukaryotic antibody expression vectors (Persic et al. 1997a, b). However, the annealing temperature required may be as low as 37°C, if failure to amplify from total cDNA is due to mutations in the primer annealing sites (Ruberti et al. 1994; Ruberti et al. 1993). Mutations in the V genes can be avoided by using high amounts of cloned V gene (1 μg) and a DNA polymerase with proofreading activity (such as T. litoralis [Vent, New England Biolabs] or P. furiosus [Stratagene]) to reduce errors introduced by PCR.

Table 2.1 gives the sequences of hinge region primers of different heavy chain isotypes, which can be used for cDNA synthesis. Additional restriction sites should be added to the 5′ end with a nucleotide tail to allow efficient digestion. Hinge-specific primers are used, as these tend to be the most isotype-specific part of the constant region genes. Nested primers that can be used are located at the 5′ end of CH1 and these are also shown in Table 2.1. There is no equivalent to hinge region primers for the light chain. As a result, irrelevant light chains will also be amplified, although one can distinguish between mouse and rat light chains, and there are slight differences between the different isotypes. The light chain cDNA synthesis primer is found at the 3′ end of the constant region and the PCR primer overlaps it with a 6 bp extension to preserve specificity.

2.2 Procedure

1. Prepare cytoplasmic mRNA from 5×10^6 hybridoma cells.
2. Synthesize cDNA[a] with the following protocol: denature 1 μg of poly (A) mRNA at 65°C for 5 min in DEPC treated water, put on ice, and then add to a mixture containing 5 μl 5×RT buffer, 10 μl RNasin (Promega), 10 pmol cDNA synthesis-specific primer (see Table 2.1), 250 μM of each of the four deoxynucleotide triphosphates (dNTPs), and 10U of Moloney murine leukemia virus reverse transcriptase in a total volume of 25 μl. The reaction mixture is incubated at 42°C for 60 min and then at 52°C for 30 min. After inactivation at 95°C for 5′ the reverse transcription mixture is diluted with 2 ml of 0.1 TE (1 mM Tris pH7.0, 0.1 mM EDTA).

Table 2.1 Primers for cDNA synthesis of mouse or rat immunoglobulin genes

cDNA primers specific for heavy chain isotypes (all priming in hinge)

RACEMOG1 (mouse IgG1)	TAT GCA AGG CTT ACA ACC ACA
RACEMOG2a (mouse IgG2a)	AGG ACA GGG CTT GAT TGT GGG
RACEMOG2b (mouse IgG2b)	AGG ACA GGG GTT GAT TGT TGA
RACEMOG3 (mouse IgG3)	GGG GGT ACT GGG CTT GGG TAT
RACERAG1 (rat IgG1)	AGG CTT GCA ATC ACC TCC ACA
RACERAG2a (rat IgG2a)	ACA AGG ATT GCA TTC CCT TGG
RACERAG2b (rat IgG2b)	GCA TTT GTG TCC AAT GCC GCC
RACERAG2c (rat IgG2c)	TCT GGG CTT GGG TCT TCT GGG

Light chain primers (all prime at 3′ end of CL)

CKFOR (mouse and rat K)	CTC ATT CCT GTT GAA GCT CTT GAC
MOCKFOR (mouse K)	CTC ATT CCT GTT GAA GCT CTT GAC AAT
RACKFOR (rat K)	CTC ATT CCT GTT GAA GCT CTT GAC GAC

MOCKFOR and RACKFOR are identical to CKFOR except for the last three bases. If a V region from a rat mouse hybrid is to be cloned, and the mouse myeloma partner expresses a light chain V region mRNA, it can be excluded by the use of RACKFOR

CL1FOR (mouse λ1 λ4; rat λ1)	ACA CTC AGC ACG GGA CAA ACT CTT CTC
CL2FOR (mouse λ2, λ3; rat λ2)	ACA CTC TGC AGG AGA CAG ACT CTT TTC

These may be used individually if the lambda isotype is known or as an equimolar mixture to prime all lambda chains.

RACE PCR primers

Heavy chain primers (all prime at 3′ end of CH1)

MOCG12FOR (mouse IgG1, IgG2a; rat IgG1, IgG2a, IgG2b)	CTC AAT TTT CTT GTC CAC CTT GGT GC
MOCG2bFOR (mouse IgG2b)	CTC AAG TTT TTT GTC CAC CGT GGT GC
RACG2cFOR (rat IgG2c)	CTC AAT TCT CTT GAT CAA GTT GCT TT
MOCG3FOR (mouse IgG3)	CTC GAT TCT CTT GAT CAA CTC AGT CT
MOCMFOR (mouse IgM)	TGG AAT GGG CAC ATG CAG ATC TCT

These may be used individually or as an equimolar mixture to prime all heavy chains.

Light chain primers (all prime at 3′ end of CL)

CKRAsp	CTC ATT CCT GTT GAA GCT CTT GAC GAC GGG

(*continued*)

Table 2.1 (continued)

(this is identical to CKFOR, except that at the 3′ end, it has 6 extra bases to increase its specificity for rat)	
CKMOsp	CTC ATT CCT GTT GAA GCT CTT GAC AAT GGG
(this is identical to CKFOR, except that at the 3′ end, it has 6 extra bases to increase its specificity for mouse)	
CL1FOR	ACA CTC AGC ACG GGA CAA ACT CTT CTC
(mouse λ1 λ4; rat λ1)	
CL2FOR	ACA CTC TGC AGG AGA CAG ACT CTT TTC
(mouse λ2, λ3; rat λ2)	
CL1FORsp	ACA CTC AGC ACG GGA CAA ACT CTT CTC CAC AGT
(mouse and rat λ1)	
CL2FORsp	ACA CTC TGC AGG AGA CAG ACT CTT TTC CAC AGT
(mouse λ2, λ3; rat λ2)	
CL4FORsp	ACA CTC AGC ACG GGA CAA ACT CTT CTC CAC ATG
(mouse λ4)	
These may be used individually or in a pooled equimolar mixture. They are identical to the corresponding CLFOR primers, except that at the 3′ end, there are 6 extra bases to increase the specificity for each λ.	
None of the constant region primers described above has restriction sites at the 5′ end included for cloning. This should be inserted according to the vector to be subsequently used.	
RACE PCR primer	
XSCTnTag	GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT
Anneals to the poly A tail are added by terminal transferase, and provide XhoI, SalI, ClaI sites at the 5′ end. This is the original primer described by Frohman et al. However, other restriction sites can also be used.	

3. Remove excess primer using a Centricon 100 spin filter (20 min at 1,000 g, twice). The first retained liquid is collected and diluted to 2 ml before repeating the Centricon concentration. The second is concentrated to 10 µl and used in the following steps.
4. Synthesize a polyA tail at the 5′ end of the cDNA by adding 4 µl 5× Tailing buffer (supplied by Promega with the enzyme), 4 µl dATP 1 mM and 10U of Terminal deoxynucleotidyl transferase (Promega). The mix is incubated for 5 min at 37°C and then 5 min at 65°C. The volume of the cDNA/tailing reaction is adjusted to 500 µl.
5. Amplify 10 µl of reaction with Vent polymerase as follows:
   1 precycle: 5 min 95°C, 5 min 60°C, 40 min 72°C; 40 cycles: 1 min 95°C, 1 min 60°C, 3 min 72°C.
   PCR is performed using the oligonucleotide XSCTnTag (Table 2.1), which hybridizes to the poly(A) tail added to the 5′ end of cDNA and one PCR primer, specific for the light or heavy chain (Table 2.1).
   [a]Although we use this protocol, which is based upon the original published method by Frohman et al. (1988), other protocols, including cDNA synthesis and RACE kits should also be effective.

2.3 Results

After following this procedure, a single band should be obtained. This can be excised and purified from the agarose gel, digested with the appropriate enzymes, and cloned into the vector of choice. Sometimes a smear may be obtained. This can be reduced by changing the PCR conditions, or can be ignored if not too strong.

2.4 Troubleshooting

We have found this method extremely successful in amplifying V regions, which cannot be amplified by V region primers. As in any PCR reaction, varying the annealing temperature, the Mg concentration or the polymerase may improve the quality of the product.

In all cases, the specificity of amplified V genes should be confirmed by functional analysis of expressed antibodies or antibody fragments after expression in either bacteria or mammalian cells. If more than one V gene is obtained, both should be tested, in combination with the other V genes, to determine the correct combination.

References

Carroll WL, Mendel E, Levy S (1988) Hybridoma fusion cell lines contain an aberrant kappa transcript. Mol Immunol 25:991–995

Coloma MJ, Larrick JW, Ayala M, Gavilondo-Cowley JV (1991) Primer design for the cloning of immunoglobulin heavy-chain leader-variable regions from mouse hybridoma cells using the PCR. Biotechniques 11(152–4):56

Doenecke A, Winnacker EL, Hallek M (1997) Rapid amplification of cDNA ends (RACE) improves the PCR-based isolation of immunoglobulin variable region genes from murine and human lymphoma cells and cell lines. Leukemia 11:1787–1792

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85:8998–9002

Gavilondo-Cowley JV, Coloma MJ, Vazquez J, Ayala M, Macias A, Fry KE, Larrick JW (1990) Specific amplification of rearranged immunoglobulin variable region genes from mouse hybridoma cells. Hybridoma 9:407–417

Honegger A, Pluckthun A (2001) The influence of the buried glutamine or glutamate residue in position 6 on the structure of immunoglobulin variable domains. J Mol Biol 309:687–699

Jung S, Spinelli S, Schimmele B, Honegger A, Pugliese L, Cambillau C, Pluckthun A (2001) The importance of framework residues H6, H7, and H10 in antibody heavy chains: experimental evidence for a new structural subclassification of antibody V(H) domains. J Mol Biol 309:701–716

Krebber A, Bornhauser S, Burmester J, Honegger A, Willuda J, Bosshard HR, Pluckthun A (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J Immunol Methods 201:35–55

McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348:552–554

Persic L, Roberts A, Wilton J, Cattaneo A, Bradbury A, Hoogenboom H (1997a) An integrated vector system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries. Gene 187:9–18

Persic L, Righi M, Roberts A, Hoogenboom HR, Cattaneo A, Bradbury A (1997b) Targeting vectors for intracellular immunisation. Gene 187:1–8

Pescatori M, Bradbury A, Bouet F, Gargano N, Mastrogiacomo A, Grasso A (1995) The cloning of a cDNA encoding a protein (latrodectin), which co-purifies with the alpha-latrotoxin from the black widow spider, Latrodectus tredecimguttatus (Theridiidae). Eur J Biochem 230:322–328

Rohatgi S, Ganju P, Sehgal D (2008) Systematic design and testing of nested (RT-)PCR primers for specific amplification of mouse rearranged/expressed immunoglobulin variable region genes from a small number of B cells. J Immunol Methods 339:205–219

Ruberti F, Bradbury A, Cattaneo A (1993) Cloning and expression of an anti-nerve growth factor (NGF) antibody for studies using the neuroantibody approach. Cell Mol Neurobiol 13:559–568

Ruberti F, Cattaneo A, Bradbury A (1994) The use of the RACE method to clone hybridoma cDNA when V region primers fail. J Immunol Methods 173:33–39

Sblattero D, Bradbury A (2000) Exploiting recombination in single bacteria to make large phage antibody libraries. Nat Biotechnol 18:75–80

Storb U, Arp B, Wilson R (1980) Myeloma with multiple rearranged immunoglobulin kappa genes: only one kappa gene codes for kappa chains. Nucleic Acids Res 8:4681–4687

Wang Z, Raifu M, Howard M, Smith L, Hansen D, Goldsby R, Ratner D (2000) Universal PCR amplification of mouse immunoglobulin gene variable regions: the design of degenerate primers and an assessment of the effect of DNA polymerase 3′ to 5′ exonuclease activity. J Immunol Methods 233:167–177

Zack DJ, Wong AL, Stempniak M, Weisbart RH (1995) Two kappa immunoglobulin light chains are secreted by an anti-DNA hybridoma: implications for isotypic exclusion. Mol Immunol 32:1345–1353

Chapter 3
Construction of scFv Fragments from Hybridoma or Spleen Cells by PCR Assembly

Jonas V. Schaefer, Annemarie Honegger, and Andreas Plückthun

Abbreviations

BSA	Bovine serum albumin
DMSO	Dimethylsulfoxide
HRP	Horse radish peroxidase
IPTG	Isopropylthiogalactoside
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
scFv	Single-chain Fv fragment
cfu	Colony forming units
tet	Tetracycline

3.1 Introduction

Today, antibodies can be obtained from naive repertoires (Winter et al. 1994; Vaughan et al. 1996) or libraries of fully synthetic genes (Knappik et al. 2000), and in the last decade, numerous libraries have been described (reviewed in Mondon et al. 2008). Nonetheless, hybridomas have remained the predominant source of antibodies, and a wealth of well characterized and even unique clones exist and are continuing to be generated. There is, thus, great interest in immortalizing these clones, in the extreme case, as a computer file of the sequences, as well as in accessing the antibody in a variety of new formats. To obtain enough material

J.V. Schaefer, A. Honegger, and A. Plückthun (✉)
Biochemisches Institut, Universität Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland
e-mail: plueckthun@bioc.uzh.ch

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_3,

for detailed biochemical and biophysical analyses of the deduced antibodies after immunization, their cloning into formats compatible with recombinant expression is beneficial, if not essential. For this purpose, the antibody genes must be cloned, and the binding properties of the recombinant protein have to be verified. In addition to existing hybridomas, the immune response of an animal upon exposure to various antigens may often be of particular scientific interest in itself and also lead to the discovery of new and potent binders. Therefore, there is merit in immortalizing the results from new immunizations as well. In this case, it is not necessary to take the detour of first making hybridomas, but instead, mRNA isolated from spleen can be directly used for the creation of an immune library, from which binders can be subsequently isolated by phage display and their sequences determined.

Once the antibody genes have been successfully cloned and after the presumed binding properties of the recombinant antibodies have been experimentally verified, their sequences can be used for modeling (www.bioc.uzh.ch/antibody/), and their structure subsequently be determined by crystallography (Honegger et al. 2005) or NMR (Freund et al. 1994; Tugarinov et al. 2000). The recombinant single-chain Fv format (Huston et al. 1988; Glockshuber et al. 1990) is an ideal starting point for all engineering efforts, from sensors (Backmann et al. 2005; Morfill et al. 2007) to therapeutic fusion proteins (Di Paolo et al. 2003), or imaging reagents (Adams et al. 1993) to multivalent and multispecific reagents (Plückthun and Pack 1997), just to name a few illustrative examples. Recombinant expression of these proteins also allows one to evolve the affinity further than the immune system normally does, e.g., to low picomolar K_D for scFv fragments (Zahnd et al. 2004; Luginbühl et al. 2006). Finally, some natural antibodies may not be of sufficient stability, which can also be corrected by engineering (Wörn and Plückthun 2001; Ewert et al. 2004). In addition, the murine antibody can be humanized for its use in therapy – a procedure rapidly achievable at the scFv stage.

The key prerequisite for the use of recombinant antibody technologies, starting from immune repertoires or defined hybridomas, is the reliable cloning of functional immunoglobulin genes. Even though hybridomas are considered to express "monoclonal" antibodies, hybridoma clones may encode more than one functional or even nonfunctional heavy or light chains (Kütemeier et al. 1992). As has been reported previously, several kappa chain-secreting hybridomas, possessing X63Ag8.653 myeloma cells as fusion partner, also occasionally transcribe a functional lambda chain, competing with the V_κ gene for in-frame scFv antibody assembly (Krebber et al. 1997). As these false or heterogeneous genes might also be amplified and subsequently assembled into the scFv fragments, it is highly recommended to include an enrichment procedure in the cloning protocol. This step can be circumvented and replaced by screening of clones at the scFv level, but the phage enrichment is generally much faster if incorrect sequences abound. Obviously, selection by phage display or by another selection technology such as ribosome display (Hanes and Plückthun 1997; Hanes et al. 1998; for detailed protocols see Schaffitzel et al. 2005; Amstutz et al. 2006; Zahnd et al. 2007) is mandatory when starting from spleens of immunized mice.

This chapter largely follows our earlier protocols (Plückthun et al. 1996; Krebber et al. 1997; Burmester and Plückthun 2001). A number of variable antibody domains of hybridomas were accessible with those procedures and reagents whose genes could not be cloned in other experimental setups. The present protocol is based on a standard phage display system, which was optimized for robustness, vector stability, and directional cloning using a single rare cutting restriction enzyme as well as tight control of the expression of the scFv-gene III fusion (Krebber et al. 1997). As the procedures for the construction of scFv fragment libraries from immunized mice and that of cloning one specific antibody from hybridomas are essentially the same, we combined them in just one protocol. However, there are slight differences in the initial preparation of the cells, and high ligation and transformation yields for library cloning are, of course, essential, as explained under "notes."

The current version of this protocol contains improvements in the methods but, most importantly, newly designed primer sequences for the amplification of V_H and V_L genes. They are based on our analysis of a reference set of murine germline sequences found in the most recent version of the IMGT database (http://imgt.cines.fr/textes/vquest/refseqh.html), which thus incorporates most of the knowledge of the mouse genome (for a description of the original database, see Lefranc and Lefranc 2001). Our key criterion was a faithful amplification of the variable region genes preserving as much sequence identity as possible, avoiding the generation of nonnatural residue combinations, which could result in sequences problematic for folding and stability (Honegger and Plückthun 2001; Jung et al. 2001). We also tried to ensure similar annealing temperatures with the different genes, as well as keeping the degeneracy on the DNA level as small as possible. Furthermore, we avoided pronounced secondary structures within the oligonucleotides such as hairpin loops or primer-dimers (which were checked against themselves using the appropriate analysis tools in the Vector NTI software (Invitrogen)). The primers shown below are the result of this iterative process and have also been tested with a slightly different overhang.

The cloning strategy outlined in this protocol (Fig. 3.1) allows the simple conversion of the expression format from the initial scFv fragments to other formats and fusion proteins. Insertion of the assembled scFv gene into the described standard vectors pAK100 and pJB12 leads to the expression of a scFv-gene III fusion applicable for phage display, due to read-through of the amber codons whenever expressed in strains with amber suppressor tRNA such as *Escherichia coli* XL1-Blue. In bacterial strains lacking such suppressor tRNA, the amber stop codons result in translation termination and production of unfused scFv fragments. For purposes of IMAC purification or whenever other fusions will be constructed, it is, however, advantageous to reclone the fragments directly into appropriate vectors (Figs. 3.4 and 3.5) (Plückthun et al. 1996), carrying stronger translation initiation sites. Conversely, it is not advantageous to make expression too strong for phage display, as discussed below. Although not explicitly mentioned, a very similar strategy of cloning (Fig. 3.1) only requiring altered reverse primers can be used for the design of Fab versions of the desired antibodies.

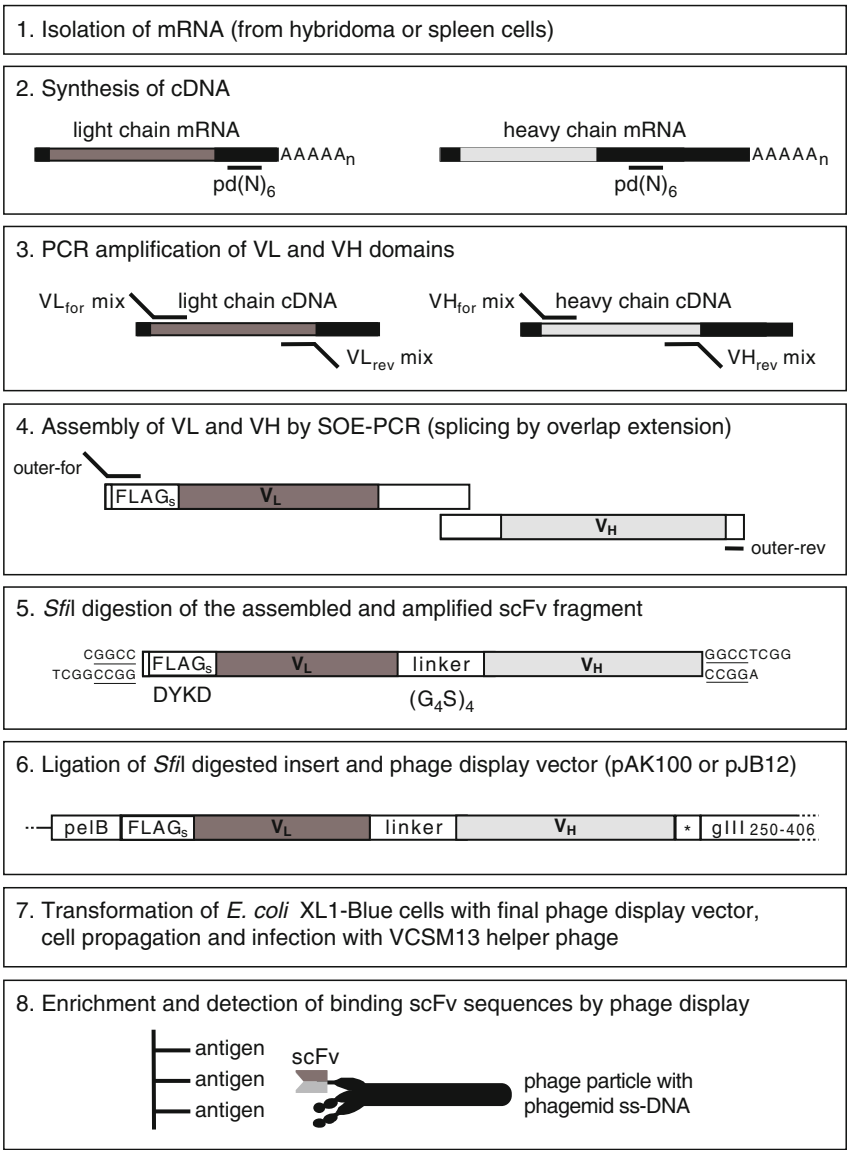


Fig. 3.1 *Schematic overview of the amplification and cloning procedure*. After its isolation from hybridoma or spleen cells, the mRNA provides the basis for cDNA synthesis, utilizing random hexamer primers. The cDNA is used afterward as template for PCR amplification of V_L and V_H domains (symbolized by the gray boxes, not drawn to scale), which are subsequently assembled by SOE-PCR into the scFv format by the outer primer pair outer-for and outer-rev. For antibody cloning into the phagemid, only the rare cutting enzyme *Sfi*I is used, guaranteeing directional cloning due to the resulting different overhangs at the cleavage site as indicated. In addition, self-ligation of insert or vector molecules is excluded by the asymmetry generated in the cut vector. $FLAG_s$ indicates the shortened *N*-terminal 4-amino acid FLAG tag (Knappik and Plückthun 1994)

3.2 Materials

- 5×10^6 cells from a growing or frozen hybridoma culture or spleen cells, respectively
- PCR primers (Figs. 3.2 and 3.3) and corresponding plasmids (Figs. 3.4 and 3.5)
- Helper phage (e.g., Stratagene VCSM13 # 200251)
- F^+, *sup*E, *rec*A strain (e.g., *E. coli* XL1-Blue) (available in electrocompetent/chemocompetent form from Stratagene)
- Anti-M13 antibody HRP-conjugate (GE Healthcare; # 27-9421-01)
- PEG 6000 (Fluka)
- Sterile, RNase-free equipment: pipet tips, tubes, RNase-free ultra high purity (UHP) water, baked nondisposable glassware, and sterile, disposable plasticware
- Standard molecular biology equipment and reagents for:
  - Determining the isotype of mAbs (Roche IsoStrip Mouse Monoclonal Antibody Isotyping Kit)
  - Purifying RNA (Invitrogen TRIzol reagent and Qiagen RNeasy Mini Kit)
  - Performing a cDNA synthesis reaction (Qiagen QuantiTect Reverse Transcription Kit)
  - Performing PCR reactions
  - Purifying PCR products (Macherey Nagel PCR clean-up Gel Extraction Kit)
  - Cutting and gel-purifying DNA (Sigma-Aldrich GenElute Gel Extraction Kit)
  - Concentrating DNA (Amicon Microcon 30 for volumes less than 500 μl)
  - Ligating and transforming DNA
  - Growing bacteria and phages
  - Conducting an Enzyme Linked Immunosorbent Assay (ELISA)
  - Performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting.

Fig. 3.1 (continued) and the asterisk symbolizes either the myc tag or the trypsin cleavage site, present in pAK100 and pJB12, respectively. After infection with VCSM13 helper phages, the transformed XL1-Blue cells produce phages, displaying the scFv antibody on their surface. The subsequent enrichment of these phages by panning against the antigen allows the selection of functional antibody sequences from the library generated from the spleen cells. In addition, this approach also supports the isolation of specific scFv fragments if the hybridoma cell line initially contained only a small fraction of mRNA coding for this particular antibody. Phage ELISA then identifies the antigen-binding clones. Subsequently, the binding properties of the unfused scFv in the absence of phage need to be verified after recloning into a more powerful expression vector (Figs. 3.4 and 3.5) and purification from *E. coli* (not shown in the diagram)

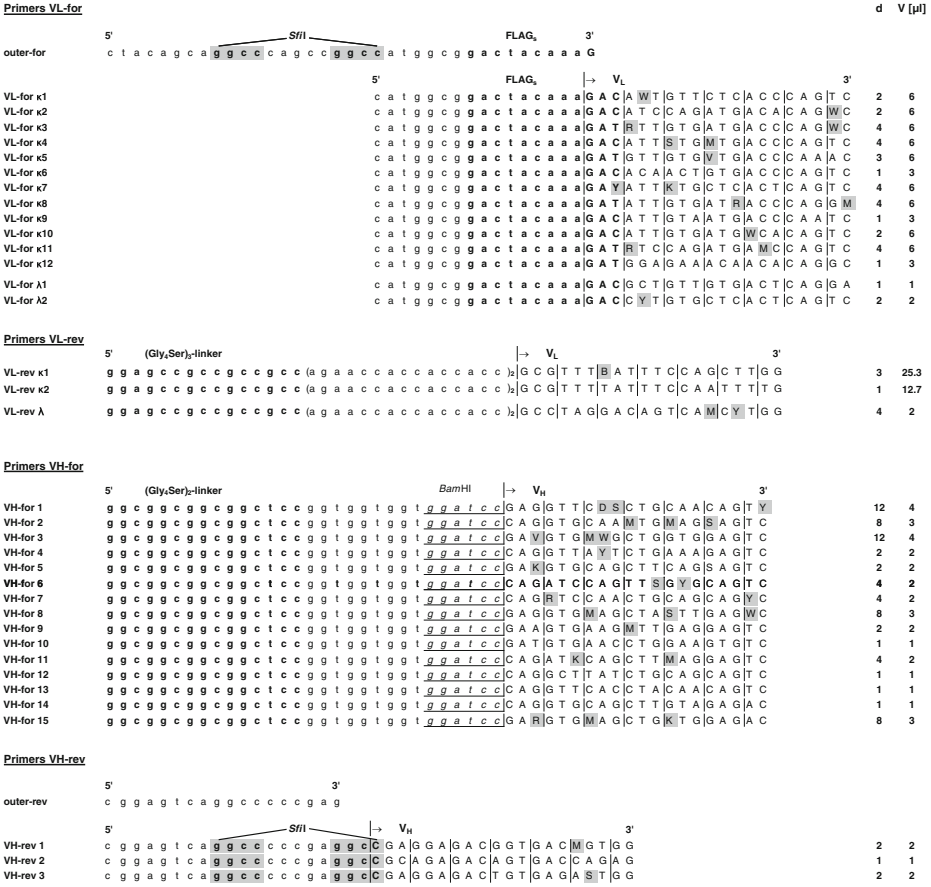


Fig. 3.2 *List of primers used for assembling mouse scFv fragments*. The depicted oligonucleotides direct the assembly of scFv fragments in the orientation V_L-$(G_4S)_4$-V_H, being compatible with the vectors presented in Fig. 3.4. They have been newly derived from an analysis of the complete set of mouse sequences, and are thus different from the previously reported sets (Burmester and Plückthun 2001; Krebber et al. 1997). The sequences are given using the IUPAC nomenclature of mixed bases (shown as capital letters with gray background, R = A or G; Y = C or T; M = A or C; K = G or T; S = C or G; W = A or T; H = A or C or T; B = C or G or T; V = A or C or G; D = A or G or T). A column lists the d-fold degeneration encoded in each primer, d being the number of unique sequences. The "VL-for" primers VL-for κ1 to VL-for κ12 encode a stretch of 20 bases, hybridizing to the mature mouse antibody κ sequences (in capital letters). The preceding sequence that encodes the shortened FLAG sequence (Knappik and Plückthun 1994) is shown in bold. Since the FLAG tag uses the fixed *N*-terminal aspartate of the mature antibody (encoded by GAY), only three additional amino acids are necessary. The FLAG codons are then preceded by the codons specifying the end of the *pelB* signal sequence. The "VL-for" primers VL-for λ1 and VL-for λ2 for mouse lambda chains are constructed in an analogous manner (the *N*-terminal glutamate of the mature mouse λ sequence is replaced by aspartate (encoded by GAC) to generate a FLAG tag). The "VL-rev" primer sequences are complementary to the J-elements of kappa or lambda chains (capital letters) and encode three repeats of the Gly_4Ser sequence, with the terminal one (bold) possessing a different codon usage to minimize incorrect overlaps during the PCR

3.3 Method

3.3.1 Isolation of mRNA and cDNA Synthesis

1. Take 5×10^6 cells from a frozen or growing hybridoma culture (for isotype determination, use the Roche IsoStrip Mouse Monoclonal Antibody Isotyping Kit) or spleen cells, respectively (see note). Perform a total RNA preparation, combining homogenization of cells in the presence of TRIzol Reagent (Invitrogen) with RNA purification using the Qiagen RNeasy Mini Kit as described by the manufacturers. According to the supplier, the latter kit can be used for up to 1×10^7 cells, but in order to get highly pure mRNA, take only 5×10^6 cells.

   Note: For RNA preparation from mouse spleen (typically yielding 5×10^7 B-cells each), first separate it from connective tissue with sharp forceps or scissors (if frozen, also cut the frozen tissue into smaller pieces and pulverize using a mortar) and homogenize it using the Tissue Lyser (Qiagen) or similar homogenizers in the presence of 1 ml TRIzol Reagent per 50 mg of tissue. Make sure not to use too many cells as spleens are typically rich in nucleases, and, therefore, enough RNAse-deactivating components from the TRIzol Reagent should be present in the solution. TRIzol Reagent is a commercial monophasic preparation of guanidinium isothiocyanate and phenol and only the addition of chloroform separates the solution in two phases. If desired, polyA^+ mRNA can subsequently be isolated from the total RNA using the Oligotex Direct mRNA Mini Kit (Qiagen) – however, in most cases, this should not be necessary for the subsequent production of cDNA. Therefore, we do not recommend including this additional purification step, as it might lead to loss of mRNAs present only in smaller quantities. Since specific amplification primers are used, we consider it rather advantageous to work with a higher total amount of RNA.
2. Separate the RNA from DNA and proteins by phenol-chloroform extraction with subsequent silica membrane purification as described by the manufacturer (Invitrogen). Transfer the upper aqueous phase to a new, RNase-free tube. Add an equal volume of 100% ethanol dropwise, as its presence is required for the RNeasy columns to bind the RNA during the initial application. Transfer up to 700 µl of the mixture, including any precipitate that may have formed,

Fig. 3.2 (continued) assembly reaction. Please note that for these primers, the two identical linker repeats are presented by a parenthesis with the subscript 2. The "VH-for" primers encode the other part of the linker (overlap with VL-rev shown in *bold*) as well as a *Bam*HI recognition site (*underlined*). The 20 bases given in capital letters hybridize with the mature mouse VH sequences. The last 20 nucleotides (nt) at the 3′ end of the "VH-rev" primers hybridize with the JH region. The first nt shown in capital letters will cause a silent mutation at the end of V_H in order to code for the first nt of the second *Sfi*I recognition site (*bold and highlighted*). The final assembly of the scFv gene by SOE-PCR is carried out with the outer-for and outer-rev primer set. The outer primer outer-for encodes the first *Sfi*I site (*bold and highlighted*). The last column lists the volume that should be used when mixing the primers (see text)

V_L	1	2	3	4	5	6	7
VL-for κ1	D	I,N	V	L	T	Q	S
VL-for κ2	D	I	Q	M	T	Q	S,T
VL-for κ3	D	I,V	V	M	T	Q	S,T
VL-for κ4	D	I	L,V	L,M	T	Q	S
VL-for κ5	D	V	V	L,M,V	T	Q	T
VL-for κ6	D	T	T	V	T	Q	S
VL-for κ7	D	I	L,V	L	T	Q	S
VL-for κ8	D	I	V	I,M	T	Q	A,D
VL-for κ9	D	I	V	M	T	Q	S
VL-for κ10	D	I	V	M	S,T	Q	S
VL-for κ11	D	I,V	Q	M	N,T	Q	S
VL-for κ12	D	G	E	T	T	Q	A
VL-for λ1	D	A	V	V	T	Q	E
VL-for λ2	D	P,V	V	L	T	Q	S

amino acid position

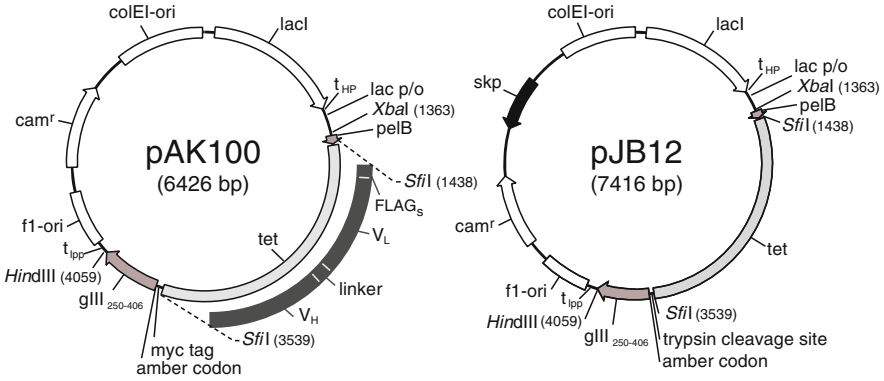
102	103	104	105	106	107	108	V_L
T	K	L	E	I,M	K	R	VL-rev κ1
T	K	L	E	I	K	R	VL-rev κ2
A,T	K,R	L,V	T	V	L	G	VL-rev λ

V_H	1	2	3	4	5	6	7
VH-for 1	E	V	H,L,Q,R	L	Q	Q	F,L,S
VH-for 2	Q	V	Q	L,M	K,Q	E,Q	S
VH-for 3	D,E	V	K,M,Q	L	V	E	S
VH-for 4	Q	V	I,T	L	K	E	S
VH-for 5	D,E	V	Q	L	Q	E,Q	S
VH-for 6	Q	I	Q	F,L	A,V	Q	S
VH-for 7	Q	I,V	Q	L	Q	Q	P,S
VH-for 8	E	V	K,Q	L	L,V	E	S,T
VH-for 9	E	V	K	I,L	E	E	S
VH-for 10	D	V	N	L	E	V	S
VH-for 11	Q	I,M	Q	L	K,Q	E	S
VH-for 12	Q	A	Y	L	Q	Q	S
VH-for 13	Q	V	H	L	Q	Q	S
VH-for 14	Q	V	Q	L	V	E	T
VH-for 15	E	V	K,Q	L	V,L	E	T

amino acid position

107	108	109	110	111	112	113	V_H
T	T	V	T	V	S	S	VH-rev 1
T	L	V	T	V	S	A	VH-rev 2
T	S,T	L	T	V	S	S	VH-rev 3

Fig. 3.3 *Deduced amino acid sequence of the V domain, hybridizing part of primers from Fig. 3.2*. The residues of V_L and V_H are numbered according to Kabat et al. (1991). All forward primers determine only the first 2 nt of residue 7, whereas the reverse primers determine the last 2 nt of position 102 or 107, respectively. Therefore, the alternative translations at those latter positions given do not indicate that they encode a mixed codon



pAK / pJB vector series		phage display	Skp coexpression	enhanced expression	trypsin cleavage site	IMAC purification	C-terminal detection	direct detection	dimerization
pAK100	XbaI SfiI SfiI EcoRI HindIII — pelB tet myc * gIII 250-406	●							
pAK300	XbaI SfiI SfiI HindIII — pelB tet (His)6					●	●		
pAK400	XbaI SD T7g10 SfiI SfiI HindIII — lac p/o pelB tet (His)6			●		●	●		
pAK500	XbaI SfiI SfiI EcoRI HindIII — pelB tet dHLX (His)5					●	●		●
pAK600	XbaI SfiI SfiI EcoRI HindIII — pelB tet alkaline phosphatase							●	●
pJB12	XbaI SfiI SfiI EcoRI HindIII — pelB tet KDIR * gIII 250-406	●	●		●				
pJB23	XbaI SfiI SfiI EcoRI HindIII — pelB tet (His)6		●			●	●		
pJB33	XbaI SD T7g10 SfiI SfiI EcoRI HindIII — lac p/o pelB tet (His)6		●	●		●	●		

Fig. 3.4 *Overview of pAK/pJB vector series*. The pAK/pJB vector series (see also Fig. 3.5) can be used either for phage display (pAK100 and pJB12) by the strategy outlined in Fig. 3.1, or for the expression of the antibody in a variety of formats. All vectors contain a chloramphenicol resistance cassette (cam^r) and additionally a tetracycline resistance "stuffer" cassette (*tetA* and *tetR*; 2,101 bp), which will be replaced by the antibody gene (the *tet* cassette allows the monitoring of complete *Sfi*I digested vector by plating of transformed DH5α cells on tetracycline plates). Furthermore, these vectors contain the *lacI* repressor gene, a strong upstream terminator (t_{HP}) to avoid read-through and premature expression, the *lac* promoter/operator and the *pelB* (pectate lyase gene of *Erwinia carotovora*) leader sequence (modified to contain a *Sfi*I site) as well as a

to one RNeasy spin column and continue according to the manufacturer's instructions.

Note: RNA in harvested tissue is not protected from degradation until the sample is mixed with TRIzol Reagent, flash-frozen or disrupted and homogenized in the presence of RNase-inhibiting or protein-denaturing reagents. Therefore, proceed with this step as fast as possible. Generally, DNase digestion is not required with RNeasy Kits since its silica membrane efficiently removes most of the DNA. However, if desired, residual DNA can be removed by optional on-column DNase digestion using the RNase-Free DNase Set (Qiagen). It is important not to overload the RNeasy spin column, as this will significantly reduce RNA yield and quality.

3. Elute the purified RNA by the addition of 30 µl RNase-free water. The mRNA solution is now ready for cDNA synthesis or can alternatively be stored at −80°C for up to one month.

   Note: Diethylpyrocarbonate (DEPC)-treated UHP water can also be used. However, as DEPC is a suspected carcinogen the use of filtrated RNase-free water is recommended.

4. For reverse transcription, take approximately 0.1–0.5 µg RNA and 1 µl random hexamer primers provided in the kit in 20 µl total reaction volume. The precise procedure is described in the QuantiTect Reverse Transcription Kit (Qiagen).

   Note: Ribonuclease H activity of Quantiscript Reverse Transcriptase specifically degrades only the RNA in RNA:DNA hybrids, but it has no effect on pure RNA. Hence, an additional RNA degradation step using another RNase H enzyme is not necessary prior to subsequent PCR reaction.

   Note: Specific primers hybridizing to the constant regions can be used as well, e.g., if only a particular antibody class should be amplified from spleen cells. In general, however, the random hexamer primers work robustly.

Fig. 3.4 (continued) downstream terminator (t_{lpp}). The rationale for these elements has been described in detail previously (Krebber et al. 1997). The origins for phage replication and plasmid replication are as described in Ge et al. (1995). The antibody gene is fused in frame either to $gIII_{250–406}$ for phage display, to a his tag for IMAC purification (Lindner et al. 1992) and *C*-terminal detection with a recombinant anti-his tag scFv-phosphatase fusion protein (Lindner et al. 1997), to dimerization helices (Pack et al. 1993, Plückthun and Pack 1997, see also chapter 7) or to alkaline phosphatase for both dimerization and direct detection (Lindner et al. 1997). In pAK100, the in-frame fusion contains a myc tag (Munro and Pelham 1986), offering an additional detection possibility next to the short *N*-terminal 4-amino acid FLAG tag (DYKD; Knappik and Plückthun 1994) present in all the vectors being encoded by the primers shown in Fig. 3.2. The plasmid pJB12 contains a trypsin cleavage site (KDIR) and can therefore be conveniently used for selection of high-affinity binders as described by Dziegiel et al. (1995) and Johansen et al. (1995). The asterisk in these two vectors pAK100 and pJB12 represents an amber codon. The scFv expression level in pAK400 and pJB33 is enhanced due to the strong Shine Dalgarno sequence SDT7g10 (from T7 phage gene 10). Because of the compatibility of the vectors, this feature can easily be introduced in all of them. In the pJB vector series the co-expressed periplasmic protein Skp (Bothmann and Plückthun 1998), encoded on this vector, can increase the functional yield of antibody fragments expressed in the periplasm without the need of cotransformation with another plasmid coding for further chaperones. This feature can also be introduced into any of the other vectors. The complete sequences of all vectors are available from the authors upon request

3.3.2 PCR Amplification and scFv Assembly

3.3.2.1 PCR Amplification of V_L and V_H Domains

1. Use the primers described in Fig. 3.2, which have been dissolved in 100 μM stock solutions in either sterile water or sterile TE buffer to prepare appropriate mixtures (VL-for mix, VL-rev mix, VH-for mix, and VH-rev mix). Mix them according to the degree of degeneration, indicated as "d" in Fig. 3.2 (equaling the number of different unique sequences encoded by mixed bases in the primer) by adding the stated volumes (in μl) towards the final primer mix. The fraction of lambda-specific primers in both the forward and reverse V_L mixture amounts for ~5% of the total volume, accounting for the low percentage of this light chain type in mouse antibodies. The nominal total primer concentration of these mixtures is still 100 μM, ranging from 3 to 40 μM for each of the individual oligonucleotides.

   Note: As described in the introduction, problems in the cloning of monoclonal antibodies can occur if the hybridoma transcribes more than one functional or even nonfunctional heavy or light chain variable region gene. Therefore, it is highly recommended to omit any lambda chain-specific primer in the PCR if the isotyping already indicates that the hybridoma of interest secretes IgGs possessing kappa light chains.
2. For PCR amplification of V_L and V_H, use the product of the completed first-strand cDNA reaction and prepare the following mixtures:

PCR mix for amplification of V_L	PCR mix for amplification of V_H
2 μl cDNA	2 μl cDNA
1 μl dNTPs (10 mM each)	1 μl dNTPs (10 mM each)
5 μl 10× ThermoPol buffer (NEB)	5 μl 10× ThermoPol buffer (NEB)
0.5 μl VL-for primer mix (100 μM)	0.5 μl VH-for primer mix (100 μM)
0.5 μl VL-rev primer mix (100 μM)	0.5 μl VH-rev primer mix (100 μM)
2.5 μl DMSO	2.5 μl DMSO
0.5 μl VentR Polymerase 2 U/μl (NEB)	0.5 μl VentR Polymerase 2 U/μl (NEB)
38 μl H_2O	38 μl H_2O

   Note: This standard protocol is optimized for VentR polymerase, a DNA polymerase with a 5–15 fold lower error rate than Taq DNA Polymerase (due to an intrinsic 3′→5′ proofreading exonuclease activity). If using other proofreading polymerases (e.g., Phusion High-Fidelity DNA Polymerase from Finnzymes), reaction and PCR program conditions might have to be adapted. If the proposed PCR mix does not lead to any product, varying the cDNA template amount might be beneficial. If the thermocycler does not have a heated cover, add one drop of mineral oil to the reaction tube to prevent evaporation.

3. Perform the following PCR cycles after an initial denaturation of the DNA template for 3 min at 95°C: 5 cycles of 30 s at 95°C, 30 s initial annealing at 55°C, and 45 s elongation at 72°C, followed by 20 cycles of 30 s at 95°C, 30 s at 63°C, and

45 s at 72°C. After the last cycle is completed, an additional 5 min elongation step at 72°C should be performed before cooling the thermocycler to 4°C.

Note: We recommend using a hot start, keeping the PCR tubes on ice and not placing them into the thermocycler until the block has reached 95°C, to minimize unspecific amplification. For successful amplification of V_L and V_H, complete annealing of the 3′-ends of the primers with the template DNA is essential. The recommended annealing temperature of 55°C should be suitable for approx. 97% of the sequences found in a reference set of murine germline sequences in the IMGT database. However, as it is not clear a priori which somatic mutations a given monoclonal antibody may carry in the primer regions, we recommend using a gradient PCR program (covering a range between 70° and 50°C in steps of 2°) to determine the optimum annealing temperature and to amplify the antibody genes without unspecific secondary bands. Alternatively, the PCR might also be run in a "touchdown" manner (Don et al. 1991), starting at an annealing temperature of 70°C and ending at 50°C. As after 5 cycles the amplified PCR product will serve itself as template DNA, the annealing temperature of the last 20 cycles can be increased to 63°C.

4. Analyze 1/10 volume of each PCR mixtures by agarose gel electrophoresis, purify the V_L and V_H genes using the PCR clean-up Gel Extraction Kit (Macherey Nagel) according to the manufacturer's instructions and determine the DNA concentration of both genes.

   Note: Using the listed primer mixtures, the expected lengths of the PCR products of V_L and V_H are between 375–402 bp and 386–440 bp, respectively. Purification of the PCR products is important to remove any residual primers which might interfere with the subsequent assembly PCR. For the case of multiple bands on the agarose gel, gel-purify the band of correct size using the GenElute Gel Extraction Kit (Sigma-Aldrich). If the final DNA concentration is too low afterward, perform a second PCR using these purified fragments as template for gaining sufficient yields of high-quality DNA.

3.3.2.2 Assembly of V_L and V_H by SOE-PCR (Splicing by Overlap Extension)

1. For the assembly PCR, use approximately 10 ng of the PCR product of both domains in a total volume of 50 µl, containing 200 µM dNTPs, 3–5% DMSO, 1 µM outer-for, and outer-rev primer (each) and 1 unit VentR DNA Polymerase (NEB). Following a 3 min 95°C step, perform 5 cycles of 1 min at 95°C, 1 min at 63°C, and 1 min at 72°C, followed by another 5 cycles of 1 min at 95°C, 30 s at 56°C, and 1 min at 72°C and finally 25 cycles of 1 min at 95°C, 90 s at 72°C.

   Note: Hot start PCR and initial assembly of V_L and V_H in the absence of the primers is usually not necessary but can be performed. It is important to include DMSO in the PCR mix as well as to keep the primer concentration as low as indicated to prevent any risk of primer-dimer formation.

   Note: The assembly, as used here, places V_L in front of V_H. This has the advantage of placing a shortened FLAG tag, consisting of only four amino acids,

at the *N*-terminus of the construct. Since its last amino acid, Asp, is the same as the first residue of the V_L domain, only three additional amino acids are needed (Knappik and Plückthun 1994) for allowing specific detection using this tag. A slight asymmetry in the V_H/V_L heterodimer with respect to the pseudo two-fold axis (Plückthun et al. 1996) is taken care of with a 20-amino acid linker, leading to monomeric scFv fragments.

3.3.3 Digestion and Cloning of scFv Genes

1. Purify the product of the assembly PCR using the PCR clean-up Gel Extraction Kit (Macherey Nagel) according to the manufacturer's instructions, eluting the product in 30 µl of the recommended buffer. In case there are several bands on the analytical agarose gel, carry out a gel purification of the correct band, as described in 3.3.3.4.
2. Perform a *Sfi*I digest of the amplified scFv gene for 3–4 h at 50°C (At 37°C, the activity of *Sfi*I would be 10 fold-lower). To the 30 µl purified PCR product, add 5 µl 10× NEbuffer 4 (NEB), 5 µl 10× BSA (final concentration, 100 µg/µl), 9 µl H_2O, and 1 µl (=20 units) *Sfi*I (NEB).
3. Digest appropriate amounts of vector (pAK100 or pJB12, see Fig. 3.4) with *Sfi*I in the presence of the above-mentioned buffer, including BSA. Use 10 units *Sfi*I for 1 µg vector in 50 µl and incubate 4 h at 50°C. Dephosphorylate the cut vector by adding Calf Intestinal Alkaline Phosphatase (CIP, NEB; 0.5 unit/µg vector) to the digestion mix after 2 h and continue incubation for another 2 h at 50°C.

   Note: Dephosphorylation should not be necessary because of the asymmetric overhangs. However, we always include this step to eliminate any risk of religation of single-cut vector.

   Note: pAK100 or pJB12 are phage display vectors (Fig. 3.4). When starting from hybridomas, one can also directly clone the V_L and V_H genes into an scFv expression vector with a stronger promoter, such as pAK400, which does not encode a fusion with gIII. However, depending on the number of additional V genes expressed in the hybridoma, a large number of clones may have to be screened from individual colonies.
4. Purify the digested scFv antibody genes and vector by preparative agarose gel electrophoresis in combination with the GenElute Gel Extraction Kit (Sigma-Aldrich).

   Note: For obtaining pure preparations of a fully digested vector, it is very important not to overload the agarose gel. Furthermore, the gel electrophoresis has to be run long enough to separate small amounts of undigested vector from the digested vector band. For large-scale vector or insert preparation, electro-elution might be an efficient and convenient alternative. If the concentration of eluted DNA is too low for further applications, Microcon 30 columns (Amicon) can be used for concentration.

5. Ligate 50 ng scFv gene fragment with the vector (molar ratio of vector to insert 1:5) with 5 units T4 DNA ligase (NEB) in the presence of 1× T4 DNA ligase buffer in 10 µl volume. Incubate for 2 h at room temperature or overnight at 16°C.

   Note: The ATP-concentration is very crucial for the successful ligation by T4 DNA ligase. Therefore, we recommend using T4 DNA ligase buffer aliquots, which have been properly stored at −20°C and not thawed repeatedly. To allow an easy subcloning of the scFv fragment into vectors for optimized soluble expression and other purposes, compatible vector sets are available (Figs. 3.4 and 3.5).
6. Transform 50 µl chemocompetent XL1-Blue cells (Stratagene) with 5 µl of the ligation mix by heat-shock for 45 s at 42°C, add 500 µl of 2× YT medium after 2 min incubation on ice, and incubate for 45 min, shaking at 37°C.

   Note: Make sure not to exceed a ratio of ligation mix/cells of 1:10 (v/v). Chemocompetent *E. coli* are used, if only a very small diversity of clones is expected, e.g., when cloning from a hybridoma. If a larger diversity and thus many clones are required (e.g., when cloning from spleen cells), follow the instructions for electroporation described in steps 3.3.5.1–3.3.5.3.
7. Plate the transformed cells on 2× YT, 1% glucose, chloramphenicol (30 µg/ml) agar plates, and incubate overnight at 37°C.

   Note: You may check the ratio of desired ligation product to background by including transformation with "religated" plasmid in the absence of any insert. Alternatively, the background signal can be analyzed by testing for tetracycline resistance after transformation of other *E. coli* strains not possessing an intrinsic *tet* resistance (like Invitrogen's DH5α) with the ligation mix. The portion of vector with unremoved or religated *tet* cassette is typically in the range of 0.01–0.1%.

3.3.4 Preparation of Electrocompetent E. coli

1. For preparation of electrocompetent *E. coli* XL1-Blue cells (Stratagene), use 2 ml of a dense overnight pre-culture to inoculate 500 ml medium (2× YT, 15 µg/ml tetracycline). Shake it at 25°C until an OD_{600} of 0.6 is reached, then chill the culture on ice as quickly as possible for 30 min (cool the whole shake flask in a large ice bath).

   Note: Sufficient agitation during growth seems to be very important for preparation of electrocompetent cells, reaching reproducible efficiencies of 3–6 × 10^9 cfu/µg pUC19 DNA. Therefore, use 5 l baffled shake flasks with only 500 ml medium and make sure that the amplitude of the shaker is high enough to vigorously circulate the medium.

   Note: The use of electrocompetent bacteria is an alternative to 3.3.3.6 and needed when a large diversity is expected, typically when cloning from spleen cells.

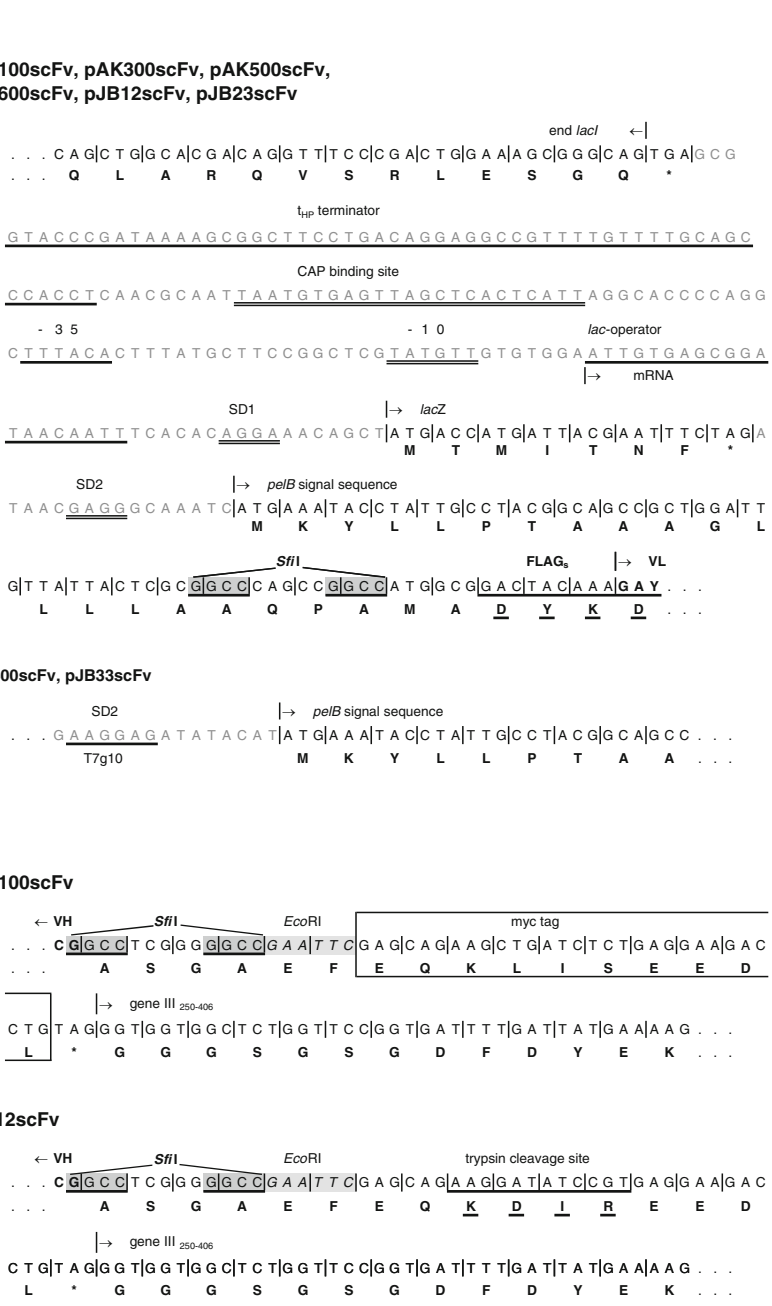


Fig. 3.5 (Continued)

Fig. 3.5 *Detailed sequences upstream and downstream of scFv clonig site*. (**a**) Upstream sequence of pAK100scFv, pAK300scFv, pAK400scFv, pAK500scFv, pAK600scFv, pJB12scFv, and pJB23scFv. The symbol scFv indicates that the vectors are shown after an scFv has been introduced, replacing the *tet* stuffer fragment. The region from the end of the *lacI* repressor gene to the beginning of the antibody V_L domain is shown. The *lacI* repressor gene, t_{HP} terminator sequence, CAP binding site, *lac* operator region, including the -35 and -10 sequence, Shine-Dalgarno (SD) sequence of *lacZ* (SD1), *lacZ* peptide, a second SD sequence (SD2), *pelB* signal

2. Centrifuge the bacterial culture in 50 ml aliquots in disposable tubes for 5 min at 5,000 *g*. Remove as much supernatant as possible (leave the tube upside down for 15–30 s on a clean tissue). Then, fill each tube with 1 volume of ice-cold distilled water (i.e., the same volume as the original culture aliquot) and remove the water immediately (the cell pellet is very solid after this first centrifugation step and will not be resuspended by the brief rinsing with distilled water).

   Note: All these steps should be carried out using ice-cold solutions and be performed in the cold room. Use only ultra pure water to wash cells and to prepare 10% glycerol, as the presence of impurities such as salts in the water might cause the subsequent transformations to fail.
3. Fill each tube with 1 vol distilled water (i.e., the same volume as the original culture aliquot), resuspend the pellet carefully and incubate for 10 min on ice.

   Note: Make sure that the cells are sufficiently solubilized to yield a homogeneous suspension. Cells are best resuspended by swirling rather than pipetting. Never vortex the cell suspension!
4. Transfer the cells into new 50 ml tubes and centrifuge at 5,000 *g* for 10 min. Carefully remove the supernatant and resuspend the pellets each in 50 ml pre-chilled 10% (v/v) glycerol (Fluka). Incubate on ice for 10 min.
5. Centrifuge resuspended cells at 5,000 *g* for 15 min and remove the supernatant (you might lose a small portion of cells – do not put the tubes upside down on tissue in this step!). Carefully resuspend the cells in 1/500 of the original culture volume (= 1 ml) 10% (v/v) glycerol, freeze the cells in 100 µl aliquots by dipping the tubes immediately into liquid nitrogen and store them at −80°C.

   Note: Electrocompetent cells can be kept at −80°C for up to 12 months.
6. To determine the transformation efficiency, add 1 µl of 10 pg/µl pUC19 DNA (in water) to 40 µl of barely thawed cells (see step 3.3.5.2). Fifty colonies per 1/1,000 of the transformation volume plated correspond to an efficiency of 5×10^9 cfu/µg pUC19 DNA.

Fig. 3.5 (continued) sequence, *N*-terminal *Sfi*I site (*underlined and highlighted*), four amino acid $FLAG_s$ tag (*underlined*), and the start of the V_L domain (sequence GAY; *bold*) are indicated above the sequence. In addition, also the corresponding amino acid sequence is shown. In pAK400 and pJB33, the 15 bp upstream from the *pelB* start codon are replaced by another sequence, including the SD sequence of the phage T7 gene10, while everything else is identical. Because of the modularity of the vectors, this feature can be easily introduced into any of the other vectors (see Fig. 3.4) (**b**) Downstream sequence of pAK100scFv and pJB12scFv. The last two bases of V_H (*bold*), the *Sfi*I and *Eco*RI restriction sites, myc tag (*boxed*) or trypsin cleavage site and the start of $geneIII_{250–406}$ are indicated above the sequence. Asterisks indicate amber stop codon, leading to scFv-gene III fusions upon expression in *E. coli* strains with amber suppressor tRNA, such as XL1-Blue. (**c**) Downstream sequence of pAK300scFv, pAK400scFv, pJB23scFv, and pJB33scFv. The last two bases of V_H (*bold*), the *Sfi*I and *Eco*RI restriction sites and $(His)_6$ tag (*boxed*) are indicated above the sequence. (**d**) Sequences of the downstream *Eco*RI/*Hin*dIII fusion cassettes as used in pAK500 and pAK600. The dHLX dimerization motif (*double underlined*) was taken from Pack et al. (1993). The complete sequence of the mature *E. coli* alkaline phosphatase (AP) gene can be found in Shuttleworth et al. (1986). For the *Eco*RI/*Hin*dIII cloning cassette the two internal *Eco*RI sites of the AP gene have been removed by silent mutations. The complete sequences of all vectors are available from the authors upon request

3.3.5 Library Preparation/Construction

1. For desalting the DNA prior to electroporation, apply the ligation mix to StrataClean Resin (Stratagene; hydroxylated silica, binding proteins with a high affinity, while having a low affinity for DNA at near neutral pH), followed by precipitation in 70% ethanol. Since most salts and small organic molecules are soluble in 70% ethanol, they can be separated from DNA by centrifugation. Resuspend the precipitated DNA in ultra pure water.
2. For each transformation, use desalted ligation mixtures corresponding to 20–100 ng insert. Add the DNA to 40 µl of barely thawed cells on ice and mix by flipping the tube shortly and gently. Immediately transfer the cell-DNA mix to chilled electroporation cuvette (bubble free), pulse according to the guidelines of the electroporator's manufacturer, and add 1 ml of SOC medium (20 g/l bacto-tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose, 10 mM $MgCl_2$, 10 mM $MgSO_4$) to cells immediately after the pulse.

   Note: For efficient transformation ($\geq 10^8$ clones per µg insert DNA), the time constant using 2 mm cuvettes should be ≥5 ms, reflecting properly washed cells. Also, make sure that no air-bubbles are trapped in the cell-DNA mix as they will interfere with the electroporation.
3. Resuspend cells completely in SOC medium and shake for 1 h at 37°C. Afterward, plate dilutions on 2× YT, 1% glucose, chloramphenicol (30 µg/ml) agar plates. Use a sterile spreader or sterile glass beads to evenly distribute the culture over the surface of the 12 × 12 cm plate (do not exceed about 5,000 clones per square plate) and incubate overnight at 37°C. The next day, scrape the colonies off the plates in 3–4 ml 2× YT, containing 30% glycerol, and subsequently store them at −80°C.

   Note: Take care that your library is homogeneously mixed.
4. For phage panning as described in 3.3.6, inoculate cultures with at least tenfold more viable cells than colonies obtained after transformation, in order to have sufficient oversampling. When starting from spleen cells, perform three rounds of phage panning as described in 3.3.6 before testing single clones. When starting from a hybridoma, one round should be sufficient, and in ideal cases, single clones can be tested right away by phage ELISA.

   Note: The first panning round is the most crucial, as you might lose any desired, but less abundant antibody sequence by too extensive washing. Therefore, do not exceed ten washing steps in this first panning round. The panning procedure is analogous to the phage ELISA (3.3.6), except that a pool of phages is grown and that phages are eluted from antigen (at the end of 3.3.6.4), which are afterward added again to exponentially growing bacteria. This is described in detail elsewhere (Barbas et al. 2001; Lee et al. 2007) and also in this volume.

   Note: The screening of single clones can be performed in three ways. First, at the level of phages (phage ELISA), as described in Sect. 3.3.3.6, second, after retransforming of an suppressor tRNA-deficient strain such as, e.g., *E. coli* strain JM83 (Yanisch-Perron et al. 1985), still with the amber codon, containing

pAK100 derived plasmids, or third, after recloning into a more efficient expression vector such as pAK400 (Fig. 3.5), which carries no gene III. In second and third option, the soluble scFv is screened by ELISA.

Note: This protocol does not describe the periplasmic expression of scFv fragments in *E. coli* (Glockshuber et al. 1990) and their subsequent purification (reviewed in Plückthun et al. 1996). More details can be found in chapter 27 "Improving expression of scFv fragments by coexpression of periplasmic chaperones" in this volume.

3.3.6 Screening for Binders by Phage ELISA

1. When starting from hybridoma, pick 10 colonies (from spleen, as many as you can handle) and grow them separately at 37°C in 2 ml 2× YT, 1% glucose, chloramphenicol (30 μg/ml) until they reach an OD_{600} of 0.5. This level of glucose fully represses expression, and, thus, the growth temperature can be 37°C. Dilute 1:10 in 2× YT, 1% glucose, chloramphenicol (30 μg/ml), containing 1 mM IPTG, and 1×10^{10} pfu VCSM13 helper phage (Stratagene) per ml, and grow overnight at 26°C or 37°C (for some murine scFvs with aggregation tendencies, growth at 26°C after infection may be necessary). The phage titer after overnight incubation is in the range of 10^{11}–10^{12} cfu per ml supernatant.

   Note: XL1-Blue should be grown on agar plates and in media containing tetracycline (*tet*) as the F′-plasmid encoding for the F-pili required for infection of bacteria also carries the *tet* resistance gene. The phage titer (in cfu) should be determined in order to rule out any problems during phage production. To do so, take a log-phase culture of XL1-Blue cells (OD_{600} = 0.4–0.6) and incubate aliquots of this culture with serial dilutions of your phage preparation. After 15 min incubation at 37°C, plate appropriate amounts (30–150 cfu/plate) on 2× YT, 1% glucose, chloramphenicol (30 μg/ml) agar plates.

   Note: We are aware of the fact that the presence of such a high level of glucose during IPTG-induction is rather unusual. However, for phage display, the induction level does not have to be very high. Based on our experiences with this vector series described here, a combination of IPTG addition and the presence of glucose seems to be crucial for the successful expression of some scFv fragments, notably those with nonideal biophysical properties, and appropriate for most, but may have to be checked for each scFv individually in case of unusual properties.
2. Centrifuge the culture 10 min at 16,000 *g* and 4°C. Take 1.6 ml supernatant and mix it with 0.4 ml 20% PEG 6000 (Fluka), 2.5 M NaCl in a 2 ml Eppendorf tube in order to precipitate the phages (Sambrook and Russell 2001).

   Note: We recommend that the PEG solution be freshly prepared.
3. Incubate on ice for 30–60 min and centrifuge for 15 min at 5,600 *g* and 4°C.

   Note: It is important not to centrifuge phages at too high a *g* force, as otherwise, it will be difficult to resuspend them homogeneously, resulting in a

decreased phage titer. The size of the white pellet does not necessarily reflect a high or low phage titer.

4. Resuspend the phage pellet in 400 µl PBS (with 10% (v/v) glycerol). For complete resuspension, incubate the phage solution on an orbital shaker at 800 rpm for 15 min at 4°C. Pellet insoluble matter (cell debris) by centrifugation for 10 min at 11,000 *g* and 4°C and transfer the phage solution to a fresh tube. Use 100 µl phage solution per well in an ELISA assay to distinguish phages displaying functional scFv antibody from those which display nonfunctional or nonproductive antibody fragments.
5. If soluble antigen is available, include a competition ELISA control showing that free antigen is able to compete with bound antigen for phage binding to distinguish nonspecific "sticky" from specifically binding phages. In principle, the same ELISA protocol that was used for the hybridoma screening procedure can be used.

   Note: For weak binders, it might be important to use more phages for ELISA analysis. In this case, the culture volume should be increased ten times. If no functional clone shows up in ELISA of single clones, perform one round of phage panning in order to enrich the functional binders. The enrichment should be checked by comparison of eluted phages from a specific surface versus a surface without antigen. In addition, it is recommended to analyze the phage solution by immunoblot, using an anti-M13 HRP-conjugated antibody (GE Healthcare), to ensure the correct fusion of the scFv to the gIII-protein as well as its correct display on the phage surface.

3.4 Troubleshooting

This part of the protocol contains general comment about potential pitfalls of the recommended standard method. The most critical steps were already highlighted directly following the instructions in the different subsections.

(a) In case of low transformation yields, check whether the problem is the transformation itself or rather the ligation. To investigate the quality of ligation, analyzing an aliquot by agarose gel electrophoresis might indicate any problems caused by nucleases. Furthermore, it might be informative to compare the ligation efficiency of *Sfi*I digested PCR product with inserts derived from plasmid digestion. In order to check both the ligation and the transformation efficiency, a defined amount of pUC19 DNA can be added to the ligation mixture. Because of the chloramphenicol resistance of the cloning vector and the ampicillin resistance of pUC19 DNA, it is possible to calculate the ligation efficiency by plating double transformed cells on ampicillin or chloramphenicol plates, respectively, and comparing the number of clones. The transformation efficiency (in presence of the ligation mixture) can be judged by comparison of the colony number after transformation with pUC19 DNA alone.

(b) The quality of the oligonucleotides used in this procedure is crucial for the successful and reliable amplification of various antibody genes as well as their subsequent assembly into scFvs. The number of proposed primers is important for a broad representation of the immune response, as any sequence absent from the complex mixture will obviously decrease the functional library size. We also strongly recommend using primers that have been accurately purified after their synthesis (either by HPLC or, for longer primers, by PAGE) to ensure that no single-base deletions are present in any of the oligonucleotides. These deletions as well as any insertions would cause frameshifts in the final gene assembly, resulting in a number of nonfunctional library members. Therefore, we also suggest – especially for library cloning – to sequence the genes of several random clones as well as to check for full-length scFv by western blot analysis detecting its fusion partner gene III (see note at 3.3.6.5).

(c) In case of severe problems in the PCR amplification of the V_H and V_L genes (steps 3.3.2), it might be worth considering to divide this reaction into two separate ones. Using the proposed primers without any overhang at their 5′-end (which either codes for the $FLAG_s$-tag and the *Sfi*I cleavage site, or the $(Gly_4Ser)_4$-linker), the pure antibody DNA should be amplified in a first PCR reaction, and, subsequently, a second PCR should be performed for reamplification and introduction of the appropriate overhangs with the original full-length primers. This procedure increases the degree of matching in both reactions, and might therefore help in the annealing step of the primers.

(d) Whenever expression of the scFv gene is not required, the bacteria should be grown in the presence of 1% glucose. Glucose will cause a tight suppression of the *lac* promoter, thereby ensuring the genetic stability of the inserted scFv genes. Likewise, we suggest growing XL1-Blue always on agar plates and in media containing tetracycline (*tet*) to keep the bacteria infective, as the *tet* resistance is located on the F′-plasmid that also contains the genes encoding F-pilus formation. Always use fresh XL1-Blue colonies, as subcloning might occasionally lead to the formation of *tet* resistant cells, which are no longer infectable. As the F-pilus expression is reduced when the bacteria are past log phase as well as when grown at temperatures below 34°C, we also recommend growing them at 37°C to OD_{600} = 0.4–0.6.

(e) When working with libraries, double transformants can and will occur (Goldsmith et al. 2007). It is thus highly recommended that the scFv fragments of interest be recloned into a new vector (e.g., from pAK100 to pAK400), when they are analyzed at the level of pure unfused protein, thereby also introducing a stronger translation initiation region. It should be noted that diluted retransformation cannot resolve plasmid mixtures, as in *E. coli,* plasmids can form reversible concatamers.

Acknowledgements This protocol has evolved over the years, and heavily relies on the original versions developed by Anke Krebber and Jörg Burmester, with important discussions and contributions to the reagents and procedures gradually added from Peter Lindner, Lutz Jermutus, Jörg Willuda, Daniel Steiner, Barbara Klinger, and Cornelia Rinderknecht.

References

Adams GP, McCartney JE, Tai MS, Oppermann H, Huston JS, Stafford WF 3rd, Bookman MA, Fand I, Houston LL, Weiner LM (1993) Highly specific in vivo tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single-chain Fv. Cancer Res 53:4026–4034

Amstutz P, Binz HK, Zahnd C, Plückthun A (2006) Ribosome display: in vitro selection of protein-protein interactions. In: Celis J (ed) Cell biology: a laboratory handbook, 3rd edn. Elsevier, Amsterdam, pp 497–509

Backmann N, Zahnd C, Huber F, Bietsch A, Plückthun A, Lang HP, Güntherodt HJ, Hegner M, Gerber C (2005) A label-free immunosensor array using single-chain antibody fragments. Proc Natl Acad Sci USA 102:14587–14592

Barbas C, Burton D, Scott J, Silverman G (2001) Phage display: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Bothmann H, Plückthun A (1998) Selection for a periplasmic factor improving phage display and functional periplasmic expression. Nat Biotechnol 16:376–380

Burmester J, Plückthun A (2001) Construction of scFv fragments from hybridoma or spleen cells by PCR assembly. In: Kontermann R, Dübel S (eds) Antibody engineering. Springer, Berlin, pp 19–40

Di Paolo C, Willuda J, Kubetzko S, Lauffer I, Tschudi D, Waibel R, Plückthun A, Stahel RA, Zangemeister-Wittke U (2003) A recombinant immunotoxin derived from a humanized epithelial cell adhesion molecule-specific single-chain antibody fragment has potent and selective antitumor activity. Clin Cancer Res 9:2837–2848

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008

Dziegiel M, Nielsen LK, Andersen PS, Blancher A, Dickmeiss E, Engberg J (1995) Phage display used for gene cloning of human recombinant antibody against the erythrocyte surface antigen, rhesus D. J Immunol Methods 182:7–19

Ewert S, Honegger A, Plückthun A (2004) Stability improvement of antibodies for extracellular and intracellular applications: CDR grafting to stable frameworks and structure-based framework engineering. Methods 34:184–199

Freund C, Ross A, Plückthun A, Holak TA (1994) Structural and dynamic properties of the Fv fragment and the single-chain Fv fragment of an antibody in solution investigated by heteronuclear three-dimensional NMR spectroscopy. Biochemistry 33:3296–3303

Ge L, Knappik A, Pack P, Freund C, Plückthun A (1995) Expressing antibodies in Escherichia coli. In: Borrebaeck C (ed) Antibody engineering, 2nd edn. Oxford University Press, Oxford, pp 229–236

Glockshuber R, Malia M, Pfitzinger I, Plückthun A (1990) A comparison of strategies to stabilize immunoglobulin Fv-fragments. Biochemistry 29:1362–1367

Goldsmith M, Kiss C, Bradbury AR, Tawfik DS (2007) Avoiding and controlling double transformation artifacts. Protein Eng Des Sel 20:315–318

Hanes J, Plückthun A (1997) *In vitro* selection and evolution of functional proteins by using ribosome display. Proc Natl Acad Sci USA 94:4937–4942

Hanes J, Jermutus L, Weber-Bornhauser S, Bosshard HR, Plückthun A (1998) Ribosome display efficiently selects and evolves high-affinity antibodies in vitro from immune libraries. Proc Natl Acad Sci USA 95:14130–14135

Honegger A, Plückthun A (2001) The influence of the buried glutamine or glutamate residue in position 6 on the structure of immunoglobulin variable domains. J Mol Biol 309:687–699

Honegger A, Spinelli S, Cambillau C, Plückthun A (2005) A mutation designed to alter crystal packing permits structural analysis of a tight-binding fluorescein-scFv complex. Protein Sci 14:2537–2549

Huston JS, Levinson D, Mudgett-Hunter M, Tai MS, Novotny J, Margolies MN, Ridge RJ, Bruccoleri RE, Haber E, Crea R, Oppermann H (1988) Protein engineering of antibody binding

sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. Proc Natl Acad Sci USA 85:5879–5883

Johansen LK, Albrechtsen B, Andersen HW, Engberg J (1995) pFab60: a new, efficient vector for expression of antibody Fab fragments displayed on phage. Protein Eng 8:1063–1067

Jung S, Spinelli S, Schimmele B, Honegger A, Pugliese L, Cambillau C, Plückthun A (2001) The importance of framework residue H6, H7, and H10 in antibody heavy chains: Experimental evidence for a new structural subclassification of antibody V_H domains. J Mol Biol 309:701–716

Kabat E, Wu T, Reid-Miller M, Perry H, Gottesman K, Foeller C (1991) Sequences of proteins of immunological interest, 5th edn. US Department of Health and Human Services, Public Service, NIH

Knappik A, Plückthun A (1994) An improved affinity tag, based on the FLAG peptide for the detection and purification of recombinant antibody fragments. Biotechniques 17:754–761

Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellnhofer G, Hoess A, Wölle J, Plückthun A, Virnekäs B (2000) Fully synthetic human combinatorial antibody libraries (HuCAL), based on modular consensus frameworks and CDRs randomized with trinucleotides. J Mol Biol 296:57–86

Krebber A, Bornhauser S, Burmester J, Honegger A, Willuda J, Bosshard HR, Plückthun A (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J Immunol Methods 201:35–55

Kütemeier G, Harloff C, Mocikat R (1992) Rapid isolation of immunoglobulin variable genes from cell lysates of rat hybridomas by polymerase chain reaction. Hybridoma 11:23–32

Lee CM, Iorno N, Sierro F, Christ D (2007) Selection of human antibody fragments by phage display. Nat Protoc 2:3001–3008

Lefranc M, Lefranc G (2001) The Immunoglobulin FactsBook. Academic Press, London

Lindner P, Guth B, Wülfing C, Krebber C, Steipe B, Müller F, Plückthun A (1992) Purification of native proteins from the cytoplasm and periplasm of Escherichia coli using IMAC and histidine tails: a comparison of proteins and protocols. Methods 4:41–56

Lindner P, Bauer K, Krebber A, Nieba L, Kremmer E, Krebber C, Honegger A, Klinger B, Mocikat R, Plückthun A (1997) Specific detection of his-tagged proteins with recombinant anti-His tag scFv-phosphatase or scFv-phage fusions. Biotechniques 22:140–149

Luginbühl B, Kanyo Z, Jones RM, Fletterick RJ, Prusiner SB, Cohen FE, Williamson RA, Burton DR, Plückthun A (2006) Directed evolution of an anti-prion protein scFv fragment to an affinity of 1 pM and its structural interpretation. J Mol Biol 363:75–97

Mondon P, Dubreuil O, Bouayadi K, Kharrat H (2008) Human antibody libraries: a race to engineer and explore a larger diversity. Front Biosci 13:1117–1129

Morfill J, Blank K, Zahnd C, Luginbühl B, Kuhner F, Gottschalk KE, Plückthun A, Gaub HE (2007) Affinity-matured recombinant antibody fragments analyzed by single-molecule force spectroscopy. Biophys J 93:3583–3590

Munro S, Pelham HR (1986) An Hsp70-like protein in the ER: identity with the 78 kD glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46:291–300

Pack P, Kujau M, Schroeckh V, Knüpfer U, Wenderoth R, Riesenberg D, Plückthun A (1993) Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high cell density fermentation of Escherichia coli. Biotechnology (N Y) 11:1271–1277

Plückthun A, Pack P (1997) New protein engineering approaches to multivalent and bispecific antibody fragments. Immunotechnology 3:83–105

Plückthun A, Krebber A, Krebber C, Horn U, Knüpfer U, Wenderoth R, Nieba L, Proba K, Riesenberg D (1996) Producing antibodies in Eschericia coli: Fom PCR to fermentation. In: McCafferty J, Hoogenboom H (eds) Antibody engineering: a practical approach. IRL press, Oxford, pp 203–252

Sambrook J, Russell D (2001) Molecular cloning. A laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schaffitzel C, Zahnd C, Amstutz P, Luginbühl B, Plückthun A (2005) In vitro selection and evolution of protein-ligand interactions by ribosome display. In: Golemis E, Adams P (eds) Protein-protein interactions: a molecular cloning manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 517–548

Shuttleworth H, Taylor J, Minton N (1986) Sequence of the gene for alkaline phosphatase from Escherichia coli JM83. Nucleic Acids Res 14:8689

Tugarinov V, Zvi A, Levy R, Hayek Y, Matsushita S, Anglister J (2000) NMR structure of an anti-gp120 antibody complex with a V3 peptide reveals a surface important for co-receptor binding. Structure 8:385–395

Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, McCafferty J, Hodits RA, Wilton J, Johnson KS (1996) Human antibodies with sub-nanomolar affinities isolated from a large nonimmunized phage display library. Nat Biotechnol 14:309–314

Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR (1994) Making antibodies by phage display technology. Annu Rev Immunol 12:433–455

Wörn A, Plückthun A (2001) Stability engineering of antibody single-chain F_v fragments. J Mol Biol 305:989–1010

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Zahnd C, Spinelli S, Luginbühl B, Amstutz P, Cambillau C, Plückthun A (2004) Directed in vitro evolution and crystallographic analysis of a peptide binding scFv antibody with low picomolar affinity. J Biol Chem 279:18870–18877

Zahnd C, Amstutz P, Plückthun A (2007) Ribosome display: selecting and evolving proteins in vitro that specifically bind to a target. Nat Methods 4:269–279

Part II
Generation of Antibody Repertoires

Chapter 4
Mouse Immune Libraries for the Generation of ScFv Fragments Directed Against Human Cell Surface Antigens

Christian Kellner, Sahar Mohseni Nodehi, and Matthias Peipp

4.1 Introduction

In various applications and different settings, phage display has been demonstrated as a powerful technique in generating highly specific, high affinity binders to a variety of antigens. Antibody libraries have especially been used for the isolation of antibody fragments against potential target structures on the cell surface of different types of cancer (Winter et al. 1994; Hoogenboom 2005; Thie et al. 2008). The genes for the variable regions of antibodies (V-regions) from different mammalian species, such as mouse, rabbit, and human, served as sources for the generation of antibody phage display libraries. F(ab) as well as scFv libraries have been successfully screened and delivered valuable antibodies for many applications.

Independent from the source of V-regions and the antibody format, two general strategies have been pursued in generating antibody phage display libraries. Either nonimmune libraries such as the so-called naïve (or synthetic) libraries or repertoires from immunized animals (or patients) were generated. Most published naïve or synthetic libraries contain 10^9–10^{12} independent clones, making it difficult to maintain diversity during propagation in a routine laboratory (Nissim et al. 1994; Knappik et al. 2000). These highly complex libraries, in contrast to immune libraries, theoretically allow the isolation of antibody specificities against any given antigen from one library. However, screening these large libraries still does not guarantee the isolation of high affinity binders, and frequently additional in vitro affinity maturation steps have to be applied to obtain binders with sufficient affinities (Osbourn et al. 2005; Groves et al. 2006). Compared to naïve or synthetic libraries, immune libraries have the advantage that a smaller library size in terms of

C. Kellner, S.Mohseni Nodehi and M. Peipp (✉)
Division of Stem Cell Transplantation and Immunotherapy, 2nd Department of Medicine, Christian-Albrechts University, Kiel, Germany
e-mail: c.kellner@med2.uni-kiel.de; s.mohseni@med2.uni-kiel.de; m.peipp@med2.uni-kiel.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_4,

independent clones represented in the library is sufficient to isolate high affinity binders. It has been demonstrated that libraries with $5 \times 10^5 - 1 \times 10^6$ clones (Peipp et al. 2001; Schwemmlein et al. 2006) allowed the isolation of high affinity scFv fragments with equilibrium binding constants in the low nanomolar range. This is achieved primarily by consecutive rounds of immunization accompanied by affinity maturation by the murine immune system. In addition, a high frequency of antigen-specific B-cells, producing large amounts of antibody, and the corresponding RNA used for the generation of the library is likely to result in higher frequencies of phages with the desired binding specificity (Winter et al. 1994).

Immunized mice as a starting point for the generation of phage display libraries are an attractive approach because many classical immunization protocols using protein antigens as well as DNA immunization protocols are available, have been validated, and used successfully in combination with a phage display technique (Chowdhury et al. 1998; Peipp et al. 2001; Schwemmlein et al. 2006). Although mouse immune libraries have been used successfully in a variety of settings, this approach has several limitations. A prerequisite for success is the immunogenicity of the desired target antigen. If low or nonimmunogenic proteins are used, because of the high homology to endogenous host proteins, the likelihood in isolating high affinity binders is dramatically reduced. Moreover, when proteins are used for immunizations that are toxic to the host, this procedure is not applicable.

Another possible problem in using mouse immune libraries for the generation of antibody specificities for therapeutic applications is the potential immunogenicity associated with murine protein sequences in a clinical setting. Antibodies derived from libraries of human origin might reduce the immunogenic potential in humans, although this might not be a general rule. Adalimumab, an affinity maturated human IgG1 antibody with human V-regions derived by guided selection, is immunogenic in humans, whereas Rituximab, a chimeric IgG1 antibody with murine V-regions, demonstrated little immunogenicity (most likely due to its B-cell depleting activity, potentially preventing an immune response) (Hwang and Foote 2005). Therefore, murine V-regions may still represent valuable building blocks for therapeutic substances, depending on the final clinical setting.

In the following protocols, two general strategies to generate scFv fragments that recognize surface antigens in their native states using mouse immune libraries are described (Fig. 4.1).

For the isolation of scFv fragments directed against the extracellular domains of membrane receptors, an important point to consider is that the antigen used for immunization should resemble the properties of the targeted receptor as close as possible. Secondary protein modifications such as glycosylation might critically affect protein folding and accessibility of epitopes potentially recognized by isolated antibody fragments. Pure receptors in their native state can be obtained by direct purification from cell lines, but often these procedures are cumbersome and deliver only small amounts of protein. A promising strategy that has been described uses the extracellular domains of transmembrane receptors for the generation of Fc fusion proteins (Fig. 4.1; strategy 1). When expressed by secretion from

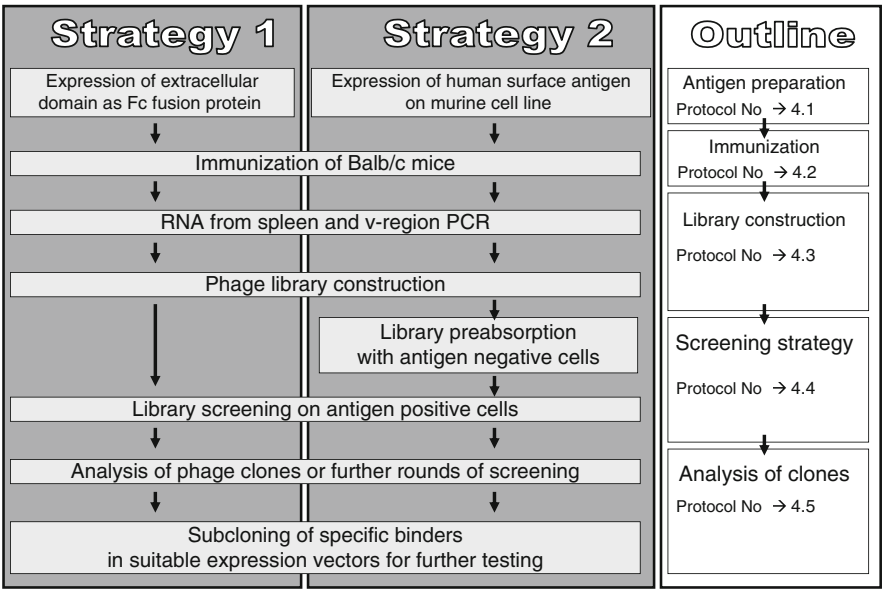


Fig. 4.1 Outline of the two strategies for the isolation of scFv fragments against human cell surface antigens

mammalian cell lines, these proteins usually are glycosylated and often closely resemble the native state of the receptor. This strategy is applicable to type I and type II membrane proteins (Peipp et al. 2001; Schwemmlein et al. 2006). In cases in which this approach is not applicable because of miss folding of the receptors´ extracellular domains as fusion proteins, an alternative strategy for the isolation of specific antibody fragments at high frequencies is outlined as strategy 2 (Fig. 4.1). The full-length cDNA for the desired human membrane receptor is transfected in a murine cell line, allowing receptor expression at the cell surface. Plasma membrane fractions are prepared under native conditions and used to immunize mice. By using this strategy, the human receptor should be preferentially recognized as foreign by the murine immune system, while most of the other proteins present in the membrane fraction should be less immunogenic due to their murine origin, leading to a preferential immune response against the desired human protein.

After several booster immunizations, affinity maturation and class switch during secondary immune responses should have led to an IgG immune response with high affinity antibodies. After test bleedings, the spleens of animals with high antibody titers are used for the generation of phage display libraries.

To further enhance the likelihood of isolating antibody fragments that bind to the membrane receptor in its natural environment and to ensure effective enrichment of the desired specific binders, a different source of target antigen should be used for the screening procedure compared to that used for immunization. Ideally, primary

human cells or tumor cell lines expressing the desired receptor at high levels should be used.

These two strategies were used successfully for the isolation of scFv fragments against several CD antigens (cluster of differentiation antigens), and they should, in theory, be applicable to most known human surface receptors that are immunogenic in mice.

4.2 Materials (Key Components)

Materials Used in: 4.3.1 Preparation of Antigen

- HEK293T (LGC Standards GmbH; Wesel; Germany);
- Culture medium: DMEM Glutamax-I / 10% FCS / 1% penicillin-streptomycin
- Fetal calf serum (FCS; Invitrogen); Optional: low IgG FCS (Invitrogen)
- 2× HBS buffer: 1.5 mM Na_2HPO_4/50 mM HEPES/280 mM NaCl; pH 7.05
- Chloroquine (Sigma-Aldrich)
- Dounce homogenizer pestle A (Wheaton)
- Protein A column (GE Healthcare)
- Protein A washing buffer: 100 mM Tris/HCl; pH 8
- Protein A elution buffer: 0.1 M glycine; pH 3.0
- HisTrap HP columns and buffers (GE Healthcare).

Materials used in: 4.3.2 Immunization of mice and test bleeding

- TiterMax Gold Adjuvant (Sigma-Aldrich).

Materials Used in: 4.3.3 cloning of VL- and VH-regions

- Trizol Reagent (Invitrogen)
- Oligo$(dT)_{15}$ primer (Roche)
- Superscript III reverse transcriptase (Invitrogen)
- Light and heavy chain V-region primers 100 μM (Krebber et al. 1997)
- Scfor and scback primers 100 μM (Krebber et al. 1997)
- pAK100 vector ((Krebber et al. 1997); kindly provided by Prof. A. Plückthun)
- peqGold Pwo polymerase (PeqLab)
- peqGold Taq polymerase (PeqLab)
- T4-DNA-Ligase (Roche)
- Electrocompetent *Escherichia coli* TG1 (Stratagene).

Materials Used in: 4.3.4 Propagation and Screening of the Library

- BstNI restriction enzyme (New England Biolabs)
- 2× YT medium (16 g Bacto Tryptone, 10 g yeast extract, 5 g NaCl; pH 7.2)
- SB medium (30 g Bacto Tryptone, 20 g yeast extract, 10 g MOPS; pH 7.0)
- E. coli TG1 (Stratagene)
- Helperphage M13KO7 (New England Biolabs).

Materials Used in: 3.5 Analysis of Isolated Phage Clones

- Anti-M13-HRP conjugate (GE Healthcare)
- ABTS ELISA substrate (Roche).

4.3 Protocols

4.3.1 Preparation of Antigen

4.3.1.1 Production of Fc Fusion Proteins by Transient Transfection of HEK-293T cells (Ca–Phosphate Transfection)

1. Seed 3–5 × 10^6 HEK-293T cells in 100 mm dishes and grow overnight.
2. After 24 h, remove medium and add 8 ml of prewarmed culture medium.
   Note: 50–70% confluent plates are optimal for transfection. Depending on the expressed protein, 30–50 plates usually represent a good starting point to generate enough recombinant protein for initial immunization steps.
3. Prepare DNA mix (n= number of tissue culture plates):

   (n+1) × 20 µg plasmid DNA in 895 µl sterile water.
   (n+1) × 100 µl 2.5 M $CaCl_2$.
   (n+1) × 5 µl 100 mM Chloroquine (final 50–100 µM).

4. Continuously bubble air through (n+1) × 1 ml 2×HBS buffer in a 15 or 50 ml Falcon tube and add the DNA/Ca/Chloroquine solution dropwise.
5. Vortex shortly and add 2 ml of the transfection solution dropwise to each tissue culture plate.
6. Incubate for 9–10 h in a humidified incubator at 37°C and 5–6% CO_2.
7. Change transfection media by prewarmed tissue culture media.
8. Collect supernatant every 24 h for 5–7 d.

Purification of Fc Fusion Proteins

Step 1: Protein A Purification

1. Add 1/10 vol of 1 M Tris/HCl pH 8.0 to collected supernatant.
2. Perform purification of Fc fusion proteins according to protocols described for human IgG1 antibodies ((Harlow and Lane 1988); buffer composition see above).
3. Neutralize elution fractions immediately by adding 1/5 vol 1 M Tris/HCl pH 8

Step 2: Ni-NTA Purification

1. Pool elution fractions (4-5 ml) isolated in step 1 and add His-Tag binding buffer to a final volume of 40 ml. Adjust to pH 7.4.
2. Perform purification according to manufacturers protocols (GE Healthcare).

3. Pool elution fractions and exchange elution buffer by PBS by dialysis.
4. Determine the protein concentration and store at 4°C.
   Note: The second purification step may not be necessary when using low IgG FCS, because usually bovine immunoglobulins are the sole major contaminants after protein A purification

4.3.1.2 Generation of Stably Transfected Murine Cell Lines Expressing Human Surface Receptors and Purification of Plasma Membrane Fractions

1. Use a suitable mammalian expression vector that drives receptor expression under the control of a strong promoter (e.g., pCDNA series of vectors; Invitrogen) and that carries a selectable marker for eukaryotic cells (geneticin or hygromycin) to transfect NIH/3T3 cells using standard procedures such as lipofection (e.g., Lipofectamine; Invitrogen or Superfect reagent; Qiagen). As an alternative, use the murine Ba/F3 cell line and perform transfection, using the Amaxa Transfection System according to the manufacturers´ protocols.
2. Check expression by flow cytometry or western blot analysis.
   Note: In case no commercial antibody is available that allows detection of expression, the receptor can be C-terminally tagged.
3. Use either high expressing cell clones isolated by limiting dilution or a mixed cell strain with good overall antigen expression for the purification of plasma membrane fractions.
4. Resuspend 5–10 $\times$ 10^8 cells in prechilled lysis buffer (1 mM $NaHCO_3$ pH 8.0, 1 mM MgCl, 100 mM Pefablock proteinase inhibitor (Roth, Karlsruhe, Germany)).
5. Incubate on ice for 15–20 min.
6. Disrupt cells in a dounce homogenizer. Check cell integrity by microscopy.
7. Clear the lysate by centrifugation.
8. Keep the supernatant and discard pellet (nuclei and non-disrupted cells).
9. Add succrose to a final concentration of 1.59 M.
10. Overlay 10-ml aliquots in SW28 disposable rotor tubes (Beckman, Munich, Germany) with 9 ml of 1.175 M sucrose in carbonate buffer (1 mM $NaHCO_3$; pH 8.0). Add a second layer of 7 ml of 0.98 M sucrose in carbonate buffer and a third layer of 2 ml of 0.8 M sucrose in carbonate buffer.
11. Perform gradient centrifugation for 16 h and 4°C at 70,000$\times$ g in an SW28 swing-out rotor.
12. Collect membrane fractions (visible as white bands).
13. Dilute collected fractions in at least five volumes of phosphate buffered saline (PBS), and sediment for 1 h and 4°C at 70,000$\times$ g
14. Resuspend membrane pellets in 100 µl of PBS.
15. Quantify protein concentration by the Lowry method with trichloric acid precipitation.

16. Identify antigen containing fractions by ELISA.
    Note: for more detailed information on preparation of plasma membrane fractions, refer to reference (Eylar and Hagopian 1971). Commercial kits are also available for subcellular fractionation.

4.3.2 Immunization of Mice and Test Bleeding

4.3.2.1 Immunization

1. Mix purified Fc-fusion proteins or membrane fractions with TiterMax Gold adjvant according to the manufacturers' instructions.
2. Inject groups of 5–10 mice per protein using varying amounts of antigen (see Table 4.1 for examples that have been used successfully).
3. Perform test bleeds at certain time points during the immunization procedure.
4. Perform booster immunizations (using smaller amounts of antigen) to provoke secondary immune responses and affinity maturation. (See Table 4.1 for details).

4.3.2.2 Test Bleeding and Serum Preparation to Analyze Antibody Titers

1. Draw test bleedings according to local regulations in animal care.
2. Incubate blood sample at 37°C for 1 h to allow agglutination.
3. Spin down in a microcentrifuge at max. speed for 10 min.

Table 4.1 Immunization schemes

A Immunization scheme: CD33-Fc fusion protein					
Day	Injection (route of application)	Mouse 1 (µg prot.)	Mouse 2 (µg prot.)	Mouse 3 (µg prot.)	Test bleeding
0	1. inject. (i.p.)	60	60	60	
26	1. boost (s.c.)	30	30	30	
41	2. boost (s.c.)	20	20	20	
63					all mice
75	3. final boost (i.p.)	40			
79		X			final bleed
84	3. final boost (i.p.)		40		
88			X		final bleed
126	3. final boost (i.p.)			40	
130				X	final bleed
B Immunization scheme: CD13 membrane fractions					
0	1. inject. (s.c.)	500	500	500	
25	1. boost (s.c.)	300	300	150	
45					all mice
49	2. boost (s.c.)	300	300	150	
57					all mice
60	3. final boost (i.p.)	150	150	150	
64		X	X	X	final bleed

4. Carefully remove the clear serum without disturbing cellular compounds and transfer to a sterile test tube.
5. Add sodium-azide to a final concentration of 0.02%.
6. Store at 4°C until use.
7. Test for the presence of antigen-specific antibodies by ELISA or flow cytometry using serial dilutions and IgG specific secondary antibodies.
   Note: after several booster immunizations, usually serum dilutions of 1:5,000–1:100,000 show specific signals 2–3 fold over background staining and indicate an efficient immune response.

4.3.3 Cloning of VL- and VH-Regions

4.3.3.1 RNA Isolation from Spleen

1. Cut spleen into pieces and homogenize in 5 ml of TRIZOL Reagent with a dounce homogenizer until spleen is completely dissolved.
2. Prepare total RNA according to the manufacturers' instructions. Yields of 0.2–1 mg total RNA per spleen can be expected.

4.3.3.2 Generation of First Strand cDNA by Reverse Transcription

1. Add 10–20 µg of total RNA and 1 µg oligo $(dT)_{15}$ primer to a sterile 1.5 ml tube and adjust the volume to 28 µl with nuclease-free water.
2. Incubate at 70°C for 10 min to resolve secondary structures of template RNA.
3. Cool down on ice and spin sample.
4. Add 2 µl RNAse inhibitor RNAsin (40 U/µl; Promega), 10 µl 5× Reverse Transcriptase buffer (provided with enzyme), 3 µl dNTP (25 mM each), 5 µl 0.1 M DTT, and nuclease-free water to a final volume of 48 µl.
5. Add 2 µl Reverse Transcriptase Superscript III (Invitrogen).
6. Incubate at room temperature for 10 min, then at 42°C for 50 min.
7. Terminate the reaction by incubating the sample at 90°C for 5 min, then at 4°C for 10 min.
8. Add 1 µl Rnase H (1 U/µl) and incubate the sample at 37°C for 20 min.

4.3.3.3 PCR Amplification of VL and VH Antibody Regions and Assembly of scFvs by Splicing by Overlap Extension PCR (SOE-PCR)

1. Prepare a 4:1 or 8:1 polymerase mix of peqGOLD Pwo-DNA-Polymerase and peqGOLD Taq-DNA-Polymerase.

2. Use the first-strand cDNA as template for the amplification of variable regions with published sets of primers specific for the VL and VH regions. Set up the following PCR reactions:

PCR mix for VL-regions	
cDNA	5 µl
10× Pwo complete reaction buffer	5 µl
dNTP (10 mM each)	1 µl
$MgSO_4$ (25 mM)	4 µl
DMSO	2 µl
Light chain Back primer mix (100 µM)	1 µl
Light chain Forward primer mix (100 µM)	1 µl
Polymerase mix	2.5 µl
H_2O	28.5 µl
PCR mix for VH-regions	
cDNA	5 µl
10× Pwo complete reaction buffer	5 µl
dNTP (10 mM each)	1 µl
$MgSO_4$ (25 mM)	4 µl
DMSO	2 µl
Heavy chain Back primer mix (100 µM)	1 µl
Heavy chain Forward primer mix (100 µM)	1 µl
Polymerase mix	2.5 µl
H_2O	28.5 µl

3. Run PCR program:

   1× 5 min, 92°C
   7×: 1 min, 92°C/30 s, 63°C/50 s, 58°C/1 min, 72°C
   23×: 1 min, 92°C/1 min, 63°C/1 min, 72°C

4. Purify the PCR products for VL and VH PCR-products by preparative gel electrophoresis and determine their concentrations.
5. Assemble scFvs by splicing by overlap extension (SOE) PCR using published primer sets (Krebber et al. 1997).

SOE PCR mix:	
VL PCR product	10 ng
VH PCR product	10 ng
10× Pwo complete reaction buffer	5 µl
dNTP (10 mM each)	1 µl
scfor (50 µM)	1 µl
scback (50 µM)	1 µl
Polymerase mix	1 µl
H_2O	ad 50 µl

6. Run PCR program:

   1×: 3 min, 92°C
   7×: 1 min, 92°C/30 s, 63°C/50 s, 58°C/1 min, 72°C
   23×: 1 min, 92°C/30 s, 63°C/1 min, 72°C

7. Gel-purify the scFv PCR products.
8. Extract DNA using a gel elution kit (e.g., Qiagen; use 50 µl sterile water for elution).

4.3.3.4 Ligation of scFv cDNA Sequences into the Vector pAK100

1. Set up restriction digest for the scFv PCR product. Add 1-2 µl SfiI, 6 µl of 10× enzyme buffer, 0.6 µl 100× BSA, and 2.4 µl H_2O to the 50 µl of the purified PCR product.
2. Digest 1–2 µg of pAK100 vector with SfiI in 30 µl volume.
3. Overlay both samples with mineral oil and incubate the digests for at least 3 h in a water bath at 50°C (alternatively incubate reactions overnight).
4. Gel-purify the digested vector and the scFv. Analyze 1/10 volume of both purified DNA fragments by gel electrophoresis and determine the concentrations.
5. Ligate 50–100 ng vector and the digested scFv fragments at a vector to insert ratio of 1:3 with 1 U T4-DNA-ligase (Roche) in a volume of 20 µl. Incubate the reactions overnight at 4°C.
   Note: Variations in vector to target ratios might lead to higher ligation frequency. Ratios from 1.5:1 to 1:3 could be tested. About 1×10^6–5×10^6 independent clones can be expected per 1 µg of digested Vector.
6. Inactivate ligase according to the manufacturers´ instructions.
7. Desalt ligation reaction by n-butanol precipitation and resolve the pelleted DNA with 5 µl of H_2O (alternatively use Amicon desalting columns).
8. Transform 2× 2.5 µl of the ligation into competent E.coli XL1-Blue (Stratagene) cells by electroporation, outgrow at 37°C for 1–2 h and plate the bacteria on 2×YT, 1% glucose and 30 µg/ml chloramphenicol agar plates. Incubate overnight at 30°C. (Prepare serial dilutions of a small aliquot and spread out on titration plates).
9. Analyze the proportion of clones carrying inserts with SfiI control digests and analyze diversity by BstNI fingerprinting (see protocol 4.3.3.5).
10. Wash colonies from agar plates with freezing media (2×YT containing 15–20% glycerol).
11. Pellet bacteria by centrifugation, resuspend bacteria in a small volume of freezing media, and shock frost with liquid nitrogen.
12. Store library at −80°C until use.

4.3.3.5 PCR-Screening and BstNI Fingerprint (Analysis of Library Diversity and Clone Diversity after Individual Screening Rounds)

1. For screening purposes a PCR master mix contains:

   (n+1)× 14.8 µl sterile water
   (n+1)× 2.0 µl 10× PCR buffer

(n+1)× 1.0 µl dNTPs (10 mM; each)
(n+1)× 1.0 µl scFor primer (at 10 pmol/µl)
(n+1)× 1.0 µl scBack primer (at 10 pmol/µl)
(n+1)× 0.2 µl Taq polymerase (0.5–1 unit)

2. Aliquot 20 µl into appropriate PCR tubes
3. Pick individual colonies from titer plates using sterile toothpicks or pipette tips
4. Carefully wash tip or toothpick into individual PCR tubes
5. Tip or toothpick may be transferred into appropriate test tube with 2x YT medium for reamplification and phage preparation.
6. Place tubes in an appropriate thermo cycler
7. Cycler program:

   1×: 10 min, 94°C
   30×: 1 min, 94°C/1 min, 55°C/1 min, 72°C

8. PCR reactions may be analyzed by gel electrophoresis to screen for clones with inserts or directly move to step 9.
9. For BstNI fingerprinting, prepare a restriction enzyme mix of the following:

   (n+1)× 17.5 µl sterile water
   (n+1)× 2.0 µl NEB buffer 2
   (n+1)× 0.5 µl BstNI (10u/µl)

10. Add 20 µl of the mix to each PCR reaction
11. Incubate at 60°C for 2– 4 h (alternatively: overnight)
12. Analyze on a 2–3% agarose gel.

4.3.4 Propagation and Screening of the Library

4.3.4.1 Library Propagation and Preparation of Phages

1. Inoculate a small fraction of the library in 500 ml SB medium (1% glucose, 30 µg/ml chloramphenicol) (adjust OD_{600} to < 0.1) and grow at 37°C until OD_{600} = 0.5–1.0 is reached.
2. Infect 50 ml of bacteria culture with M13KO7 helper phages at a 20-fold excess over the bacteria. Add IPTG to a final concentration of 0.5 mM and incubate at 37°C without shaking for 30 min.
3. Add 500 ml SB medium (1% glucose; 30 µg/ml chloramphenicol; 0.5 mM IPTG;) and grow for 1–2 h 37°C.
4. Add kanamycin (final concentration: 25 µg/ml) and grow at 30° overnight.
5. Pellet bacteria from overnight culture by centrifugation. Transfer the supernatant to an appropriate centrifuge tube and add ¼ volume 20% PEG 6,000/2.5 M NaCl.
6. Mix gently and incubate on ice for 30 min.

7. Centrifugate for 20 min 9,000 rpm 4°C (JA-14 Rotor). Discard the supernatant and air dry the pellet for 5–10 min at room temperature.
8. Resuspend the pellet in 2 ml TBS or PBS.
9. Spin down residual bacterial debris in a table top centrifuge for 3 min and transfer the supernatant into a new reaction tube.
10. Titrate phage library and store at 4°C until use (for prolonged storage add sodium azide at a final concentration of 0.02%).

4.3.4.2 Screening the Library for Binders on Transfected Cells or Tumor Cells

Strategy 1: Start with step 5.
Strategy 2: Preabsorption of the phage library on irrelevant cell line to remove phage clones directed against nondesired antigens present in the membrane fraction used for immunization.

1. Incubate 1–2 $\times$ 10^7 nontransfected cells in 1–2 ml of blocking buffer (4% nonfat dry milk in PBS) for 1 h at room temperature (with slow agitation).
2. Add 50–500 µl phage library (~1 $\times$ 10^{12} – 1$\times$ 10^{13} phage particles) and incubate at room temperature for 1–2 h.
3. Pellet cells by centrifugation in a table top centrifuge.
4. Transfer supernatant to a test tube.
5. Suspend 1–2 $\times$ 10^6 antigen-positive cells in 500 µl blocking buffer (4% nonfat dry milk in PBS) and incubate at room temperature for 30 min with gentle shaking.
6. Add 100–500 µl of phage library or preabsorbed library and incubate 1.5–2 h at room temperature with gentle shaking.
7. Wash cells 10 times with 5 ml washing buffer (2% nonfat dry milk in PBS) and two times with 5 ml PBS and pellet cells by centrifugation.
8. Add 1.5 ml 50 mM HCl (alternatively 100 mM Triethylamine could be used) and incubate the cells for 10 min at room temperature with gentle shaking for elution of cell-bound phages.
9. Neutralize with 0.5 ml 1 M Tris pH 7.5 and pellet cells/cellular debris by centrifugation.
10. Transfer eluted phages to a new reaction tube.
11. Infect 8–10 ml exponentially growing E.coli TG1 cells with 1 ml of eluted phages for 30 min at 37°C without shaking.
    Note: Infected bacteria might be plated on agar plates (30 µg/ml chloramphenicol; 1% glucose) and incubated at 30°C overnight. For further rounds of panning, the colonies can be washed from agar plates, as described for the original library. This procedure might prevent the loss of rare clones with slower growth rates.
12. Add 20 ml 2xYT (1% glucose, 30 µg/ml chloramphenicol) and grow bacteria at 37°C for 2 h.

13. Add 1 × 10^{12} pfu of helper phage and IPTG to a final concentration of 0.5 mM and incubate 30 min at 37°C without shaking.
14. Add 100 ml 2xYT Medium (30 μg/ml chloramphenicol; 0.5 mM IPTG; 1% glucose) and incubate 1 h at 37°C with shaking.
15. Add kanamycin to a final concentration of 25 μg/ml and shake overnight at 30°C. Continue with preparation of phages as described (above) and repeat the screening procedure two more times.
16. Titrate phage particles.

4.3.5 Analysis of Isolated Phage Clones

4.3.5.1 BstNI Fingerprint

→ See protocol no. 4.3.3.5.

4.3.5.2 Analysis: Whole Cell Phage ELISA

1. Aliquot 0.5 × 10^{6} antigen-positive cells (150 μl 2% non-fat dry milk in PBS) per well in a 96-well plate and incubate at room temperature for 30 min with gentle shaking.
2. Centrifugate (3 min 1,800 rpm in a Megafuge). Discard the supernatant.
3. Add 1 × 10^{11}–1 × 10^{12} phages in 50 μl of PBS containing 2% nonfat dry milk to each well. Resuspend the cells and incubate for 1 h at room temperature.
4. Wash 5–10 times with 0.1% nonfat dry milk in PBS and 2 times with PBS.
5. Add 50 μl of HRP-conjugated anti-M13 antibody (GE Healthcare) at a dilution of 1:2,000 in 1% nonfat dry milk in PBS. Incubate at room temperature for 1 h with gentle shaking.
6. Wash three times with 0.1% dry milk powder in PBS and two times with PBS.
7. Add 60–80 μl ABTS-solution
8. Add stop solution when a good signal to background staining is detectable (usually 5–30 min).

4.4 Results

For the preparation of antigen used for immunization, the extracellular domains of type I or type II transmembrane receptors were cloned into plasmid vectors, allowing expression in fusion with IgG1-Fc (Fig. 4.2).

The resulting expression vectors were transiently transfected in 293T cells by the calcium phosphate method. Collected supernatant were applied to protein

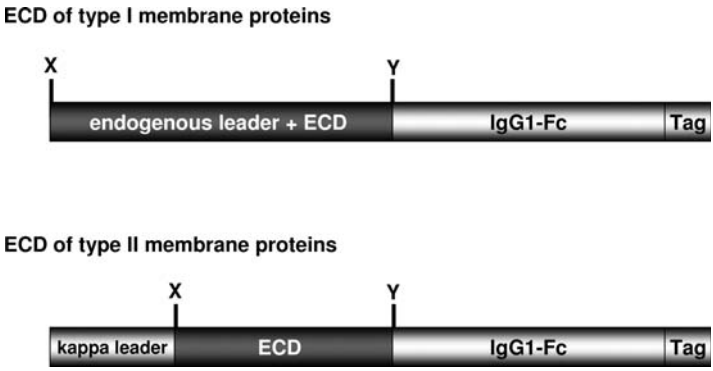


Fig. 4.2 Cloning scheme of ECD-Fc fusion proteins. ECDs from type I transmembrane proteins usually are expressed well with the endogenous secretion leader, while ECDs from type II membrane receptors are fused to a murine kappa chain leader sequence (pSEC-Tag2-Hygro vectors; Invitrogen) and the stop codon is removed during the cloning process to allow in frame fusion of the IgG1-Fc part. X,Y = appropriate restriction sites; Tag = myc-his tag; ECD = extracellular domain

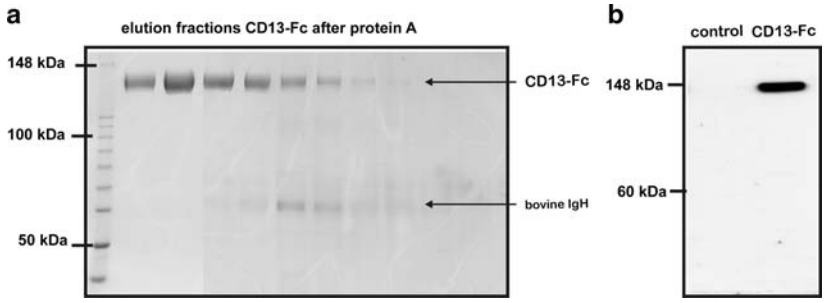


Fig. 4.3 Purification of Fc fusion proteins. CD13-Fc was transiently expressed in 293T cells and supernatants were collected for 5 days. Low IgG FCS was used for the preparation of culture medium. (**a**) Affinity chromatography was performed using a protein A column. Elution fractions (peak fractions) were analyzed by SDS-PAGE and choomassie staining. (**b**) Western blot analysis using a human Fc specific HRP-conjugated antibody further demonstrated that antigen Fc-fusion protein was isolated

A chromatography and elution fractions are analyzed by coomassie blue staining. A typical result for CD13, a type II transmembrane protein is shown in Fig. 4.3.

Mice were immunized with recombinant protein or membrane fractions mixed with TiterMax Gold adjuvant. After several booster immunizations (see Table 4.1) mice were sacrificed and total RNA from spleen was prepared. From cDNA, the VL and VH regions were amplified (Fig. 4.4a) and used for the assembly of scFvs by SOE-PCR (Fig. 4.4b). Results from mouse 5 and 7 are shown. Some nonspecific amplimers might be visible at 500–600 bp.

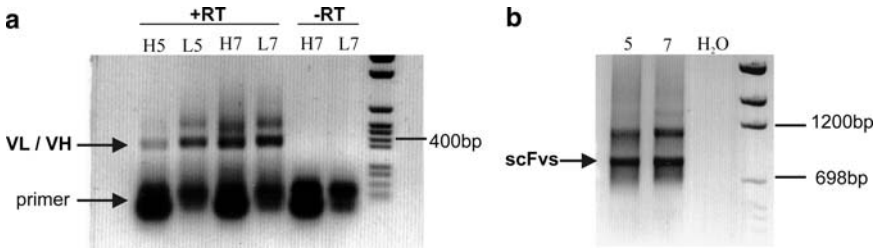


Fig. 4.4 Amplification of VL and VH regions and SOE-PCR**.** Total RNA from spleen of immunized mice (mouse 5 and 7) was used for reverse transcription. (**a**) cDNA was used as template for PCR amplification of the VL and VH repertoire. The Size of VL and VH regions is 380–400 bp (**b**) After gel purification VL and VH fragments were used in SOE-PCR for random assembly of VL and VH fragments in scFv fragments of approx. 800 bp

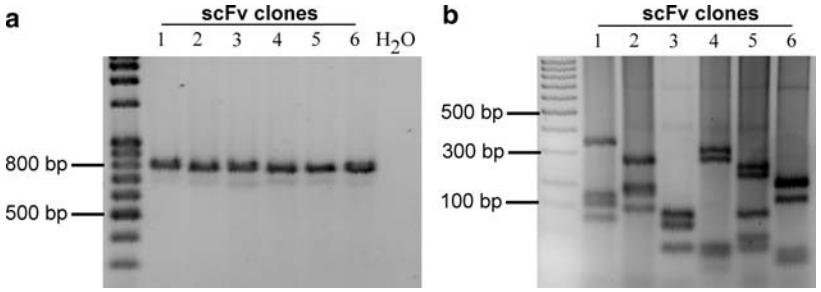


Fig. 4.5 DNA fingerprint analysis of individual scFv clones. (a) The coding sequences for six different scFv clones were amplified by PCR with primers scfor and scback, resulting in fragments of approx. 800 bp. (**b**) The PCR-products were digested with BstNI and analyzed on a 2.5% agarose gel

The scFv phage display library can be analyzed by DNA fingerprint analysis. The PCR amplified scFv inserts from different clones were analyzed with the restriction enzyme BstNI (Fig. 4.5). Because individual scFv clones are characterized by a unique banding pattern, this technique allows to discriminate between different scFv clones and to estimate the diversity of the library. DNA fingerprint analysis is also an easy method to monitor the enrichment of individual clones during subsequent rounds of panning and to identify different scFv clones after the final round.

Panning was performed with antigen-positive tumor cells, and after three consecutive rounds of screening, polyclonal phage preparations were tested in whole cell ELISA experiments using an equal number of phage particles from every phage preparation. Figure 4.6 illustrates a typical result from a whole cell ELISA experiment with polyclonal phage preparations on antigen-positive and -negative cells.

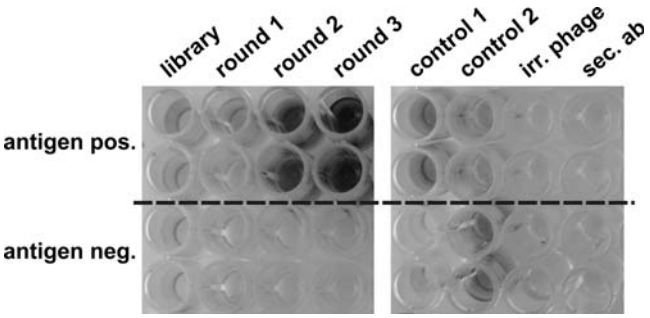


Fig. 4.6 Whole cell ELISA after three rounds of panning on antigen pos. cells. After three consecutive panning cycles 1×10^{12} phage particles were used in whole cell ELISA. 1×10^{6} Antigen-positive or antigen-negative cells were blocked and stained with polyclonal phages from every panning cycle. Bound phages were detected using HRP-conjugated anti-M13 antibodies. The ELISA was developed by adding ABTS and 15–30 min incubation at room temperature. Control 1 = control antibody against surface antigen on antigen-positive cells; control 2 = antibody preferentially recognizing antigen-negative cells; irr. phage = control phage that does not bind to either cell line; sec. ab. = HRP anti-M13 conjugate

References

Chowdhury PS, Viner JL, Beers R, Pastan I (1998) Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. Proc Natl Acad Sci USA 95:669–674

Eylar E, Hagopian A (1971) Isolation of plasma membranes from mammalian cells. In: Jakoby WB (ed) Methods in enzymology, vol XXII. Academic, London

Groves M, Lane S, Douthwaite J, Lowne D, Rees DG, Edwards B, Jackson RH (2006) Affinity maturation of phage display antibody populations using ribosome display. J Immunol Methods 313:129–139

Harlow E, Lane D (1988) Antibodies – A laboratory manual. Cold Spring Harbor Laboratory Press, New York

Hoogenboom HR (2005) Selecting and screening recombinant antibody libraries. Nat Biotechnol 23:1105–1116

Hwang WY, Foote J (2005) Immunogenicity of engineered antibodies. Methods 36:3–10

Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellnhofer G, Hoess A, Wolle J, Pluckthun A, Virnekas B (2000) Fully synthetic human combinatorial antibody libraries (HuCAL), based on modular consensus frameworks and CDRs randomized with trinucleotides. J Mol Biol 296:57–86

Krebber A, Bornhauser S, Burmester J, Honegger A, Willuda J, Bosshard HR, Pluckthun A (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J Immunol Methods 201:35–55

Nissim A, Hoogenboom HR, Tomlinson IM, Flynn G, Midgley C, Lane D, Winter G (1994) Antibody fragments from a 'single pot' phage display library as immunochemical reagents. EMBO J 13:692–698

Osbourn J, Groves M, Vaughan T (2005) From rodent reagents to human therapeutics using antibody guided selection. Methods 36:61–68

Peipp M, Simon N, Loichinger A, Baum W, Mahr K, Zunino SJ, Fey GH (2001) An improved procedure for the generation of recombinant single-chain Fv antibody fragments reacting with human CD13 on intact cells. J Immunol Methods 251:161–176

Schwemmlein M, Peipp M, Barbin K, Saul D, Stockmeyer B, Repp R, Birkmann J, Oduncu F, Emmerich B, Fey GH (2006) A CD33-specific single-chain immunotoxin mediates potent apoptosis of cultured human myeloid leukaemia cells. Br J Haematol 133:141–151

Thie H, Meyer T, Schirrmann T, Hust M, Dübel S (2008) Phage display derived therapeutic antibodies. Curr Pharm Biotechnol 9:439–446

Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR (1994) Making antibodies by phage display technology. Annu Rev Immunol 12:433–455

Chapter 5
Human Antibody Gene Libraries

Michael Hust and Stefan Dübel

5.1 Introduction

Antibodies are valuable tools for basic research (Konthur et al. 2005), diagnostics (M. D. Sørensen et al. 2006), and therapy (Dübel 2007). For more than 100 years, polyclonal antisera have been produced by immunization of animals (von Behring and Kitasato 1890) With the advent of hybridoma technology, it was possible to produce a monoclonal antibody of a defined antigen specificity (Köhler and Milstein 1975) in mice and rats, but only very few human antibodies were generated.

Currently, two alternative strategies are used for the generation of human antibodies: transgenic mice and *in vitro* selection technologies. Transgenic mice contain the human immunoglobulin gene repertoire instead of the murine, allowing the generation of human antibodies by hybridoma technology (Fishwild et al. 1996; Lonberg and Huszar 1995; Jakobovits 1995). An advantage of transgenic mice is the *in vivo* affinity maturation after immunization. Transgenic mice already yielded a significant number of antibodies under clinical evaluation. As immunization is required, this method is limited in respect of toxic and highly conserved antigens (Winter and Milstein 1991).

The alternative is the generation of human antibodies by antibody phage display done completely independent from any immune system by an *in vitro* selection process called "panning." The first antibody gene repertoires in phage were generated and screened by using the lytic phage Lambda (Huse et al. 1989), however, with limited success. The display method most commonly used today is based on the groundbreaking work of Georg P. Smith (1985) on filamentous phage display. Here, the genotype and phenotype of oligo-peptides were linked by fusing the corresponding gene fragments to the minor coat protein III gene of the filamentous

M. Hust (✉) and S. Dübel
Abteilung Biotechnologie, Institut für Biochemie und Biotechnologie, Technische Universität Braunschweig, Spielmannstr. 7, Braunschweig 38106, Germany
e-mail: m.hust@tu-bs.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_5,

bacteriophage M13. The resulting peptide::pIII fusion protein is expressed on the surface of the phage, allowing the affinity purification of the peptide together with its corresponding gene. In the same way, antibody fragments fused to pIII can be presented on the surface of M13 phage (Breitling et al. 1991; Clackson et al. 1991; Hoogenboom et al. 1991; Marks et al. 1991; Barbas et al. 1991; McCafferty et al. 1990). Because of limitations of the *Escherichia coli* folding machinery, complete IgG molecules can only be produced or displayed on the surface of phage in rare cases (Mazor et al. 2007; Simmons et al. 2002). Mainly, smaller antibody fragments are used for antibody phage display: the Fab fragment or the single chain Fv fragment (scFv). Fab fragments consist of two chains, the variable (V_H) and first constant region of the heavy chain (CH1) and the light chain (LC) of the antibody, both linked by a disulphide bond. In contrast, scFv fragments consist of only one polypeptide chain, composed of the variable region of the the heavy chain (VH) and the variable region of the light chain (VL) fused by a short peptide linker. Two different genetic systems have been developed for the expression of the antibody:: pIII fusion proteins for phage display. First, the antibody genes can be directly inserted into the phage genome, fused to the wildtype pIII gene (McCafferty et al. 1990). However, most of the successful systems uncouple antibody expression from phage propagation by providing the genes encoding the antibody::pIII fusion proteins on a separate plasmid (called "phagemid"), containing a phage morphogenetic signal for packaging the vector into the assembled phage particles (Breitling et al. 1991; Clackson et al. 1991; Barbas et al. 1991; Hoogenboom et al. 1991; Marks et al. 1991). The affinity of antibodies selected by phage display can be increased by additional *in vitro* affinity maturation steps (Thie et al. 2009). Despite other in vitro methods such as ribosomal display (Hanes and Plückthun 1997; He and Taussig 1997), puromycin display (Roberts and Szostak 1997), or yeast surface display (Boder and Wittrup 1997), antibody phage display today is the most widely used selection method for human antibodies. An overview about phage display derived human therapeutic antibodies in the clinical development is given by Thie et al. (2008).

Depending on the scientific or medical applications, different types of antibody gene libraries can be constructed and used. Immune libraries are constructed from antibody V-genes isolated from IgG secreting plasma cells of immunized donors (Clackson et al. 1991; Pelat et al. 2007). Immune libraries are typically generated and used in medical research to get an antibody against one particular target antigen, e.g., of an infectious pathogen or a tumor marker. Alternatively, naive, semi-synthetic, and synthetic libraries have been subsumed as "single-pot" or "universal" libraries, as they are designed to isolate antibody fragments binding to every possible antigen, at least in theory. Naive libraries are constructed from rearranged V genes from B cells (IgM) of nonimmunized donors. An example for this library type is the naive human Fab library constructed by de Haard et al. (1999). Semisynthetic libraries are derived from unrearranged V genes from pre B cells (germline cells) or from one antibody framework with genetically randomized complementary determining region (CDR) 3 regions, as described by Pini et al. (1998). A combination of naive and synthetic repertoire was used by Hoet et al. (2005). They combined

light chains from autoimmune patients with an fd fragment, containing synthetic CDR1 and CDR2 in the human VH3-23 framework and naive CDR3 regions originated from autoimmune patients. The fully synthetic libraries have a human framework with randomly integrated CDR cassettes (Hayashi et al. 1994; Knappik et al. 2000). All library types – immune, naïve, synthetic, and their intermediates – are valuable sources for the selection of antibodies for diagnostic and therapeutic purposes. To date, "single-pot" antibody libraries with a theoretical diversity of up to 10^{11} independent clones have been generated (Sblattero and Bradbury 2000) to serve as a molecular repertoire for phage display selection procedures. An overview of antibody gene libraries is given by Hust and Dübel (2004, 2005), Hust et al. (2007a).

Various methods have been employed to clone the genetic diversity of antibody repertoires. After isolation of mRNA from B-lymphocytes and preparation of cDNA, the construction of immune libraries is usually done by a two-step cloning or assembly PCR (see below). Very large "single pot" naïve antibody gene libraries are generally constructed by two or three separate cloning steps. In the two-step cloning strategy, the amplified repertoire of light chain genes is cloned into the phage display vector first. Since the heavy chain contributes more to diversity, because of its highly variable CDRH3, the heavy chain gene repertoire is cloned in the second step into the phagemids containing the light chain gene repertoire (Welschof et al. 1997; Johansen et al. 1995; Little et al. 1999; Pelat et al. 2007; Kirsch et al. 2008). In the three-step cloning strategy, separate heavy and light chain libraries are engineered. The V_H gene repertoire has then to be excised and cloned into the phage display vector containing the repertoire of VL genes (de Haard et al. 1999). Another common method used for the cloning of naïve (McCafferty et al. 1994; Vaughan et al. 1996), immune (Clackson et al. 1991), or hybridoma (Krebber et al. 1997) scFv phage display libraries is the assembly PCR. The VH and VL genes, including the additional linker sequence are amplified separately and fused by assembly PCR, before the scFv encoding gene fragments are cloned into the vector. Since the CDRH3 is a major source of sequence variety in antibodies (Shirai et al. 1999), the assembly PCR can be combined with a randomization of the CDR3 regions, leading to semi-synthetic libraries. Here, oligonucleotide primers encoding various CDR3 and J gene segments were used for the amplication of the V gene segments from the human germline (Akamatsu et al. 1993). Hoogenboom and Winter (1992) as well as Nissim et al. (1994) used degenerated CDRH3 oligonucleotide primers to generate a semi-synthetic heavy chain repertoire derived from human V gene germline segments. Afterwards, this VH repertoire was combined with an anti-BSA light chain. In some cases, a framework of a well known/robust antibody was used as scaffold for the integration of randomly created CDRH3 and CDRL3 (Desiderio et al. 2001; Barbas et al. 1992). Jirholt et al. (1998) and Söderlind et al. (2000) amplified all CDR regions derived from B cells before shuffling them into this antibody framework by assembly PCR. An entirely synthetic library was described by Knappik et al. (2000), who utilized seven different VH and VL germline master frameworks combined with six synthetically created CDR cassettes. The construction of large naïve and semi-synthetic libraries (Hust

et al. 2007b; Hoet et al. 2005; Løset et al. 2005; Little et al. 1999; Sheets et al. 1998; Vaughan et al. 1996) requires significant effort to tunnel the genetic diversity through the bottleneck of *E. coli* transformation, e.g., 600 transformations were necessary for the generation of a 3.5×10^{10} phage library (Hoet et al. 2005).

To overcome the bottleneck of *E. coli* transformation, the Cre-lox or lambda phage recombination system has also been employed for library generation (Waterhouse et al. 1993; Griffiths et al. 1994; Geoffroy et al. 1994; Sblattero and Bradbury 2000). However, since libraries with more than 10^{10} independent clones have also been accomplished by conventional transformation, most of these complicated methods are rendered unnecessary, particularly as they may result in decreased genetic stability.

In summary, antibodies can be selected from either type of library, naive, or synthetic. If the assembly by cloning or PCR and preservation of molecular complexity is carefully controlled at every step of its construction, libraries of more than 10^{10} independent clones can be generated.

5.2 Outline

The methods describe the generation of human naïve or immune scFv antibody gene libraries by a two-step cloning strategy already proven for different naïve (Hust et al. 2007a, b) and immune libraries (Pelat et al. 2007; Kirsch et al. 2008). In a first step, lymphocytes will be prepared for mRNA or total RNA isolation. The isolated mRNA will be reverse transcribed into cDNA. A set of human antibody gene oligonucleotide primers will be used for the amplification of VH and light chains by PCR. In a second PCR, restriction sites for the cloning in pHAL14 will be added. The cloning will be performed in two steps, first VL will be cloned, followed by cloning of VH. In the end, the antibody gene libraries will be packaged using a helperphage and analyzed by immunoblot. The schema of the total procedure is shown in Fig. 5.1.

5.3 Materials

5.3.1 Isolation of Lymphocytes

- Phosphate buffered saline (PBS) pH 7.4 (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4*$2H_2O$, 0.24 g KH_2PO_4 in 1 L)
- Lymphoprep (Progen, Heidelberg)
- mRNA isolation Kit (QuickPrep micro mRNA Purification Kit, GE Healthcare, Munich) or Trizol (Invitrogen, Karlsruhe) for total RNA.

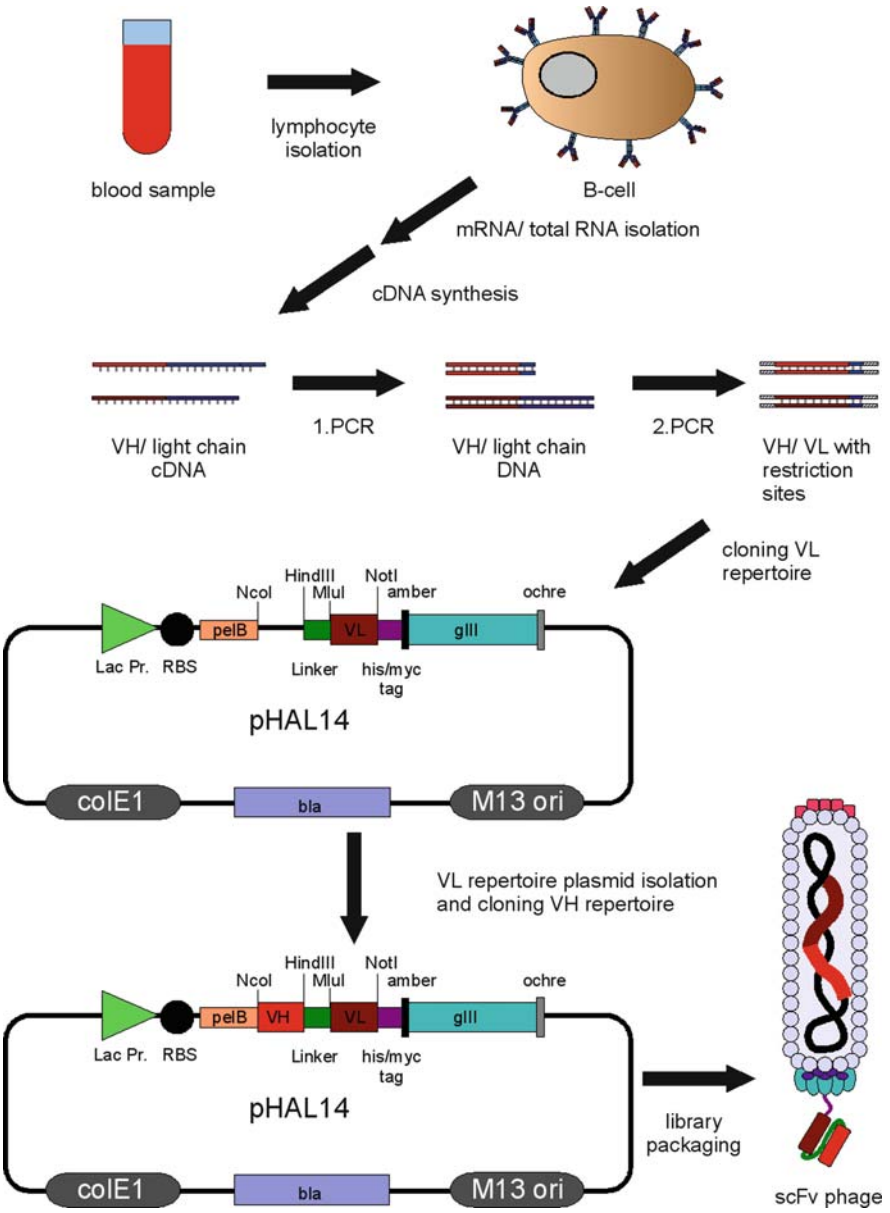


Fig. 5.1 Schematic overview about of the construction of antibody gene libraries

5.3.2 cDNA Synthesis

- Superscript II (Invitrogen) + 5 × RT buffer + 0.1 m DTT
- Random hexamer oligonucleotide primer (dN_6)
- dNTP mix (2.5 mM each).

5.3.3 First and Second Antibody Gene PCR

- Red Taq (Sigma, Hamburg) + 10 × buffer
- dNTP mix (10 mM each)
- Oligonucleotide primer (see Table 5.1)
- Agarose (Serva, Heidelberg)
- TAE-buffer 50 × (2 M TrisHCl, 1 M acetic acid, 0.05 M EDTA pH 8)
- Nucleospin Extract 2 Kit (Macherey-Nagel, Düren).

5.3.4 First Cloning Step: VL

- MluI (NEB, Frankfurt)
- NotI (NEB)
- Buffer 3 (NEB)
- BSA (NEB)
- Calf intestine phosphatase (CIP) (MBI Fermentas, St. Leon-Rot)
- T4 ligase (Promega, Mannheim)
- 3M sodium acetate pH5.2
- *E. coli* XL1-Blue MRF'(Stratagene, Amsterdam), genotype: Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *pro*AB *lac*I^qZΔM15 Tn10 (Tet^r)]
- Electroporator MicroPulser (BIO-RAD, München)
- 2 M Glucose (sterile filtered)
- 2 M Magnesium solution (1 M MgCl, 1 M $MgSO_4$) (autoclaved)
- SOC medium pH 7.0 (2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.05% (w/v) NaCl, 20 mM Mg solution, 20 mM glucose) (sterilize magnesium and glucose separately, and add solutions after autoclavation)
- 2xYT-medium pH 7,0 (1.6% (w/v) Tryptone, 1% (w/v) Hefe Extrakt, 0.5% (w/v) NaCl)
- 2xYT-GAT (2xYT + 100 mM Glucose + 100 μg/mL ampiciline + 20 μg/mL tetracycline)
- Ampiciline (100 mg/mL stock)
- Tetracycline (10 mg/mL stock)
- 9 cm Petri dishes
- 25 × 25 cm square Petri dishes ("pizza plates")

Table 5.1 Primers used for first and second PCR of antibody genes for antibody gene library construction using phagemids like pHAL14. Restriction sites are underlined

Primer	5′ to 3′ Sequence
First antibody gene PCR VH	
MHVH1_f	cag gtb cag ctg gtg cag tct gg
MHVH1/7_f	car rts cag ctg gtr car tct gg
MHVH2_f	cag rtc acc ttg aag gag tct gg
MHVH3_f1	sar gtg cag ctg gtg gag tct gg
MHVH3_f2	gag gtg cag ctg ktg gag wcy sg
MHVH4_f1	cag gtg car ctg cag gag tcg gg
MHVH4_f2	cag stg cag ctr cag sag tss gg
MHVH5_f	gar gtg cag ctg gtg cag tct gg
MHVH6_f	cag gta cag ctg cag cag tca gg
MHIgMCH1_r	aag ggt tgg ggc gga tgc act
MHIgGCH1_r	gac cga tgg gcc ctt ggt gga
First antibody gene PCR kappa	
MHVK1_f1	gac atc cag atg acc cag tct cc
MHVK1_f2	gmc atc crg wtg acc cag tct cc
MHVK2_f	gat rtt gtg atg acy cag wct cc
MHVK3_f	gaa atw gtg wtg acr cag tct cc
MHVK4_f	gac atc gtg atg acc cag tct cc
MHVK5_f	gaa acg aca ctc acg cag tct cc
MHVK6_f	gaw rtt gtg mtg acw cag tct cc
MHkappaCL_r	aca ctc tcc cct gtt gaa gct ctt
First antibody gene PCR lambda	
MHVL1_f1	cag tct gtg ctg act cag cca cc
MHVL1_f2	cag tct gtg ytg acg cag ccg cc
MHVL2_f	cag tct gcc ctg act cag cct
MHVL3_f1	tcc tat gwg ctg acw cag cca cc
MHVL3_f2	tct tct gag ctg act cag gac cc
MHVL4_f1	ctg cct gtg ctg act cag ccc
MHVL4_f2	cag cyt gtg ctg act caa tcr yc
MHVL5_f	cag sct gtg ctg act cag cc
MHVL6_f	aat ttt atg ctg act cag ccc ca
MHVL7/8_f	cag rct gtg gtg acy cag gag cc
MHVL9/10_f	cag scw gkg ctg act cag cca cc
MHlambdaCL_r	tga aca ttc tgt agg ggc cac tg
MHlambdaCL_r2	tga aca ttc cgt agg ggc aac tg
Second antibody gene PCR VH	
MHVH1-NcoI_f	gtcctcgca cc atg gcc cag gtb cag ctg gtg cag tct gg
MHVH1/7-NcoI_f	gtcctcgca cc atg gcc car rts cag ctg gtr car tct gg
MHVH2-NcoI_f	gtcctcgca cc atg gcc cag rtc acc ttg aag gag tct gg
MHVH3-NcoI_f1	gtcctcgca cc atg gcc sar gtg cag ctg gtg gag tct gg
MHVH3-NcoI_f2	gtcctcgca cc atg gcc gag gtg cag ctg ktg gag wcy sg
MHVH4-NcoI_f1	gtcctcgca cc atg gcc cag gtg car ctg cag gag tcg gg
MHVH4-NcoI_f2	gtcctcgca cc atg gcc cag stg cag ctr cag sag tss gg
MHVH5-NcoI_f	gtcctcgca cc atg gcc gar gtg cag ctg gtg cag tct gg
MHVH6-NcoI_f	gtcctcgca cc atg gcc cag gta cag ctg cag cag tca gg
MHIgMCH1scFv-HindIII_r	gtcctcgca aag ctt tgg ggc gga tgc act
MHIgGCH1scFv-HindIII_r	gtcctcgca aag ctt gac cga tgg gcc ctt ggt gga
Second antibody gene PCR kappa	
MHVK1-MluI_f1	accgcctcc a cgc gta gac atc cag atg acc cag tct cc
MHVK1-MluI_f2	accgcctcc a cgc gta gmc atc crg wtg acc cag tct cc

(*continued*)

Table 5.1 (continued)

Primer	5′ to 3′ Sequence
MHVK2-MluI_f	accgcctcc a cgc gta gat rtt gtg atg acy cag wct cc
MHVK3-MluI_f	accgcctcc a cgc gta gaa atw gtg wtg acr cag tct cc
MHVK4-MluI_f	accgcctcc a cgc gta gac atc gtg atg acc cag tct cc
MHVK5-MluI_f	accgcctcc a cgc gta gaa acg aca ctc acg cag tct cc
MHVK6-MluI_f	accgcctcc a cgc gta gaw rtt gtg mtg acw cag tct cc
MHkappaCLscFv-NotI_r	accgcctcc gc ggc cgc gaa gac aga tgg tgc agc cac agt
Second antibody gene PCR lambda	
MHVL1-MluI_f1	accgcctcc a cgc gta cag tct gtg ctg act cag cca cc
MHVL1-MluI_f2	accgcctcc a cgc gta cag tct gtg ytg acg cag ccg cc
MHVL2-MluI_f	accgcctcc a cgc gta cag tct gcc ctg act cag cct
MHVL3-MluI_f1	accgcctcc a cgc gta tcc tat gwg ctg acw cag cca cc
MHVL3-MluI_f2	accgcctcc a cgc gta tct tct gag ctg act cag gac cc
MHVL4-MluI_f1	accgcctcc a cgc gta ctg cct gtg ctg act cag ccc
MHVL4-MluI_f2	accgcctcc a cgc gta cag cyt gtg ctg act caa tcr yc
MHVL5-MluI_f	accgcctcc a cgc gta cag sct gtg ctg act cag cc
MHVL6-MluI_f	accgcctcc a cgc gta aat ttt atg ctg act cag ccc ca
MHVL7/8-MluI_f	accgcctcc a cgc gta cag rct gtg gtg acy cag gag cc
MHVL9/10-MluI_f	accgcctcc a cgc gta cag scw gkg ctg act cag cca cc
MHLambdaCLscFv-NotI_r	accgcctcc gc ggc cgc aga gga sgg ygg gaa cag agt gac
Primer for colony PCR and sequencing	
MHLacZPro_f	ggctcgtatgttgtgtgg
MHgIII_r	ctaaagttttgtcgtctttcc

- 2xYT-GAT agar plates (2xYT-GAT, 1.5% (w/v) agar-agar)
- Nucleobond Plasmid Midi Kit (Macherey-Nagel).

5.3.5 Second Cloning Step: VH

- NcoI (NEB)
- HindIII (NEB)
- Buffer 2 (NEB)
- Glycerol of 99.5% (Roth, Karlsruhe).

5.3.6 Colony PCR

- Oligonucleotide primer (see Table 5.1).

5.3.7 Library Packaging and scFv Phage Production

- 2xTY media pH 7.0 (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl)
- 2xTY-GA (2xTY, 100 mM glucose, 100 μg/mL ampicillin)

- M13K07 Helperphage for monovalent display (Stratagene)
- Hyperphage for oligovalent display (Progen, Heidelberg)
- 2xTY-AK (2xTY, containing 100 μg/mL ampicillin, 50 μg/mL kanamycin)
- Sorval Centrifuge RC5B Plus, rotor GS3 and SS34 (Thermo Scientific, Waltham)
- Polyethylenglycol (PEG) solution (20% (w/v) PEG 6000, 2.5 M NaCl)
- Phage dilution buffer (10 mM TrisHCl pH7.5, 20 mM NaCl, 2 mM EDTA)
- Mouse α-pIII monoclonal antibody PSKAN3 (Mobitec, Göttingen)
- Goat α-mouse IgG alcaline phosphatase (AP) conjugate (Sigma, Hamburg).

5.4 Procedure

5.4.1 Isolation of Lymphocytes (Peripheral Blood Mononuclear Cells (PBMC))

1. Mix 20 mL fresh blood or EDTA/citric acid treated blood (~2×10^7 cells) of each donor with 20 mL PBS.
2. Fill 10 mL Lymphoprep into a 50 mL polypropylen tube. Carefully overlay Lymphoprep with 40 mL of the diluted blood using a plastic pipette.
   Note: Human blood samples are potentially infectious (HIV, hepatitis, etc.)!
3. Centrifuge the blood with 800× *g* for 20 min at RT (without brake!).
4. The lymphocytes form an opaque phase separation layer between the Lymphoprep and the medium, whereas the erythrocytes and granulocytes will be pelleted. Carefully aspirate the lymphocytes using a plastic pipette and transfer to a new 50 mL polypropylen tube.
5. Fill up with 50 mL PBS and pellet the lymphocytes with 250 × g for 10 min at RT. Discard the supernatant (be careful, the lymphocyte pellet is not solid).
6. Repeat this washing step to remove most of the thrombocytes.
7. Resuspend the lymphocytes pellet in the supplied extraction buffer of the mRNA isolation kit according to the manufacturers' instructions or use 0.5 mL Trizol for total RNA isolation. Both methods, mRNA or total RNA isolation, work well. After resuspension using the mRNA extraction buffer or Trizol, the RNA pellet can be stored at −80°C.

5.4.2 cDNA Synthesis

1. Set up mixture for the first strand cDNA synthesis:

Solution or component	Volume	Final concentration
mRNA or total RNA	9 μL	50–250 ng (mRNA) or 2–20 μg (total RNA)
random hexamer oligonucleotid primer (dN_6) (10 μM)	2.5 μL	1.5 μM
dNTP-Mix (2,5 mM each)	5 μL	500 μM

2. Denature the RNA for 5 min at 70°C. Afterward, directly chill down on ice for 5 min.
3. Add following components:

Solution or component	Volume	Final concentration
RT buffer (5×)	5 μL	1×
0.1 M DTT	2.5 μL	10 mM
Superscript II reverse transcriptase (200 U/μl)	1 μL	200 U

4. Incubate the 25 μL mixture for 10 min at 25°C for primer annealing. Then incubate 50 min at 42°C for first strand synthesis.
5. Denature the RNA/DNA hybrids and the enzyme for 15 min at 70°C. Store at −20°C.
   Note: Check the cDNA quality, using standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH) oligonucleotide primers. Do not use cDNA preparations if the GAPDH fragment could not be amplified by PCR.

5.4.3 First Antibody Gene PCR

1. The cDNA derived from 50–250 ng mRNA or 2–20 μg total RNA will be used as template to amplify VH and the light chain. Set up the PCR reactions as follows (30 × mastermix for 27 PCR reactions):

Solution or component	Volume	Final concentration
dH_2O	1,230 μL	–
Buffer (10×)	150 μL	1×
dNTPs (10 mM each)	30 μL	200 μM each
cDNA	25 μL	complete first strand synthesis reaction
RedTaq 1 U/μl	60 μL	2 U

2. Divide the master mix in 450 μL for VH, 350 μL for kappa and 550 μL for lambda.
3. Add to each of the three reactions, the corresponding reverse primers (see also Table 5.1) as follows:

Antibody gene	Primer	Volume	Final concentration
VH	MHIgMCH1_r or MHIgGCH1_r (10 μM)	18 μL	0.4 μM
kappa	MHkappaCL_r (10 μM)	14 μL	0.4 μM
lambda	MHlambdaCL_r1/_r2 mix (9:1) (10 μM)	22 μL	0.4 μM

Note: Use the IgM primer for naïve antibody gene libraries or the IgG primer for immune antibody gene libraries.

4. Divide the mixture to 9 (VH), 7 (Kappa), and 11 (Lambda) PCR reactions each with 48 µL and add 2 µL (10 µM, 0.4 µM final concentration) of the subfamily specific forward primer (see also Table 5.1):

VH:	(1) MHVH1_f, (2) MHVH1/7_f, (3) MHVH2_f, (4) MHVH3_f1, (5) MHVH3_f2, (6) MHVH4_f1, (7) MHVH4_f2, (8) MHVH5_f, (9) MHVH6_f
Vkappa:	(10) MHVK1_f1, (11) MHVK1_f2, (12) MHVK2_f, (13) MHVK3_f, (14) MHVK4_f, (15) MHVK5_f, (16) MHVK6_f
Vlambda:	(17) MHVL1_f1, (18) MHVL1_f2, (19) MHVL2_f, (20) MHVL3_f1, (21) MHVL3_f2, (22) MHVL4_f1, (23) MHVL4_f2, (24) MHVL5_f, (25) MHVL6_f, (26) MHVL7/8_f, (27) MHVL9/10_f

5. Carry out the PCR using the following program:

94°C	1 min
94°C	1 min
55°C	1 min
72°C	2 min
72°C	10 min

30x

6. Separate PCR products by 1.5% TAE agarose gel electrophoresis (an example is given in Fig. 5.2), cut out the amplified antibody genes (VH: ~380 bp, kappa/lambda: ~650 bp), and purify the PCR products using a gel extraction kit according to the manufacturers' instructions. Pool all VH, kappa, and lambda subfamilies separately. Determine the DNA concentration. Store the three purified first PCR pools at −20°C.
   Note: The VH amplifications of VH subfamilies sometimes results in additional longer PCR products. Avoid those by cutting out only the ~380 bp fragment. The amplifications of kappa subfamilies should always give a clear ~650 bp fragment. When amplifying lambda subfamilies, often other PCR products are generated, especially the amplification of the lambda2 subfamily may result in slushy bands. If some subfamilies are badly amplified and no clear ~650 bp fragment is detectable, use only the ~650 bp fragments from the well amplified subfamilies. Additional comment: since the first PCR amplifies the full LC, it can be used also to construct a Fab or scFab (Hust et al. 2007a,b) libraries from this material.

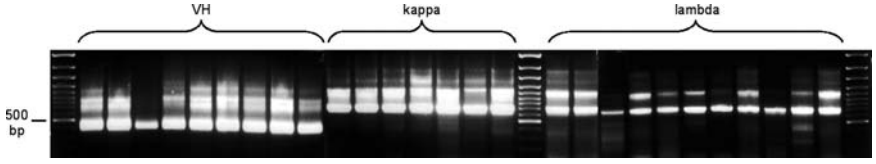


Fig. 5.2 Agarose gelelectrophoresis of the first PCR. The DNA bands are in the order of the VH, kappa, and lambda forward oligonukleotid primers given in Table 5.1. In the first PCR VH PCR products have a length of about 380 bp and kappa or lambda lightchains have a length of about 650 bp. The second PCR looks similiar to the first PCR, but the length of the VH PCR products is due to the added restriction sites about 400 bp and the length of kappa or lambda VL is about 400 bp

5.4.4 Second Antibody Gene PCR

1. In the second PCR, the restriction sites for library cloning will be added. Set up the PCR reactions as follows (30 × mastermix for 27 PCR reactions):

Solution or component	Volume	Final concentration
dH_2O	2,500 µL	–
RedTaq buffer (10×)	300 µL	1×
dNTPs (10 mM each)	60 µL	200 µM each
RedTaq 1 U/µl	120 µL	2 U

Note: For a very large naive antibody gene library, perform as many PCRs as sufficient to perform 20 light chains ligations/transformations and about 100 VH ligations. For an immune library, 4 light chains ligations/transformations and 8 VH ligations are usually sufficient. Also, prepare and digest adequate amounts of pHAL14 and VL for the first cloning step and pHAL14-VL library and VH for the second cloning step. Keep kappa and lambda in all steps (cloning, packaging) separately and mix only after phage production before panning.

2. Divide the master mix in 900 µL for VH, 700 µL for kappa and 1,100 µL for lambda.
3. Add to each of the three reactions the corresponding reverse primers (see also Table 5.1) as follows:

Antibody gene	Primer	Volume	Final concentration
VH	MHIgMCH1scFv-HindIII_r or MHIgGCH1scFv-HindIII_r (10 µM)	18 µL	0.2 µM
kappa	MHKappaCLscFv-NotI_r (10 µM)	14 µL	0.2 µM
lambda	MHLambdaCLscFv-NotI_r (10 µM)	22 µL	0.2 µM

Note: Use the IgM primer for naive antibody gene libraries or the IgG primer for immune antibody gene libraries.

4. Add the corresponding PCR products of the first PCR as follows:

VH	900 ng
Kappa	700 ng
Lambda	1,100 ng

5. Divide the solutions to 9 (VH), 7 (Kappa) and 11 (Lambda) PCR reactions, each with 98 µl and add 2 µL (10 µM, 0.2 µM final concentration) the subfamily specific forward primer (see also Table 5.1):

VH:	(1) MHVH1-NcoI_f, (2) MHVH2-NcoI_f, (3) MHVH1/7-NcoI_f, (4) MHVH3-NcoI_f1, (5) MHVH3-NcoI_f2, (6) MHVH4-NcoI_f1, (7) MHVH4-NcoI_f2, (8) MHVH5-NcoI_f, (9) MHVH6-NcoI_f
Vkappa:	(10) MHVK1-MluI_f1, (11) MHVK1-MluI_f2, (12) MHVK2-MluI_f, (13) MHVK3-MluI_f, (14) MHVK4-MluI_f, (15) MHVK5-MluI_f, (16) MHVK6-MluI_f
Vlambda:	(17) MHVL1-MluI_f1, (18) MHVL1-MluI_f2, (19) MHVL2-MluI_f, (20) MHVL3-MluI_f1, (21) MHVL3-MluI_f2, (22) MHVL4-MluI_f1, (23) MHVL4-MluI_f2, (24) MHVL5-MluI_f, (25) MHVL6-MluI_f, (26) MHVL7/8-MluI_f, (27) MHVL9/10-MluI_f

6. Carry out the PCR using the following program:

94°C	1 min	20x
94°C	1 min	
57°C	1 min	
72°C	2 min	
72°C	10 min	

7. Separate the PCR products by 1.5% TAE agarose gel electrophoresis, cut out the amplified antibody genes (VH: ~400 bp, kappa/lambda: ~400 bp), and purify the PCR products using a gel extraction kit according to the manufacturers' instructions. Pool all VH, kappa, and lambda subfamilies separately. Determine the DNA concentration. Store the three purified second PCR pools at −20°C.

5.4.5 First Cloning Step: VL

1. Prepare a plasmid preparation of pHAL14 vector for library cloning.
2. Digest the vector and the VL PCR products:

Solution or component	Volume	Final concentration
dH_2O	83-x μL	–
pHAL14 or VL	x μL	5 μg or 2 μg
NEB buffer 3 (10×)	10 μL	1×
BSA (100×)	1 μL	1×
NEB MluI (10 U/μl)	3 μL	30 U
NEB NotI (10 U/μl)	3 μL	30 U

Note: Always perform single digests using only one enzyme in parallel to control the success of the restriction reaction. Confirm the correct digestion by TAE agarose gelelectophoresis in comparison to the undigested plasmid. Use only material where both single digests are successful and where no degradation is visible in the double digest.

3. Incubate at 37°C for 2 h. Control the digest of the vector by using a 1 μL aliquot on 1.5% TAE agarose gelelectrophoresis. If the vector is not fully digested, extend the incubation time.
4. Inactivate the enzymes at 65°C for 10 min.
5. Add 0.5 μL CIP (1 U/μL) and incubate at 37°C for 30 min. Repeat this step once.
6. Purify the vector and the PCR product using a PCR purification kit according the manufacturers' instructions and elute with 50 μL elution buffer or water. The short stuffer fragment, containing multiple stop codons between MluI and NotI in pHAL14, will be removed. Determine the DNA concentration.
7. Ligate the vector pHAL14 (4,255 bp) and VL (~380 bp) as follows:

Solution or component	Volume	Final concentration
dH_2O	89-x-y μL	–
pHAL14	x μL	1,000 ng
VL (kappa or lambda)	y μL	270 ng
T4 ligase buffer (10×)	10 μL	1×
T4 ligase (3 U/μL)	1 μL	3 U

8. Incubate at 16°C overnight.
9. Inactivate the ligation at 65°C for 10 min.
10. Precipitate the ligation with 10 μL 3 M sodium acetate pH 5.2 and 250 μL ethanol, incubate for 2 min at RT, and centrifuge for 5 min at 16,000 × g and 4°C.
11. Wash the pellet with 500 μL 70% (v/v) ethanol and pellet the DNA for 2 min at 16,000 × g and 4°C. Repeat this step once and resolve the DNA pellet in 35 μL dH_2O.
12. Thaw 25 μL electrocompetent XL1-Blue MRF′ on ice and mix with the ligation reaction.
13. Transfer the 60 μL mix to a prechilled 0.1 cm cuvette. Dry the electrode of the cuvette with a tissue paper.
14. Perform a 1.7 kV pulse using an electroporator. The pulse time should be between 4–5 ms for optimal electroporation efficiency. Immediately, add 1 mL 37°C prewarmed SOC medium, transfer the suspension to a 2 mL cap, and shake for 1 h at 600 rpm and 37°C.
15. To determine the amount of transformants, use 10 μL ($=10^{-2}$ dilution) of the transformation and perform a dilution series down to 10^{-6} dilution. Plate out a 10^{-6} dilution on 2XYT-GAT agar plates and incubate overnight at 37°C.
16. Plate out the remaining 990 μL on a 25 × 25 cm 2XYT-GAT agar plate and incubate overnight at 37°C.
17. Calculate the amount of transformants, and that should be 1×10^6–5×10^8 cfu. Control colonies for full size insert by colony PCR.
18. Wash off the colonies on the 25 × 25 cm plate with 40 mL 2XYT medium using a drigalsky spatula. Use 5 mL bacteria solution for "midi" plasmid preparation according to the manufacturers' instructions. Determine the DNA concentration.

5.4.6 Second Cloning Step: VH

1. Digest the pHAL14-VL repertoire and the VH PCR products (always perform single enzyme digest of the vector in parallel):

Solution or component	Volume	Final concentration
dH_2O	81-x μL	–
pHAL14-VL or VH	x μL	5 μg or 2 μg
NEB buffer 2 (10×)	10 μL	1×
BSA (100×)	1 μL	1×
NEB NcoI (10 U/μl)	3 μL	30 U
NEB HindIII (20 U/μl)	5 μL	100 U

2. Incubate at 37°C for 2 h. Control the digest of the vector by using a 1 μL aliquot on 1.5% agarose gelelectrophoresis.

Note: Often the HindIII digestion is incomplete after 2 h. Then, inactivate the enzymes by heating up to 65°C for 10 min, add additional 5 μL of HindIII, and incubate overnight. Alternative: perform the NcoI digest first for 2 h, inactive the digest, and afterward perform the HindIII digest.

3. Inactivate the digestion at 65°C for 10 min.
4. Add 0.5 μL CIP (1 U/μL) and incubate at 37°C for 30 min. Repeat this step once.
5. Purify the vector and the PCR product using a PCR purification Kit according the manufacturers' instructions and elute with 50 μL elution buffer or water. The short stuffer fragment between NcoI and HindII in pHAL14 will be removed. Determine the DNA concentration.
6. Ligate the vector pHAL14-VL (~4,610 bp) and VH (~380 bp) as follows:

Solution or component	Volume	Final concentration
dH_2O	89-x-y μL	–
pHAL14	x μL	1,000 ng
VH	y μL	250 ng
T4 ligase buffer (10×)	10 μL	1×
T4 ligase (3 U/μL)	1 μL	3 U

7. Incubate at 16°C overnight.
8. Inactivate the ligation at 65°C for 10 min.
9. Precipitate the ligation with 10 μL 3 M pH5.2 sodium aceteate and 250 μL ethanol, incubate for 2 min at RT, and centrifuge for 5 min at 16,000 × *g* and 4°C.
10. Wash the pellet with 500 μL 70% (v/v) ethanol and pellet the DNA for 2 min at 16,000 × g and 4°C. Repeat this step once and resolve the pellet in 35 μL dH_2O.
11. Thaw 25 μL electrocompetent XL1-Blue MRF′ on ice and mix with the ligation reaction.
12. Transfer the 60 μL mix to a prechilled 0.1 cm cuvette. Dry the electrode of the cuvette with a tissue paper.
13. Perform a 1.7 kV pulse using a electroporator. The pulse time should be between 4–5 ms for optimal electroporation efficiency. Immediately, add 1 mL 37°C prewarmed SOC medium, transfer to a 2 mL cap, and incubate for 1 h at 600 rpm.
14. To determine the amount of transformants, use 10 μL ($=10^{-2}$ dilution) of the transformation and perform a dilution series down to 10^{-6} dilution. Plate out a 10^{-6} dilution on 2xYT-GAT agar plates and incubate overnight at 37°C.
15. Plate out the remaining 990 μL on 25 × 25 cm 2xYT-GAT agar plate and incubate overnight at 37°C.
16. Calculate the amount of transformants (1×10^6–5×10^8 should be reached to be included into the final library). Control colonies for full size insert by colony PCR.
17. Wash off the colonies on the 25 × 25 cm plate with 40 mL 2xYT medium using a drigalsky spatula. Use 800 μL bacteria solution and 200 μL glycerol

for glycerol stocks. Make 5–25 glycerol stocks per sublibrary and store at −80°C.

18. When all transformations are done, thaw one aliquot of each sublibrary on ice, mix all sublibraries, and make new aliquots for storage at −80°C.
    Note: Keep kappa and lambda in all steps (cloning, packaging) separately and mix only after phage production before panning. To minimize loss of diversity, avoid too many freeze and thaw steps, e.g., when constructing an immune library, make eight transformations in parallel and directly package the immune library.

5.4.7 Colony PCR

1. Choose 10–20 single colonies per transformation. Set up the PCR reaction per colony as follows (see Table 5.1 for primer sequences):

Solution or component	Volume	Final concentration
dH_2O	16.7 µl-x µl	
RedTaq buffer (10×)	2 µl	1×
dNTPs (10 mM each)	0.4 µl	je 200 µM
MHLacZPro_f 10 µM	0.2 µl	0,1 µM
MHgIII_r 10 µM	0.2 µl	0,1 µM
RedTag (1 U/µl)	0.5 µl	1 U
template	picked colony from dilution plate	

2. Control the PCR by 1.5% TAE agarose gelelectrophoresis.
3. The PCR products should be ~1,100 bp when including VH and VL, ~750 bp when including only VL or VH, and 375 bp if the vector contains no insert. Each used sublibrary should have more than 75% full-size inserts to be included into the final library.

5.4.8 ScFv Phage Production

1. Inoculate 400 mL 2xTY-GA in a 1 L Erlenmeyer flask with 1 mL antibody gene library stock. Grow at 250 rpm at 37°C up to an $O.D._{600\ nm}$ ~0.5.
2. Infect 25 mL bacteria culture (~1.25×10^{10} cells) with 2.5×10^{11} colony, forming units (cfu) of the helper phage M13K07 or Hyperphage according to a multiplicity of infection (moi) = 1:20. Incubate 30 min without shaking and the following 30 min with 250 rpm at 37°C.
   Note: The use of Hyperphage as helperphage instead of M13K07 offers oligovalent phage display, facilitates the selection of specific binders in the first and most critical panning round by avidity effect (Rondot et al. 2001; Soltes et al. 2007; Pelat et al. 2007; Kirsch et al. 2008). The Hyperphage should be only used for initial library packaging. For the following panning rounds, use M13K07 to enhance the stringency of the panning process.

3. To remove the glucose, which represses the lac promoter of pHAL14, and therefore the scFv::pIII fusion protein production, harvest the cells by centrifugation for 10 min at 3,200 × g in 50 mL polypropylene tubes.
4. Resuspend the pellet in 400 mL 2xTY-AK in a 1 L Erlenmeyer flask. Produce scFv-phage overnight at 250 rpm and 30°C.
5. Pellet the bacteria by centrifugation for 10 min at 10,000 × *g* in two GS3 centrifuge tubes. If the supernatant is not clear, centrifuge again to remove remaining bacteria.
6. Precipate the phage from the supernatant by adding 1/5 volume PEG solution in two GS3 tubes. Incubate for 1 h at 4°C with gentle shaking, followed by centrifugation for 1 h at 10,000 × g.
7. Discard the supernatant, resolve each pellet in 10 mL phage dilution buffer in SS34 centrifuge tubes, and add 1/5 volume PEG solution.
8. Incubate on ice for 20 min and pellet the phage by centrifugation for 30 min at 10,000 × g.
9. Discard the supernatant and put the open tubes upside down on tissue paper. Let the viscous PEG solution move out completely. Resuspend the phage pellet in 1 mL phage dilution buffer. Titre the phage preparation. Store the packaged antibody phage library at 4°C.
10. The library packaging should be controlled by 10% SDS-PAGE, Western-Blot, and anti-pIII immunostain (mouse anti-pIII 1:2000, goat anti-mouse IgG AP conjugate 1:10000). Wildtype pIII has a calculated molecular mass of 42.5 kDa, but it runs at an apparent molecular mass of 65 kDa in SDS-PAGE. Accordingly, the scFv::pIII fusion protein runs at about 95 kDa (an example is given in Fig. 5.3).
    Note: Instead of one scFv::pIII protein, normally two proteins with different molecular mass will be detected at about 95 kDa (Løset et al. 2005; Kirsch et al. 2008). The wildtype pIII is expected to be the dominant band in phage preparations not packaged with Hyperphage but M13K07.
11. The Panning of libraries using the pHAL14 vector system is described in the chapter "Phage display and selection in microtitre plates"

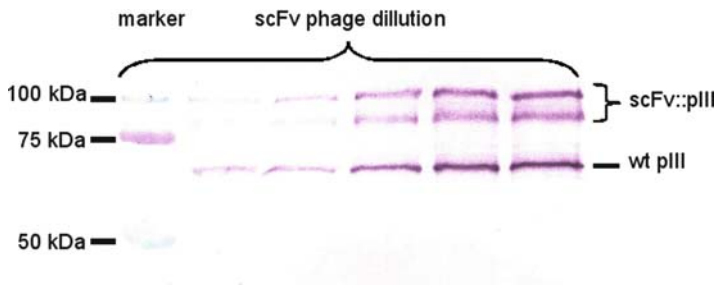


Fig. 5.3 Immunoblot of a Hyperphage packaged immune library (serial dilution from 1 × 10^8 to 1 × 10^9 scFv phage particles)

References

Akamatsu Y, Cole MS, Tso JY, Tsurushita N (1993) Construction of a human Ig combinatorial library from genomic V segments and synthetic CDR3 fragments. J Immunol 151:4651–4659

Barbas CF, Kang AS, Lerner RA, Benkovic SJ (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci USA 88:7978–7982

Barbas CF, Bain JD, Hoekstra DM, Lerner RA (1992) Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. Proc Natl Acad Sci USA 89:4457–4461

Boder ET, Wittrup KD (1997) Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol 15:553–557

Breitling F, Dübel S, Seehaus T, Klewinghaus I, Little M (1991) A surface expression vector for antibody screening. Gene 104:147–153

Clackson T, Hoogenboom HR, Griffiths AD, Winter G (1991) Making antibody fragments using phage display libraries. Nature 352:624–628

de Haard HJ, van Neer N, Reurs A, Hufton SE, Roovers RC, Henderikx P, de Bruïne AP, Arends JW, Hoogenboom HR (1999) A large nonimmunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J Biol Chem 274:18218–18230

Desiderio A, Franconi R, Lopez M, Villani ME, Viti F, Chiaraluce R, Consalvi V, Neri D, Benvenuto E (2001) A semisynthetic repertoire of intrinsically stable antibody fragments derived from a single-framework scaffold. J Mol Biol 310:603–615

Dübel S (2007) Recombinant therapeutic antibodies. Appl Microbiol Biotechnol 74:723–729

Fishwild DM et al (1996) High-avidity human IgG kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. Nat Biotechnol 14:845–851

Geoffroy F, Sodoyer R, Aujame L (1994) A new phage display system to construct multicombinatorial libraries of very large antibody repertoires. Gene 151:109–113

Griffiths AD et al (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J 13:3245–3260

Hanes J, Plückthun A (1997) In vitro selection and evolution of functional proteins by using ribosome display. Proc Natl Acad Sci USA 94:4937–4942

Hayashi N, Welschof M, Zewe M, Braunagel M, Dübel S, Breitling F, Little M (1994) Simultaneous mutagenesis of antibody CDR regions by overlap extension and PCR. Biotechniques 17, 310, 312:314–315

He M, Taussig MJ (1997) Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites. Nucleic Acids Res 25:5132–5134

Hoet RM et al (2005) Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. Nat Biotechnol 23:344–348

Hoogenboom HR, Winter G (1992) By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. J Mol Biol 227:381–388

Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, Winter G (1991) Multi subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res 19:4133–4137

Huse WD, Sastry L, Iverson SA, Kang AS, Alting-Mees M, Burton DR, Benkovic SJ, Lerner RA (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. Science 246:1275–1281

Hust M, Dübel S (2004) Mating antibody phage display with proteomics. Trends Biotechnol 22:8–14

Hust M, Dübel S (2005) Phage display vectors for the in vitro generation of human antibody fragments. Methods Mol Biol 295:71–96

Hust M, Jostock T, Menzel C, Voedisch B, Mohr A, Brenneis M, Kirsch MI, Meier D, Dübel S (2007a) Single chain Fab (scFab) fragment. BMC Biotechnol 7:14

Hust M, Toleikis L, Dübel S (2007b) Antibody phage display. In Handbook of therapeutic antibodies. Wiley-VCH, Weinheim

Jakobovits A (1995) Production of fully human antibodies by transgenic mice. Curr Opin Biotechnol 6:561–566

Jirholt P, Ohlin M, Borrebaeck CA, Söderlind E (1998) Exploiting sequence space: shuffling in vivo formed complementarity, determining regions into a master framework. Gene 215:471–476

Johansen LK, Albrechtsen B, Andersen HW, Engberg J (1995) pFab60: a new, efficient vector for expression of antibody Fab fragments displayed on phage. Protein Eng 8:1063–1067

Kirsch M, Hülseweh B, Nacke C, Rülker T, Schirrmann T, Marschall H, Hust M, Dübel S (2008) Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV). BMC Biotechnol 8:66

Knappik A et al (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. J Mol Biol 296:57–86

Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497

Konthur Z, Hust M, Dübel S (2005) Perspectives for systematic in vitro antibody generation. Gene 364:19–29

Krebber A, Bornhauser S, Burmester J, Honegger A, Willuda J, Bosshard HR, Plückthun A (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires, employing a reengineered phage display system. J Immunol Methods 201:35–55

Little M et al (1999) Generation of a large complex antibody library from multiple donors. J Immunol Methods 231:3–9

Lonberg N, Huszar D (1995) Human antibodies from transgenic mice. Int Rev Immunol 13:65–93

Løset GA et al (2005) Construction, evaluation, and refinement of a large human antibody phage library, based on the IgD and IgM variable gene repertoire. J Immunol Methods 299:47–62

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol 222:581–597

Mazor Y, Van Blarcom T, Mabry R, Iverson BL, Georgiou G (2007) Isolation of engineered, full-length antibodies from libraries expressed in Escherichia coli. Nat Biotechnol 25:563–565

McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348:552–554

McCafferty J, Fitzgerald KJ, Earnshaw J, Chiswell DJ, Link J, Smith R, Kenten J (1994) Selection and rapid purification of murine antibody fragments that bind a transition-state analog by phage display. Appl Biochem Biotechnol 47:157–171 discussion 171–3

Nissim A, Hoogenboom HR, Tomlinson IM, Flynn G, Midgley C, Lane D, Winter G (1994) Antibody fragments from a "single pot" phage display library as immunochemical reagents. EMBO J 13:692–698

Pelat T, Hust M, Laffly E, Condemine F, Bottex C, Vidal D, Lefranc M, Dübel S, Thullier P (2007) High-affinity, human antibody-like antibody fragment (single-chain variable fragment) neutralizing the lethal factor (LF) of Bacillus anthracis by inhibiting protective antigen-LF complex formation. Antimicrob Agents Chemother 51:2758–2764

Pini A, Viti F, Santucci A, Carnemolla B, Zardi L, Neri P, Neri D (1998) Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. J Biol Chem 273:21769–21776

Roberts RW, Szostak JW (1997) RNA-peptide fusions for the in vitro selection of peptides and proteins. Proc Natl Acad Sci USA 94:12297–12302

Rondot S, Koch J, Breitling F, Dübel S (2001) A helper phage to improve single-chain antibody presentation in phage display. Nat Biotechnol 19:75–78

Sblattero D, Bradbury A (2000) Exploiting recombination in single bacteria to make large phage antibody libraries. Nat Biotechnol 18:75–80

Sheets MD et al (1998) Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. Proc Natl Acad Sci USA 95:6157–6162

Shirai H, Kidera A, Nakamura H (1999) H3-rules: identification of CDR-H3 structures in antibodies. FEBS Lett 455:188–197

Simmons LC et al (2002) Expression of full-length immunoglobulins in Escherichia coli: rapid and efficient production of aglycosylated antibodies. J Immunol Methods 263:133–147

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228:1315–1317

Söderlind E et al (2000) Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. Nat Biotechnol 18:852–856

Soltes G, Hust M, Ng KKY, Bansal A, Field J, Stewart DIH, Dübel S, Cha S, Wiersma EJ (2007) On the influence of vector design on antibody phage display. J Biotechnol 127:626–637

Sørensen MD, Sørensen B, Gonzalez-Dosal R, Melchjorsen CJ, Weibel J, Wang J, Jun CW, Huanming Y, Kristensen P (2006) Severe acute respiratory syndrome (SARS): development of diagnostics and antivirals. Ann NY Acad Sci 1067:500–505

Thie H, Meyer T, Schirrmann T, Hust M, Dübel S (2008) Phage display derived therapeutic antibodies. Curr Pharm Biotechnol 9:439–446

Thie H, Voedisch B, Dübel S, Hust M, Schirrmann T (2009) Affinity maturation by phage display. Methods Mol Biol 525:1–14

Vaughan TJ et al (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat Biotechnol 14:309–314

von Behring E, Kitasato S (1890) Über das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren. Deutsche Medizinische Wochenzeitschrift 16:1113–1114

Waterhouse P, Griffiths AD, Johnson KS, Winter G (1993) Combinatorial infection and in vivo recombination: a strategy for making large phage antibody repertoires. Nucleic Acids Res 21:2265–2266

Welschof M, Terness P, Kipriyanov SM, Stanescu D, Breitling F, Dörsam H, Dübel S, Little M, Opelz G (1997) The antigen-binding domain of a human IgG-anti-F(ab′)2 autoantibody. Proc Natl Acad Sci USA 94:1902–1907

Winter G, Milstein C (1991) Man-made antibodies. Nature 349:293–299

Chapter 6
Synthetic Antibody Libraries

Pierre Martineau

6.1 Introduction

In recent years, antibodies have proved their value as therapeutic molecules (Adams and Weiner 2005). Immunoglobulins (Ig) are however large and complex molecules, difficult and expensive to produce in large quantities and not well suited for some applications. For instance, tumor penetration is limited by the Ig size and it is difficult to make the bispecific molecules that have very interesting clinical applications. Antibody fragments, such as scFv, Fab, or isolated VH domains, are much easier to produce and can be easily coupled with other proteins such as toxins or another antibody fragment to make bifunctional and bispecific molecules. This has prompted the development of new therapeutic approaches based on such fragments that are now entering clinical evaluation (Holliger and Hudson 2005).

There are large differences in the characteristics of the antibody fragments that can be selected, in terms of stability, sequence, physical properties, or expression levels (Demarest and Glaser 2008). A simple solution to this problem would be to generate a naive antibody library using only some (or even only one) well-characterized antibody frameworks. This has been done now by several groups with great success and the possibility to generate antibody molecules against any antigen from such a repertoire is now clearly demonstrated (Knappik et al. 2000; Philibert et al. 2007; Silacci et al. 2005). In addition, several studies have shown

P. Martineau
IRCM, Institut de Recherche en Cancérologie de Montpellier, Montpellier 34298, France
INSERM, U896, Montpellier 34298, France
Université Montpellier1, Montpellier 34298, France
CRLC Val d'Aurelle Paul Lamarque, Montpellier 34298, France
e-mail: pierre.martineau@valdorel.fnclcc.fr

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_6,

that the framework stability and folding properties are at least partially conserved upon loop grafting to confer a new specificity (Donini et al. 2003; Jung and Pluckthun 1997). These findings show that it is possible to construct a scFv library based on a single optimized framework.

The natural diversity of antibodies is mainly due to the sequence variability of the three CDR loops, but also to the VH-VL interface. Among these sources of diversity, CDR3 loops are by far the most variable part of the paratope, and are thus more likely to be highly tolerant to sequence and length variations.

In this protocol, we will describe the construction of a phage-displayed scFv library based on a single framework, called 13R4 (Martineau et al. 1998) (*see Note* 1). We will introduce diversity only in the CDR3 loops by using degenerate oligonucleotides. We will use, as an example, a completely random oligonucleotide to introduce the diversity, but more clever designs are possible and suitable to optimize the proportion of correctly folded and active clones in the final library (*see Note* 2). We will also use a single CDR3 length but the reader will understand easily that it is possible to use the same protocol to introduce loops of different lengths by using a mix of oligonucleotides. Having a single framework for the construction of a library should allow more comparable expression levels between clones since most of their sequences are conserved. In addition, since the library is based on a single framework, it should be fairly easy to improve the affinity of a selected scFv by using, for instance, chain shuffling, error-prone mutagenesis, or by optimization of the CDR1 and CDR2 loops (*see* Vol. 2 Sect. 39.2.9).

6.2 Materials

All buffers must be prepared with ultra-pure water and ACS grade chemicals unless otherwise indicated.

6.2.1 Introducing Diversity

- Thermocycler.
- Phusion High-Fidelity DNA Polymerase and reagents (Finnzymes #F-530) (*see Note* 3)
- Oligonucleotides (Table 6.1 and Fig. 6.1) (*see Notes* 1 & 2)
- SYBR Safe™ DNA Gel Stain 10,000X concentrate (Invitrogen #S33102) (*see Note* 4)
- Safe Imager™ blue light transilluminator (Invitrogen #S37102) (*see Note* 4)
- TAE Buffer. TAE×50: 242 g Tris, 57.1 ml glacial acetic acid, 37.2 g Na_2 EDTA.$2H_2O$, make up to 1 l (pH~8.5)

Table 6.1 Oligonucleotides[a]

Name	Sequence (5′→3′)
M13.rev	GAGCGGATAACAATTTCACACAGG
M13.uni	AGGGTTTTCCCAGTCACGACGTT
VH.FR4.for	TGGGGCAGAGGCACCCT
VL.FR3.rev	GCAGTAATAATCAGCCTCGTCC
H3.rev[b]	AGGGTGCCTCTGCCCCA $(MNN)_{5–20}$ TCTCACACAGTAATAAACAGCCG
L3.for[b]	GGACGAGGCTGATTATTACTGC $(NNK)_{9–11}$ TTCGGCGGAGGGACCAAG

[a]The Tm of the oligonucleotides are all between 60 and 70°C
[b]Design of the degenerated oligonucleotides is explained in Fig. 6.2 (*see Note* 2)

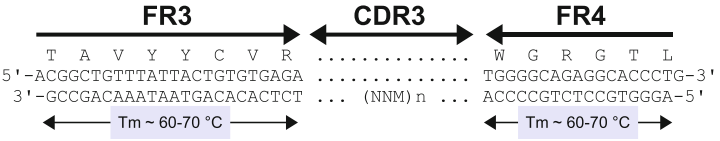


Fig. 6.1 Design of the mutagenic oligonucleotides. The H3.rev degenerated oligonucleotide (Table 6.1) for introducing diversity in the CDR3 VH loop is designed in three parts: (1) A 5′ sequence that matches the reverse of the 5′ sequence of the FR4 VH gene with a Tm of 60–70°C (usually 18–25 bp); (2) A degenerated core that will encode the CDR3 loop. We use here a fully degenerated sequence encoded by NNK codons (NNM on the reverse strand) (*see Note* 2); (3) A 3′ sequence that matches the reverse of the 3′ sequence of the FR4 VH gene with a Tm of 60–70°C. The L3.for (Table 6.1) oligonucleotide was designed following the same principles, except that is was complementary to the reverse strand. In the case of the VL, it is however usually better not to degenerate the 5′ part of the loop since it is germline encoded and not highly variable (Knappik et al. 2000; Silacci et al. 2005). Tm values were calculated using Finnzymes recommended method (https://www.finnzymes.fi/tm_determination.html)

- 1% agarose gel: add 1 g of agarose to 100 ml of TAE buffer, boil in a microwave oven until completely melted, cool down to 45–50°C, add 10 µL of SYBR Safe™
- Macherey-Nagel NucleoSpin Extract II (#740609)

6.2.2 Cloning

- *Nco*I (10 u/µL, NEB #R0193) and *Not*I (10 u/µL, NEB #R0189) enzymes. 10×NEB3 buffer and 100×BSA from NEB
- 100 bp and 1 kb DNA Ladder (NEB #N3231 and #N3232) (*see Note* 5)
- pCANTAB6 vector (1 µg/µL) (*see Note* 6)
- T4 DNA Ligase (6 Weiss u/µL) (Fermentas #EL0014) (*see Note* 7)
- Glucose 40% (w/v), autoclaved
- LBGA plates: 10 g tryptone (peptone), 5 g yeast extract, 10 g NaCl, make up to 1 l with water, adjust pH to 7.0 with 5 N NaOH, add 15 g of agar, and autoclave. Allow the solution to cool to 55–60°C, add 50 ml of 40% glucose solution, 100 µg/ml ampicillin, then pour the plates

6.2.3 Transformation in Electrocompetent Bacteria

- LB/Tet plates: prepare as for LBGA but instead of glucose and ampicillin add 1 ml of a 12 mg/ml tetracycline solution in 70% ethanol
- Cmax5αF′, freshly streaked on a LB/Tet plate (*see Note* 8)
- 2xTY: 16 g tryptone (peptone), 10 g yeast extract, 5 g NaCl, make up to 1 l with water, adjust pH to 7.0 with 5 N NaOH, and autoclave
- Hepes 1M: weight 2.38 g of Hepes, add 8 ml of H_2O, adjust pH to 7.0 and the volume to 10 ml. Sterilize by filtration
- Glycerol/Hepes: Weight 10 g of glycerol, make up to 1 l with water, and autoclave. Add 1 ml of sterile Hepes 1M
- H_2O/Hepes: Add 1 ml of sterile Hepes 1M to 1 l of autoclaved ultra-pure water
- SOC: 20 g tryptone (peptone), 5 g yeast extract, 0.5 g NaCl, 10 ml of 250 mM KCl (18.6 g/l), make up to 1 l with water, adjust pH to 7.0 with 5 N NaOH, and autoclave. Allow to cool and add 5 ml of sterile 2M $MgCl_2$ (190.4 g/l, autoclaved) and 9 ml of sterile 40% glucose
- Magnetic stir bars, 2–3 cm long. Sterilize by autoclaving and store at 4°C
- Biorad GENE PULSER II and 0.2 cm gap cuvettes (*see Note* 9)
- Large 245 mm × 245 mm square Petri dishes (Nunc #240835). Use 250 ml of LBGA per plate
- 14 ml sterile polypropylene round-bottom culture tubes (17 mm × 100 mm. BD Falcon #352059)
- Glycerol 40%: Weight 40 g of glycerol, make up to 0.1 l with water, and autoclave

6.3 Methods

6.3.1 Introducing Diversity

1. Prepare 3 PCR by mixing on ice (*see Note* 10)

Template (pAB1-13R4)	10 ng
HF buffer (×5)	5 µL
dNTP (10 mM)	0.5 µL
Primer 1 (10 Mol/µL)	1.25 µL
Primer 2 (10 pMol/µL)	1.25 µL
H2O	to 24.75 µL
Phusion	0.25 µL

   Using the 3 primer pairs (Fig. 6.2): M13.rev/H3.rev; VH.FR4.for/VL.FR3.rev; L3.for/M13.uni
2. Cycle 30 times: 98°C 30 s/(98°C 10 s/65°C 10 s/72°C 15 s) × 30/72°C 5 min/4°C hold (*see Note* 11)
3. Separate the PCR on a 1% agarose gel. Cut the three bands and pool them

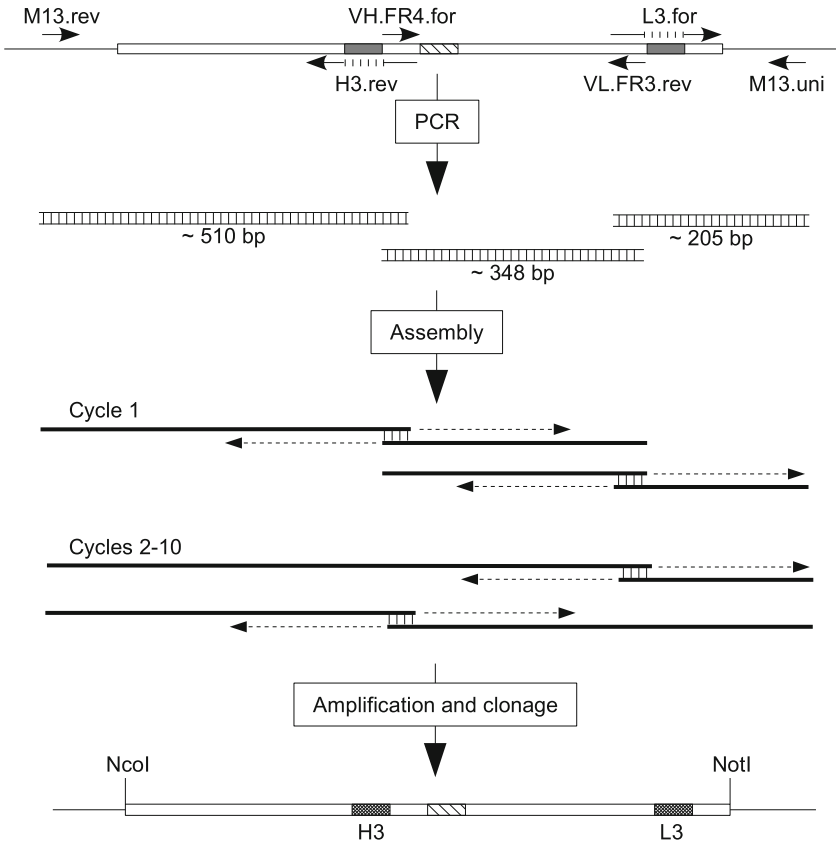


Fig. 6.2 Library construction. The figure outlines the main steps to introduce diversity in the CDR3 loops of a single scFv. Several variations of the protocol are possible. For instance, diversity could also be introduced in other CDR loops by designing additional oligonucleotide pairs and we have successfully assembled scFv genes from ten fragments using this method. The first PCR could also be omitted and replaced by synthetic oligonucleotides, resulting in a fully synthetic scFv library

4. Purify the mix on a column of NucleoSpin Extract II. Elute in 19.25 μL
5. Add 5 μL of HF Buffer (×5), 0.5 μL of dNTP (10 mM), 0.25 μL of Phusion (Final volume of 25 μL)
6. Cycle 10 times: 98°C 30 s/ (98°C 10 s/65°C 10s/72°C 15s) × 10/72°C 5 min/ 4°C hold
7. Add in the following order on ice: 310 μL H_20, 100 μL HF (×5) buffer, 10 μL dNTP (10 mM), 25 μL M13.rev, 25 μL M13.uni, and 5 μL of Phusion (Final volume of 500 μL)
8. Distribute in 10 PCR tubes and cycle 30 times: 98°C 30 s/(98°C 10 s/70°C 30 s / 72°C 15 s) × 10/72°C 5 min/4°C hold

9. Pool the 10 tubes and analyze 2 μL on a 1% agarose gel using 0.5 μg of 100 bp DNA Ladder as marker
10. Purify on a NucleoSpin Extract II column using the "Protocol for PCR clean-up." Elute in 40.5 μL

6.3.2 Cloning in Phage-Display Vector

6.3.2.1 Insert Digestion

1. Add to the 40.5 μL of insert prepared in Sect. 6.3.1: 5 μL of 10×NEB3 buffer, 0.5 μL of 100×BSA, 2 μL of *Nco*I, and 2 μL of *Not*I. Incubate for 3 h at 37°C
2. Purify on a 1% agarose gel using a NucleoSpin Extract II column. Elute in 100 μL
3. Analyze 1 μL on a 1% agarose gel using 0.5 μg of 100 bp DNA Ladder as marker
4. Quantify the insert concentration using, as reference, the marker. (*see Note* 12)

6.3.2.2 Vector Digestion

1. Mix the following: 20 μL of pCANTAB6 vector (20 μg), 5 μL of 10×NEB3 buffer, 0.5 μL of 100×BSA, 2 μL of *Nco*I, and 2 μL of *Not*I, 20.5 μL of H_2O. Incubate for 3 h at 37°C
2. Purify on a 1% agarose gel using a NucleoSpin Extract II column. Elute in 100 μL
3. Quantify the vector concentration using, as reference, 1 μg of the 1 kb DNA ladder (*see Note* 12)

6.3.2.3 Ligation

Prepare 3 ligations using 1:0.5, 1:1 and 1:2 vector:insert molar ratios (*see Note* 13).

1. Prepare 3 ligations:

Digested pCANTAB6 (4500 bp)	50 ng (0.3 – 0.5 μL)
Digested insert (750 bp)	4.15 ng/8.3 ng/16.7 ng
Ligase buffer (×10)	0.5 μL
Ligase diluted 1/10 in 1× ligase buffer	1 μL (0.5 Weiss units)
H_20	to 5 μL

2. Incubate overnight at 16°C in a water bath
3. Heat for 10 min at 65°C
4. Transform 2 μL of the ligation by electroporation (see Sect. 6.3.3 and *Note* 14)
5. Plate 100 μL of $10^{-2}/10^{-3}/10^{-4}$ dilutions on LBGA plates and incubate overnight at 37°C
6. Count the clones and measure the cloning efficiency by colony PCR (M13.rev/M13.for) or restriction (*see Note* 15)

7. Prepare the final ligation in 500 µL using the best determined ratio:

Digested pCANTAB6 (4,500 bp)	5 µg
Digested insert (750 bp)	0.42, 0.83 or 1.67 µg
Ligase buffer (×10)	50 µL
Ligase (5 u/µL)	10 µL (50 Weiss units)
H_20	to 500 µL

8. Incubate overnight at 16°C in a water bath. Heat for 10 min at 65°C
9. Purify on a NucleoSpin Extract II column using the "Protocol for PCR clean-up." Elute in 80 µL

6.3.3 Transformation in Electrocompetent Bacteria

We have always obtained better results with freshly prepared cells than with frozen ones, including commercial, electrocompetent cells. We routinely obtain 5.10^9–2.10^{10} transformants/µg of pUC18 plasmid. Upon freezing, the efficiency of transformation decreases five to tenfold. It is thus required to work with freshly prepared competent cells and you must exercise yourself in order to reliably prepare highly competent cells before performing Sect. 6.3.3.2 since you will have to prepare a new ligation if your transformation efficiency is not high enough.

All the material must be prechilled and kept as close to 0°C as possible in a ice/water bath throughout the preparation. If possible, work in a cold chamber. The centrifuge and the rotor must be precooled to 2°C.

6.3.3.1 Preparation of Electrocompetent Cells

1. Pick 1 *fresh* colony of Cmax5αF′ in a 50 ml flask containing 10 ml of 2xTY and 12 µg/ml of tetracycline, and grow overnight at 37°C with vigorous shaking (220 rpm) (*see Note* 8)
2. Pour the flask content in a 5 l flask containing 1 l of 2xTY and 12 µg/ml of tetracycline, and grow at 37°C with vigorous shaking until OD_{600nm} reaches 0.7 (*see Note* 16)
3. Pour the flask content in two 500 ml centrifuge bottles and cool down in an ice/water bath for 30 min Mix regularly and gently the bottles
4. Centrifuge at 5,000 g for 5 min at 2°C and discard the supernatant
5. Add a cold and sterile magnetic stir bar and 500 ml of cold H_2O/Hepes to each bottle. Resuspend the pellet on a magnetic stirrer. Start with a vigorous stirring until the pellet detaches from the bottle, then with a slower rotation rate until all the bacteria are completely resuspended. You can also gently mix the bottle by turning it upside down several times
6. Centrifuge at 5,000 g for 10 min at 2°C and discard the supernatant, paying attention not to disturb the pellet *containing the stir bar*
7. Repeat steps 5 and 6

8. Resuspend, as in step 5, in 50 ml of cold glycerol/Hepes. Pool the two bottles in a new centrifuge bottle. *Do not transfer the stir bars*
9. Centrifuge at 5,000 g for 15 min at 2°C and discard the supernatant
10. Resuspend the pellet in 1 ml of cold glycerol/Hepes *using a 10 ml pipette*. The final volume should be about 2 ml. (*see Notes* 8 & 17)

6.3.3.2 Library Electroporation

1. Prepare four sterile 100 ml Erlenmeyer flasks containing 25 ml of SOC and two 14 ml sterile polypropylene culture tubes containing 0.950 ml of SOC
2. Warm at 37°C for at least 1 h
3. Cool on ice: 5 electroporation cuvettes, 5 Eppendorf tubes, and the slide that holds the cuvette in the electroporator (*see Note* 18)
4. In a prechilled Eppendorf tube, mix 300 μL of competent cells and 20 μL of the purified ligation (Sect. 6.3.2.3)
5. Transfer the mix in a prechilled electroporation cuvette. Be sure to put all the sample to the bottom of the cuvette by gently tapping the bottom of the cuvette on a flat surface
6. Apply an electric pulse using the following conditions: 2,500 V, 25 μF, 200 Ω
7. Immediately transfer the cells to one of the prewarmed Erlenmeyer flask by washing the sample with 1 ml of outgrowth medium from a pasteur pipette. (*see Note* 19)
8. Immediately transfer the flask to a 37°C incubator and shake vigorously (220 rpm) for 1 h
9. Repeat steps 5–8 three times for the rest of the ligation (3 × 20 μL)
10. Negative control: Add 40 μL of competent cells to one of the two tubes of SOC
11. Positive control: Add 1 μL of highly purified supercoiled pUC18 (10 pg/μL) plasmid to 40 μL of competent cells in one of the prechilled Eppendorf tube. Follow steps 5–8 but resuspend in 0.95 ml of SOC using the second prewarmed tube
12. Incubate the flasks and the tubes for 1 h at 37°C with vigorous shaking
13. Pool the 4 flasks. Plate on LBGA plates: 100 μL of the negative control; 100 μL of 10^{-1} and 10^{-2} dilutions of the positive control; 100 μL of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions of the library
14. Centrifuge the 100 ml of cells at 5,000 g for 10 min at 8°C and discard the supernatant
15. Resuspend the pellet in 4 ml of SOC and plate on four large square Petri dishes of LBGA and incubate overnight at 37°C
16. Scrap the cells from each square plate in 10 ml of 2xTY/glycerol (7.5 ml 2xTY/2.5 ml 40% glycerol) and pool them
17. Measure the $OD600_{nm}$ of a 1/200 dilution in 2xTY. Calculate the number of cells/ml using the formula: A cell culture at 5.10^8 cells/ml has an $OD600_{nm}$ of 1. (*see Note* 20)
18. Calculate the size of the library using the series of dilutions plated at step 13

19. Aliquot the library at −80°C, each tube containing a number of cells of at least 20 times the library size. (*see Note* 21)
20. Measure the percentage of ampicillin-resistant clones by plating serial dilutions of the library on LBGA and LBG plates. Incubate overnight at 37°C. (*see Note* 22)

6.4 Results

The PCR assembly in Sect. 6.3.1 should give a clear band at about 1,000 bp. At step 9, the 2 µL sample can be roughly estimated using the DNA marker and should be about 30 ng. The total quantity produced is thus about 30 ng × 250 ~ 7 µg. After digestion and purification from an agarose gel, we routinely obtain about 3 µg of digested and purified band (in 100 µL in Sect. 6.3.2.1). A precise quantification at step 4 (Sect. 6.3.2.1) should thus be easy since the amount of 30 ng is comparable to the amount of the bands of the equivalent sizes in the DNA markers (*see Note* 5). Digestion and purification of the vector should give you about 10–15 µg.

It is usually easy to optimize the ligation conditions (Sect. 6.3.2.3) if you have precisely quantified your DNA bands (*see Note* 12). Most of the time, the 1:1 ratio gives the best results with about 10^5–10^6 transformants using frozen electrocompetent cells. In addition, you should obtain 100% of positive clones by colony-PCR. If it is not the case, it is presumably because your vector and/or your insert were not completely digested. In such a case, make new digestions and purifications, but increase the digestion time (5–16 h). Some authors use a phosphatase treatment to reduce vector self-ligation, but we found that this treatment decreases the number of clones obtained and is thus not suitable for making libraries. It is of course required to use two different enzyme sites for cloning, and trying to make libraries using blunt-end or two identical enzymes will always result in a much lower number of productive clones.

Finally, the last critical step is to obtain reliably highly competent electroporation cells (Sect. 6.3.3.1). The protocol described here is adapted from the method of Sidhu and collaborators (Sidhu et al. 2000). We have tested several commercial brands of competent cells and several cell lines and we have always obtained the best results with this protocol and Cmax5αF′ strain. It is possible to obtain a twofold higher efficiency with MC1061 strain and its male derivatives (*see Note* 8), but we prefer to use Cmax5αF′ because it is *recA* deficient and produces high-quality DNA (*endA*). It is very important to exercise yourself with this protocol since you cannot use frozen cells for the final library transformation. We indeed found that, whatever the method used, freezing of the competent cells results in a five to tenfold decrease in the transformation efficiency. This is not a problem for most experiments (as in Sect. 6.3.2.3.4), but would result in a smaller-than-possible library in Sect. 6.3.3. However, the amount of insert and vector produced will allow you to make about 2–3 ligations and you can thus

reproduce easily Sect. 6.3.3 if your first transformation is not good enough (*see Note* 23). Always include the positive control to measure the transformation efficiency of your cells to determine if a low number of clones is due to the electroporation experiment or to the ligation. Good electrocompetent Cmax5αF′ cells should give between 5.10^9 and 2.10^{10} transformants per μg of pUC18 plasmid.

If all the steps are correctly performed, you should obtain at least 10^9 clones. Of course, this number is estimated from the dilution plates. This may change in the future with the availability of the rapid sequencing technologies that should allow the sequencing of several millions, if not all the clones of a library. By repeating the experiment, it is presumably possible to make a library larger than 10^{10}, but you may ask yourself if such a diversity is really needed and if you have the tools to explore it.

6.5 Notes

1. ScFv13R4 is a highly expressed human scFv. The scFv gene is cloned in a pUC119-derived plasmid. You will have to modify the oligonucleotides in Table 6.1 according to your scFv and vector sequences.
2. We will introduce random sequences using degenerated codon NNK (K = T or G). This degenerated codon will code for the 20 amino acids and the amber stop codon (TAG). The advantage of such a design is that the oligonucleotide can be easily synthesized at a low cost. Clever designs are possible in order to optimize the expressed and functional clones using either tri-nucleotides (Knappik et al. 2000) or spiked oligonucleotides (Philibert et al. 2007).
3. The polymerase used must generate blunt fragments, otherwise the assembly step will fail. It is also better to use a high-fidelity polymerase to avoid mutations.
4. Using SYBR safe™ and a blue light transilluminator result in a much higher cloning efficiency. Indeed, exposition to UV light, when working with EtBr, results in DNA damage, and thus in a lower cloning efficiency. If you work with EtBr, try to use a long-wave UV transilluminator (365 nm) and/or add a pile of transparent plastic papers between the transilluminator and your gel to decrease as much as possible the UV intensity.
5. Any DNA marker can be used, but you must know precisely the amount of each band of the marker since we will use them to quantify the purified PCR and vector. For 0.5 μg of the two used markers, the band amounts are between 18 and 125 ng.
6. pCANTAB6 is a phagemid vector derived from pUC119. It allows the presentation of the scFv at the surface of the M13 filamentous phage (Philibert et al. 2007). If the vector in which you want to clone does not have the correct sites for your scFv, you can redesign oligonucleotides M13.rev and M13.uni in Table 6.1 to introduce new restriction sites at the two extremities of your

scFv gene. In such a case, check in the NEB or Fermentas catalogs that the restriction enzymes used are able to cut DNA close to the end of a DNA fragment.

7. NEB does not use Weiss but cohesive end units. You can convert between the two units using the formula: 1 Weiss unit = 200 NEB cohesive end units. Always aliquot the 10× ligase buffer since it contains ATP. You can freeze/thaw it about 10 times but it is better to use a new fresh aliquot to perform the final ligation in Sect. 6.3.2.3.7.
8. Cmax5αF′ is Φ80d*lac*ZΔM15Δ(*lac*ZYA – *arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (r−k , m+k) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1/F′ [*lac*I^Q *Tn*10 (Tet)] and can be purchased from Bio-Rad (#170-3341). Always streak on a plate the day before use. We have tested several *E. coli* strains for transformation efficiency. Cmax5αF′ has given the best results for a restriction (*hsd*R) and recombination minus (*rec*A) strain with values between 5.10^9 and 2.10^{10}/μg of pUC18 plasmid. It is much better to use a *rec*A strain to avoid library instability, but if it is not a requirement, the most efficient strain is MC1061 (http://www.bio-rad.com/cmc_upload/Literature/12864/M1652101C.pdf) (Sidhu et al. 2000). We have always obtained better results with freshly prepared cells than with commercial electrocompetent cells. In addition, commercial electrocompetent cells are very expensive since we need a high volume (1.2 ml).
9. Bio-Rad 0.2 cm gap cuvettes (#165–2086) allow electroporation of 400 μL of cells. You can get a higher efficiency with 0.1 cm gap cuvettes but you will have to perform 4–5 times more electroporation experiments (maximum possible volume of 80 μL).
10. Since high-fidelity polymerase enzymes have a 3′ → 5′ exonuclease activity, it is important to keep everything on ice and to add the polymerase at the end after the dNTP mix.
11. PCR conditions will depend on the enzyme and the oligonucleotide sequences. Adapt the conditions according to the manufacturer's instructions.
12. Visual comparison of the band intensities is usually sufficient. You can however use a software-like ImageJ (http://rsbweb.nih.gov/ij/) to make a precise quantification. The expected result of the PCR is about 30 ng/μL and 100–150 ng/μL for the digested vector, which should be easy to compare with the intensities of the bands obtained with 0.5–1 μg of the DNA markers (*see Note* 5).
13. Use a fixed amount of 50 ng of vector DNA and adjust the amount of insert according to the size of the vector. If you have precisely quantified your DNA bands, you should obtain the best results with the 1:1 ratio, but this preliminary experiment makes the protocol tolerant of small errors in Sects. 6.3.2.1.4 and 6.3.2.2.3.
14. Any batch of competent cells will be appropriate since we just want to compare the three ligation ratios. We usually use 40 μL of frozen electrocompetent cells (~10^8–10^9 transformants/μg).
15. Choose the vector:insert ratio that gives the highest number of clones with 100% of positive clones.

16. The cells must reach this absorbance in about 3 h. If it is much slower (>5 h), it is presumable that your flask contains traces of detergents. Start a new culture in a new clean sterile flask washed several times with sterile water or use a disposable plastic flask.
17. Competent cells can be frozen but the transformation efficiency will decrease 5–10 times. Aliquot in sterile prechilled 0.5 ml Eppendorf tubes, freeze in a dry ice/ethanol bath, and store at −80°C.
18. See Fig. 7 in the Biorad technical note MC1652101C (*see Note* 8).
19. The period between applying the pulse and transferring the cells to outgrowth medium is crucial for recovering *E. coli* transformants. Delaying this transfer by even 1 min causes a threefold drop in transformation efficiency.
20. The exact formula depends on the spectrophotometer and the cell type. The given value is, in most cases, close enough to the real value to be used here. You can however calibrate your spectrophotometer by measuring the $OD600_{nm}$ and measuring the real value by plating several dilutions of the cells.
21. If the library diversity is D, and the clone number in your tube N, the probability not to miss a given clone is $1-(1-1/D)^N$. If the library size is 10^9 (D), using an aliquot of 20.D ($N = 2.10^{10}$) results in a probability of 0.999999998. You can increase the aliquot size if you want to increase the chance to have at least 1 copy of each clone in every aliquot.
22. Since the library will grow to confluence on the plate, it is important to check that ampicillin has not been exhausted too early and that a substantial proportion of ampicillin-sensitive clones is present. We usually obtain close to 100% of ampicillin clones even with 10^9 clones on a single large square plate. If it is not the case, you must correct the value in step 19 (N value in *Note* 21). For instance, if you get only 50% of resistance cells, consider that your aliquots contain only 10 times the library size.
23. You may pool the result of several independent experiments to get a higher diversity. In such a case, you must pool the different libraries in amounts corresponding to their diversity. This is easy if you have always made aliquots of 20 times the library size in Sect. 6.3.3.2.19, since you can simply pool together one aliquot of each library: The diversity of the resulting library will be the sum of the diversities of the sub-libraries and the pool will contain a number of cells of 20 times the library size.

References

Adams GP, Weiner LM (2005) Monoclonal antibody therapy of cancer. Nat Biotechnol 23:1147–1157

Demarest SJ, Glaser SM (2008) Antibody therapeutics, antibody engineering, and the merits of protein stability. Curr Opin Drug Discov Devel 11:675–687

Donini M, Morea V, Desiderio A, Pashkoulov D, Villani ME, Tramontano A, Benvenuto E (2003) Engineering stable cytoplasmic intrabodies with designed specificity. J Mol Biol 330:323–332

Holliger P, Hudson PJ (2005) Engineered antibody fragments and the rise of single domains. Nat Biotechnol 23:1126–1136

Jung S, Pluckthun A (1997) Improving in vivo folding and stability of a single-chain Fv antibody fragment by loop grafting. Protein Eng 10:959–966

Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellnhofer G, Hoess A, Wolle J, Pluckthun A, Virnekas B (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. J Mol Biol 296:57–86

Martineau P, Jones P, Winter G (1998) Expression of an antibody fragment at high levels in the bacterial cytoplasm. J Mol Biol 280:117–127

Philibert P, Stoessel A, Wang W, Sibler A, Bec N, Larroque C, Saven JG, Courtete J, Weiss E, Martineau P (2007) A focused antibody library for selecting scFvs expressed at high levels in the cytoplasm. BMC Biotechnol 7:81

Sidhu SS, Lowman HB, Cunningham BC, Wells JA (2000) Phage display for selection of novel binding peptides. Methods Enzymol 328:333–363

Silacci M, Brack S, Schirru G, Marlind J, Ettorre A, Merlo A, Viti F, Neri D (2005) Design, construction, and characterization of a large synthetic human antibody phage display library. Proteomics 5:2340–2350

Chapter 7
Immune Libraries from Nonhuman Primates (NHP)

Thibaut Pelat, Michael Hust, and Philippe Thullier

7.1 Introduction

7.1.1 Choice of the Nonhuman Primate Approach

Utilizing nonhuman primates (NHP) to isolate therapeutic antibodies (Abs), or antibody fragments, is a two-sided idea: it may seem unexpected on one side, but obvious on the other. Indeed, in a field (recombinant antibodies, RecAbs) that has worked so hard to minimize the use of laboratory animals and to substitute them with naïve libraries, utilizing one of the most heavily regulated and onerous animal is not straightforward. However, the costs associated with NHP are a fraction of the costs necessary to access high-quality naïve libraries, and it should be easily recognized that NHPs have to be utilized when searching for new therapeutic molecules, in regulatory preclinical studies at least. When the apparent oddity of isolating NHP Abs for medical use is set aside, this approach is quite obvious as it derives from the similarities between human Abs and NHP Abs. The human-like character of NHP antibodies was revealed early by several studies (Andris et al. 1997; Kawamura et al. 1990, 1992; Meek et al. 1991), and it was noted (Barbas 2001) that "(immunoglobulin) gene segments of macaques ... are as closely related to human immunoglobulin genes as human (immunoglobulin) genes are to each other." Correspondingly, several "primatized IgGs," i.e., IgGs whose variable domains are of NHP origin and constant domains are of human origin, have been produced (an anti-B7 Ab, IDEC 114 and an anti-CD23 Ab, IDEC 152) and positively tested

T. Pelat and P. Thullier (✉)
Centre de Recherche du Service de Santé des Armées, Groupe de biotechnologie des anticorps, Département de biologie des agents transmissibles, La Tronche, France
e-mail: t.pelat@wanadoo.fr; pthullier@yahoo.com

M. Hust
Institut für Biochemie und Biotechnologie, Technische Universität, Braunschweig, Germany
e-mail: m.hust@tu-bs.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_7,

in clinical trials (Rosenwasser et al. 2003; Schopf 2001). They were, however, not marketed so far, for unknown reasons.

The use of NHP to isolate therapeutic antibodies is more particularly obvious when it comes to the generation of immune libraries. Immune libraries were conceived to harvest the high-affinity antibodies delivered by the adaptative immune system and, in humans, no less than five mechanisms allow bringing diversity to the IgG repertoire for that purpose, before selecting the best binders. Immunizing Humans is, however, associated with ethical and practical difficulties if immunization is not realized as a vaccination or naturally encountered after a disease. Immunizing NHP allows to alleviate these difficulties while still harvesting the potency of immune libraries, and Ab fragments with affinities from nanomolar to picomolar (Chassagne et al. 2004; Glamann and Hirsch 2000; Laffly et al. 2005; Pelat et al. 2007) have been directly isolated from NHP-immune libraries. The high affinity of NHP antibodies isolated from immune libraries may be their second quality, after their human-like characteristic, but other characteristics of NHP Abs may also appeal for their use in therapeutics. The possibility to target various regions of the immunogen is certainly of great interest when searching for neutralizing epitopes or for signaling, synergistic or new activities. An immune library of adequate size and diversity ($\geq 10^8$ clones) probably offers better chances to target any particular region of an immunogen than the far less diversity studied when screening hybridomas, for instance, or when utilizing naïve libraries of which only a small proportion might be reacting with the antigen (Amersdorfer et al. 2002). The lack of cross-reactivity with human proteins that are not targeted is perhaps the fourth highly desirable characteristics of Abs intended for medical use. This characteristic has a significant importance, as such cross-reactivity would, if detected in preclinical studies for instance, practically block further development of the antibody. Libraries made from immunized humans after re-arranged V(D) and J genes have been edited by the immune system to avoid autoimmunity, certainly offer the best warranty against such cross-reactivity – although it is not absolute as mutated genes coding IgGs are not edited. Similarly, immune libraries built from NHP also offer a level of safety against cross-reactivity, due to their editing and the human/NHP protein similarities. This safety aspect of NHP-immune libraries is in opposition to the use of synthetic libraries, in particular.

7.1.2 Legal Aspects

The strategy of obtaining antibody fragments of NHP origin for therapeutic purposes is covered by a patent in Europe (Newman et al. 1992) and by another in the United States (Newman et al. 1997). In the European patent, macaques are presented as having human-like antibodies, but all their other proteins are different from their human counterparts. As a consequence, macaques can raise an immune response against human proteins (not part of the macaque's self) and yield human

antibodies. As a second and very significant consequence, only antibodies directed against human antigens are protected by the European patent and, in particular, no rights are due if the antigen is of infectious origin. In the US, all macaque antibodies (and fragments thereof) are covered by the patent. The two patents are otherwise comparable and based on a number of comparisons of human/NHP antibodies sequences. They also claim certain primers for the amplification of macaque variable domains, which are, however, different from the primers indicated in this chapter. Additional legal advice may be sought, but scientists in Europe have already limited possibilities to enter the field of macaque antibodies and to utilize them in medicine. In the US, it might be possible to utilize NHP-derived antibodies in medicine with a license or to apply the results to medical use only after the patent will have expired.

In this chapter, detailed protocols will be given for macaque immunization, and for the first steps of library construction. Planning procedures and following steps present no peculiarities compared to other libraries constructions and will be found elsewhere in the present handbook.

7.2 Materials

7.2.1 Macaque Immunization and Bone Marrow (BM) Sampling

- A specimen of the Macaca fascicularis species (around 5 kg), often originating from Chinese breeding farms, will be bought from a qualified importer (for instance, CynoConsulting, Baziège, France). It will be delivered after quarantine, with its CITES (Convention for Trade on Endangered Species) certificate and proof that it has been tested negative at least for tuberculosis and herpes
- 0.5 mg of the antigen in the form of a soluble protein, or 10^{11} inactivated virus
- Freund's complete and incomplete adjuvants (Sigma, F5881 and F5506)
- Standard ELISA material

7.2.2 RNA Extraction

- Sonicator Sonicator: Vibracell 7240S (Bioblock scientifique)
- RNAse AWAY (Molecular Bio-products, Cat. n° 7002)
- TRi reagent (Molecular research center Inc, Cat. n° TR118)
- BCP (Molecular research center Inc, Cat. n°BP-ISI)
- Isopropanol, ethanol 75% (molecular grade)

7.2.3 RT-PCR, Library Precloning

- SuperScript II Reverse Transcriptase (Invitrogen, Cat. n°18064-014)
- Primers as indicated in Tables 7.1, 7.2 and 7.3
- Taq DNA polymerase, recombinant (Invitrogen, Cat. 10342-020)
- pGemT Vector System (Promega, Cat. A3600)
- Electroporation device and material
- Electrocompetent Sure bacteria (*Escherichia coli*) (Stratagene, Cat. 200227)
- Nucleobond AX (Macherey-Nagel, Cat. 740 573-100)

7.2.4 Amplifying VH and VL

- Red Taq (Sigma, Hamburg) + 10× buffer
- dNTP mix (10 mM each)
- Oligonucleotide primer (see Table 7.1)
- Agarose (Serva, Heidelberg)
- TAE-buffer 50× (2-M TrisHCl, 1-M acetic acid, 0.05 M EDTA pH 8)
- Nucleospin Extract 2 Kit (Macherey-Nagel, Düren)

Table 7.1 Primers used to amplify DNA coding macaque Kappa light chains and Fd (γ1) fragments

1.1.1. K 5′ primers (with *Sac*I site):
5′-GACATCGAGCTCACCCAGTCTCCA-3′
5′-GACATCGAGCTCACCCAGTCTCC-3′
5′-GATATTGAGCTCACTCAGTCTCCA-3′
5′-GAAATTGAGCTCACGCAGTCTCCA-3′
5′-GAAATTGAGCTCACACAGTCTCCA-3′
5′-GAGCCGCACGAGCCCGAGCTCCAGATGACCC AGTCTCC-3′
5′-GAGCCGCACGAGCCCGAGCTCGTGTTGACA CAGTCTCC-3′
1.1.2. K 3′ primer (with *Xba*I site) :
5′GCGCCGTCTAGAATTAACACTCTCCCC TGTTGAAGCTCTTTGTGACGGGCGAACTCAG-3′
1.2.1. Fd (γ1) 5′ primers (with *Xho*I site):
5′-CAGGTGCAGCTCGAGCAGTCTGGG-3′
5′-CAGGTGCAGCTGCTCGAGTCTGGG-3′
5′-CAGGTGCAGCTACTCGAGTCGGG-3′
5′-GAGGTGCAGCTCGAGGAGTCTGGG-3′
5′-GAGGTGCAGCTGCTCGAGTCTGGG-3′
5′-CAGGTACAGCTCGAGCAGTCAGG-3′
5′-AGGTGCAGCTGCTCGAGTCTGG-3′
5′-CAGGTGCAGCTGCTCGAGTCGGG-3′
5′-CAGGTGCAGCTACTCGAGTGGGG-3′
1.2.2. Fd (γ1) 3′ primer (with *Spe*I site):
5′-AGGTTTACTAGTACCACCACATGTTTTGATCTC′-3′

Table 7.2 Primers used to amplify DNA coding macaque Lambda variable region

2.1. λ 5′ primers
5′ CAG TCT GTG CTG ACT CAG CCA CC 3′
5′ CAG TCT GTG YTG ACG CAG CCG CC 3′
5′ CAG TCT GCC CTG ACT CAG CCT 3′
5′ TCC TAT GWG CTG ACW CAG CCA CC 3′
5′ TCT TCT GAG CTG ACT CAG GAC CC 3′
5′ CTG CCT GTG CTG ACT CAG CCC 3′
5′ CAG CYT GTG CTG ACT CAA TCR YC 3′
5′ CAG SCT GTG CTG ACT CAG CC 3′
5′ AAT TTT ATG CTG ACT CAG CCC CA 3′
5′ CAG RCT GTG GTG ACY CAG GAG CC 3′
5′ CAG SCW GKG CTG ACT CAG CCA CC 3′
2.2. λ 3′ primer
5′ TGA ACA TTC TGT AGG GGC CAC TG 3′

Table 7.3 Primers used for the 2. amplification of scFv-coding DNA for the heavy (H) or κ light (K) chain

VH Primer:
MHMacVH-NcoI_f1 : 5′ GTCCTCGCACCATGGCCSAGGTGCAGCTCGAGSAGTCTGGG 3′
MHMacVH-NcoI_f2 : 5′ GTCCTCGCACCATGGCCCAGGTGCAGCTRCTCGAGTCKGG 3′
MHMacVH-NcoI_f3 : 5′ GTCCTCGCACCATGGCCSAGGTGCAGCTGCTCGAGTCKGG 3′
MHMacVH-NcoI_f4 : 5′ GTCCTCGCACCATGGCCCAGGTACAGCTCGAGCAGTCAGG 3′
MHMacVH-NcoI_f5 : 5′ GTCCTCGCACCATGGCCAGGTGCAGCTGCTCGAGTCTGG 3′
MHMacVH-NcoI_f6 : 5′ GTCCTCGCACCATGGCCCAGGTGCAGCTACTRGAGTSGGG 3′
MHMacIgGCH1scFv-HindIII_r : 5′ GTCCTCGCAAAGCTTTGGGCCCTTGGTGGA 3′
Kappa Primer:
MHMacVK-MluI_f1 : 5′ ACCGCCTCCACGCGTAGAHATCGAGCTCCANCAGTCTCC 3′
MHMacVK-MluI_f6 : 5′ ACCGCCTCCACGCGTAGAGCTWCAGATGACMCAGTCTCC 3′
MHMacKappaCL-NotI_r : 5′ ACCGCCTCCGCGGCCGCGACAGATGGTGSAGCCAC 3′
Lambda Primer:
MHMacVL-MluI_f1 : 5′ ACCGCCTCCACGCGTACAGTCTGTGCTGACTCAGCCRCC 3′
MHMacVL-MluI_f2 : 5′ ACCGCCTCCACGCGTACAGTCTGCCCTGACTCAGCCT 3′
MHMacVL-MluI_f3 : 5′ ACCGCCTCCACGCGTATCCTATGAGCTGACWCAGCCACC 3′
MHMacVL-MluI_f4 : 5′ ACCGCCTCCACGCGTATCTTCTGAGCTGACTCAGGACCC 3′
MHMacVL-MluI_f5 : 5′ ACCGCCTCCACGCGTACWGCCTGTGCTGACTCAGCC 3′
MHMacVL-MluI_f6 : 5′ ACCGCCTCCACGCGTACAGCCGGCCTCCCTCTCAGCATCT 3′
MHMacVL-MluI_f7 : 5′ ACCGCCTCCACGCGTACAGRCTGTGGTGACYCAGGAGCC 3′
MHMacVL-MluI_f8 : 5′ ACCGCCTCCACGCGTACAGCCTGTGCTGACTCAGCCA 3′

[a]Primers' names indicate whether they hybridize to DNA encoding the variable (V) or constant (C) region for the heavy (H) or κ light (K) chain

[b]Lowercase letters indicate additional sequences to facilitate digestion; boldfaced lowercase letters indicate restriction sites; and capital letters indicate the parts encoding antibody genes

7.2.5 Cloning VH and VL

- NotI (NEB)
- MluI (NEB, Frankfurt)
- NcoI (NEB)

- HindIII (NEB)
- Buffer 3 (NEB)
- Buffer 2 (NEB)
- BSA (NEB)
- Calf intestine phosphatase (CIP) (MBI Fermentas, St. Leon-Rot)
- T4 ligase (Promega, Mannheim)
- 3M sodium acetate pH5.2
- *E. coli* XL1-Blue MRF (Stratagene, Amsterdam), genotype: Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)]
- Electroporator MicroPulser (BIO-RAD, München)
- 2M Glucose (steril filtered)
- 2M Magnesium solution (1-M MgCl, 1-M $MgSO_4$) (autoclaved)
- SOC medium pH 7.0 (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 20 mM Mg solution, 20 mM glucose) (sterilize magnesium and glucose separately, add solutions after autoclavation)
- 2xYT-medium pH 7,0 (1,6% (w/v) tryptone, 1% (w/v) yeast extract, 0,5% (w/v) NaCl)
- 2xYT-GAT (2xYT + 100 mM Glucose + 100 μg/mL ampiciline + 20 μg/mL tetracycline)
- Ampiciline (100-mg/mL stock)
- Tetracycline (10-mg/mL stock)
- 9cm Petri dishes
- 25cm square Petri dishes (“pizza plates”)
- 2xYT-GAT agar plates (2xYT-GAT, 1.5% (w/v) agar-agar)
- Nucleobond Plasmid Midi Kit (Macherey-Nagel)
- Glycerol of 99.5% (Roth, Karlsruhe)

7.2.6 Library Packaging

- 2xTY media pH 7.0 (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl)
- 2xTY-GA (2xTY, 100 mM glucose, 100 μg/mL ampicillin)
- M13K07 Helperphage for monovalent display (Stratagene)
- Hyperphage for oligovalent display (Progen, Heidelberg)
- 2xTY-AK (2xTY, containing 100 μg/mL ampicillin, 50 μg/mL kanamycin)
- Sorval Centrifuge RC5B Plus, rotor GS3 and SS34 (Thermo Scientific, Waltham)
- Polyethylenglycol (PEG) solution (20% (w/v) PEG 6000, 2.5 M NaCl)
- Phage dilution buffer (10 mM TrisHCl pH7.5, 20 mM NaCl, 2 mM EDTA)
- Mouse α-pIII monoclonal antibody PSKAN3 (Mobitec, Göttingen)
- Goat α-mouse IgG alcaline phosphatase (AP) conjugate (Sigma, Hamburg)

7.3 Methods

7.3.1 Macaque Immunization and BM Sampling

1. After arrival, the animal should be given one month for acclimation
2. 5 ml of bone marrow (BM) should be sampled at the end of the acclimation period and RNA should be extracted and utilized in RT-PCR. Work will not proceed beyond the observation of PCR products on gel, as no amplification should be observed from a healthy animal
3. After preimmune blood will have been drawn (store the serum at −20°C), prime (D0) with two simultaneous subcutaneous injections (200 μL each, representing 50 μg of immunogen – or 10^{10} inactivated virions – plus 100 μL of Freund's complete adjuvant), performed in the shaven back of the animal
4. Superficial granuloma generally appear within a few weeks of Freund's complete adjuvant injection (and, to a lesser extent, after incomplete adjuvant use) and have to be operated to prevent their ulceration.
5. At 1 month intervals, boost twice (D30, D60) as for priming, except that Freund's incomplete adjuvant will replace the complete adjuvant and immune serum will be drawn.
6. At D90, immune serum should be drawn and tested in ELISA against the antigen. Titres should have progressively increased to 1:100,000 at least. Continue immunization if needed or proceed as below according to the result.
7. The macaque should be put at rest for two additional months and until all surgical procedures ended. At the end of this period (D140), BM (5 ml) should be re-sampled and utilized as above. Slight or no amplification of H and L PCR products should be observed on gel.
8. On D150, the last boost should be performed as previously and BM should be sampled on D157 and D160 after boosting. Sites of BM sampling should be rotated between humerus and iliac crests.
9. The following dates of sampling should be adapted to the pace of the macaque's response, as seen from the gel. When the increase in response is slow, sample each week until a decrease is observed on gel; when the increase is rapid so that band intensities are expected to reach a maximal intensity in the following days, sampling twice a week for a maximum of 2 weeks maximum is not deleterious to the animal.

7.3.2 RNA Extraction

1. Treat all materials (pipet and sonicator extremities, benchtop) with RNAse AWAY (sterile plastic tubes of 2, 15, and 50 mL do not need to be treated) and wear gloves.

2. Centrifuge (4°C, 500 *g*, 10 min) the BM, and discard (or store at −20°C for future ELISA) the supernatant.
3. Resuspend the cells in 24 ml of TRI reagent
4. On ice, sonicate the cells for 1 min with pulses of 10 watts and a duration of 6 s, separated by pauses of 1 s.
5. Incubate for 5 min at RT.
6. Centrifuge (4°C, 2,500 *g*, 10 min), and transfer the supernatant in new tubes.
7. Add 2.4 mL of BCP to the supernatant, vortex (15 s).
8. Incubate for 15 min at RT.
9. Centrifuge (4°C, 17,500 *g*, 15 min), and transfer the supernatant in a new tube.
10. Add 12-mL isopropanol, vortex (15 s), and incubate for 10 min at RT.
11. Centrifuge (4°C, 17,500 *g*, 10 min), and discard the supernatant.
12. Add 1.5-mL ethanol 75%, centrifuge (4°C, 17,500 *g*, 10 min), discard the supernatant, and then let the RNA dry at RT.
13. Dissolve RNA in water, quantify on spectrometer: approximately, 200 μg of total RNA should have been purified from 5 ml of BM.

7.3.3 RT-PCR, Library Precloning

1. RT-PCR is performed according to the SuperScript II Reverse Transcriptase protocol, with 20 μg of RNA per reaction. Several (less than 5) RT reactions might be performed in parallel and utilized or stored at −20°C in case of future need because cDNA is more stable than RNA for storage.
2. For PCR, the following Fd and Kappa master mix will be prepared (optionally, for scFv libraries only, an additional Lambda master mix might be prepared to include lambda variable regions in the library) (primers are indicated on Tables 7.1 and 7.2):

(μL)	Fd master mix	Kappa master mix	Lambda master mix (optional)
Water	275	220	330
Buffer 10x	50	40	60
$MgCl_2$	15	12	18
dNTP	10	8	12
3′ primers (5 μM)	60 μL of Fd (γ1) 3′ primer (Table 1.2.2.)	48 μL of K 3′ primer (Table 1.1.2.)	72 μL of λ 3′ primer (Table 2.2.)
cDNA	20	16	24
Taq polymerase	10	8	12

3. 6 μL of each 5′ primers (5 μM) will be added to 44 μL of each corresponding master mix to obtain the following PCR reactions:

(a) Each Fd (γ1) 5′ primer (Table 1.2.1.) will be added to the Fd master mix to give nine separate PCR reactions of 50 μL each.
(b) Each K 5′ primer (Table 1.1.1.) will be added to the Kappa master mix to give seven separate PCR reactions of 50 μL each.
(c) Optionally (for scFv libraries only, and in addition to H and Kappa PCR reactions), each λ 5′ primer (Table 2.1.) will be added to the Lambda master mix to give 11 separate PCR reactions of 50 μL each.

4. The PCR is to be performed as follows:

(a) 94°C for 3 min
(b) 40 cycles of

- 94°C for 45 s
- 52°C for 30 s
- 72°C for 90 s

(c) 72°C for 5 min

5. After PCR, 5 μL of each product will be visualized on an agarose gel 0.8% and only Fd, kappa (and optional lambda) reactions showing amplification will be pooled.
6. Pooled Fd, kappa (and optional lambda) PCR products will be ligated in pGemT, with ligation reactions prepared as follows:

μL	Negative control	Fd ligation	Kappa ligation	Lambda ligation (optional)
Buffer 2x	5	5	5	5
pGemT	1	1	1	1
PCR products	–	3 μL of pooled H reactions	3 μL of pooled Kappa reactions	3 μL of pooled Lambda reactions
Ligase	1	1	1	1
Water	3	–	–	–

(PCR products that will not be ligated can be stored at −20°C)

7. The 3 (optionally 4) ligation reactions will be incubated at 4°C overnight and then stored at −20°C.
8. BM sampling, RNA extraction, and RT-PCR are performed until the macaque response decreases, as evaluated on gel (for the pace of sampling, see "macaque immunization," above). Then, the best sample should be chosen according to band intensities and to the highest number of pairs of primers allowing amplification (on Figure 1, the best sample corresponds to Day 7).
9. The three (optionally four) ligation reactions corresponding to the best sample are separately electroporated in Sure bacteria to obtain no more than 100 colonies on the negative control and between 10^4 and 10^5 colonies for each H, kappa (and optional lambda) ligations
10. Electroporated DNA is isolated by Nucleobond AX, according to the manufacturer's protocol.

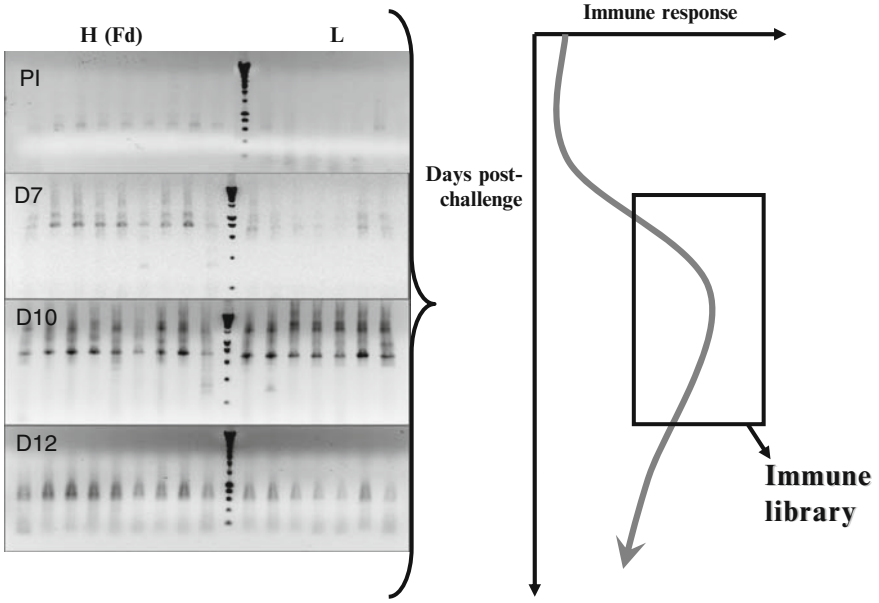


Fig. 7.1 NHP immune response, an example. Follow-up of the immune response by iterative BM sampling and RT-PCR. RT-PCR was performed with primers specific for Fd and kappa light chain amplification

7.3.4 Amplifying VH and VL

1. The amplified DNA can be cut with enzymes corresponding to the restriction sites present in the primers for cloning in vectors bearing corresponding cloning sites (pComb family of vectors, for instance, to be requested from Pr C. Barbas, see notes) in order to obtain Fab libraries. To obtain a scFv library, cDNA (needs two amplifications, first, amplifying the VH/VL genes and second, reamplifying VH/VL and adding the restriction sites) or the precloned material can be used as starting material. Here, the procedure using precloned VH/VL is described.
2. In this PCR, the restriction sites for library cloning will be added. Set up the PCR reactions as follows (18 × 100 µL master mix for 16 PCR reactions):

Solution or component	Volume	Final concentration
dH_2O	1,500 µL	–
RedTaq buffer (10×)	180 µL	1×
dNTPs (10 mM each)	36 µL	200 µM each
RedTaq 1 U/µl	72 µL	2 U

Note: For an immune library four light chains ligations/transformations and eight VH ligations are usually sufficient. Also prepare and digest adequate

amounts of pHAL14 and VL for the first cloning step and pHAL14-VL library and VH for the second cloning step. Keep kappa and lambda in all steps (cloning, packaging) separately and mix only after phage production before panning.

3. Divide the master mix in 600 μL for VH, 200 μL for kappa and 800 μL for lambda.
4. Add the corresponding reverse primers (see also Table 1) to each of the three reactions as follows:

Antibody gene	Primer	Volume	Final concentration
VH	MHMacIgGCH1scFv-HindIII_r (10 μM)	12 μL	0.2 μM
kappa	MHMacKappaCL-NotI_r (10 μM)	4 μL	0.2 μM
lambda	MHMacLambdaCL-NotI_r (10 μM)	16 μL	0.2 μM

5. Add the precloned VH/VL genes of the first PCR as follows:

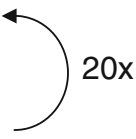
VH	600 ng
Kappa	200 ng
Lambda	1,600 ng

6. Divide the solutions to six (VH), two (kappa) and eight (lambda) PCR reactions, each with 98 μl and add 2 μL (10 μM, 0.2 μM final concentration) to the subfamily-specific forward primer (see also Table 3):

VH:	(1) MHMacVH-NcoI_f1, (2) MHMacVH-NcoI_f2, (3) MHMacVH-NcoI_f3, (4) MHMacVH-NcoI_f4 (5) MHMacVH-NcoI_f5, (6) MHMacVH-NcoI_f6
Vkappa:	(7) MHMacVK-MluI_f1, (8) MHMacVK-MluI_f6
Vlambda:	(9) MHMacVL-MluI_f1, (10) MHMacVL-MluI_f2, (11) MHMacVL-MluI_f3, (12) MHMacVL-MluI_f4, (13) MHMacVL-MluI_f5, (14) MHMacVL-MluI_f6 (15) MHMacVL-MluI_f7, (16) MHMacVL-MluI_f8

7. Carry out the PCR using the following program:

94°C	1 min
94°C	1 min
57°C	1 min
72°C	2 min
72°C	10 min



8. Separate the PCR products by 1.5% TAE agarose gel electrophoresis, cut out the amplified antibody genes (VH: ~400 bp, kappa/lambda: ~400 bp) and purify the PCR products using a gel-extraction kit according to the manufacturer's instructions. Pool all VH, kappa and lambda subfamilies separately. Determine the DNA concentration. Store the three purified second PCR pools at −20°C.

7.3.5 Cloning VH and VL

1. In the first step, prepare pHAL14 vector for library cloning
2. Digest the vector and the VL PCR products: (Pelat et al. 2007, Kirsch et al. 2008)

Solution or component	Volume	Final concentration
dH_2O	83−x μL	–
pHAL14 or VL	x μL	5 μg or 2 μg
NEB buffer 3 (10×)	10 μL	1×
BSA (100×)	1 μL	1×
NEB MluI (10 U/μl)	3 μL	30 U
NEB NotI (10 U/μl)	3 μL	30 U

Note: Always perform single digests using only one enzyme in parallel to control the success of the restriction reaction. Analyze the digestion by TAE agarose gel electrophoresis by comparing with the undigested plasmid. Use only material where both single digests are successful and where no degradation is visible in the double digest

3. Incubate the digestion at 37°C for 2 h. Control the digest of the vector by using a 1-μL aliquot on 1.5% TAE agarose gel electrophoresis. If the vector is not fully digested, extend the incubation time
4. Inactivate the enzymes at 65°C for 10 min
5. Add 0.5 μL CIP (1 U/μL) and incubate at 37°C for 30 min Repeat this step once
6. Purify the vector and the PCR product using a PCR purification Kit according to the manufacturers instructions and elute with 50-μL elution buffer or water. The short stuffer fragment containing multiple stop codons between MluI and NotI in pHAL14 will be removed. Determine the DNA concentration
7. Ligate the vector pHAL14 (4,255 bp) and VL (~380 bp) as follows:

Solution or component	Volume	Final concentration
dH_2O	89-x-y μL	–
pHAL14	x μL	1,000 ng
VL (kappa or lambda)	y μL	270 ng
T4 ligase buffer (10x)	10 μL	1×
T4 ligase (3 U/μL)	1 μL	3 U

8. Incubate at 16°C overnight
9. Inactivate the ligation at 65°C for 10 min
10. Precipitate the ligation with 10-μL 3-M sodium acetate pH 5.2 and 250-μL ethanol, incubate for 2 min at RT and centrifuge for 5 min at 16,000× *g* and 4°C
11. Wash the pellet with 500-μL 70% (v/v) ethanol and pellet the DNA for 2min at 16,000× g and 4°C. Repeat this step once and resolve the DNA pellet in 35-μL dH_2O
12. Thaw 25-μL electrocompetent XL1-Blue MRF' on ice and mix with the ligation reaction

Table 7.4 Primers for colony PCR

MHLacZ-Pro_f : 5′ GGCTCGTATGTTGTGTGG 3′
MHgIII_r : 5′ CTAAAGTTTTGTCGTCTTTCC 3′

13. Transfer the 60-μL mix to a prechilled 0.1-cm cuvette. Dry the electrode of the cuvette with a tissue paper
14. Perform a 1.7 kV pulse using an electroporator. The pulse time should be between 4 and 5 ms for optimal electroporation efficiency. Immediately, add 1 mL 37°C prewarmed SOC medium, transfer the suspension to a 2-mL cap and shake for 1 h at 600 rpm and 37°C
15. To determine the amount of transformants, use 10 μL ($=10^{-2}$ dilution) of the transformation and perform a dilution series down to 10^{-6} dilution. Plate out a 10^{-6} dilution on 2xYT-GAT agar plates and incubate overnight at 37°C
16. Plate out the remaining 990 μL on 2xYT-GAT agar "pizza plate" and incubate overnight at 37°C
17. Calculate the amount of transformants, that should be 1×10^6–5×108 cfu. Control colonies for a full size insert by colony PCR using the primers MHLacZ-Pro_f and MHgIII_r (Table 3). The PCR product should be about 740 bp with VL and 375 bp for pHAL14 without insert. Throw away sublibraries with less than 90% inserts
18. Float-off the colonies on the "pizza plate" with 40 mL 2xYT medium using a drigalsky spatula. Use a 5-mL bacteria solution for midi plasmid preparation according to the manufacturer's instructions. Determine the DNA concentration
19. For the second cloning step, digest the pHAL14-VL repertoire and the VH PCR products (always perform-single enzyme digest of the vector in parallel):
20. Incubate at 37°C for 2 h. Control the digest of the vector by using a 1 μL aliquot on 1.5% agarose gel electrophoresis
21. Inactivate the digestion at 65°C for 10 min
22. Add 0.5 μL CIP (1 U/μL) and incubate at 37°C for 30 min Repeat this step once
23. Purify the vector and the PCR product using a PCR purification Kit according to the manufacturer's instructions and elute with 50 μL elution buffer or water. The short stuffer fragment between NcoI and HindII in pHAL14 will be removed. Determine the DNA concentration
24. Ligate the vector pHAL14-VL (~4,610 bp) and VH (~380 bp) as follows:

Solution or component	Volume	Final concentration
dH_2O	89-x-y μL	–
pHAL14	x μL	1,000 ng
VH	y μL	250 ng
T4 ligase buffer (10×)	10 μL	1×
T4 ligase (3 U/μL)	1 μL	3 U

25. Incubate at 16°C overnight
26. Inactivate the ligation at 65°C for 10 min

27. Precipitate the ligation with 10 µL 3 M pH5.2 sodium acetate and 250 µL ethanol, incubate for 2 min at RT and centrifuge for 5 min at 16,000× *g* and 4°C
28. Wash the pellet with 500 µL 70% (v/v) ethanol and pellet the DNA for 2 min at 16,000× g and 4°C. Repeat this step once and resolve the pellet in 35 µL dH_2O
29. Thaw 25 µL electrocompetent XL1-Blue MRF' on ice and mix with the ligation reaction
30. Transfer the 60 µL mix to a prechilled 0.1 cm cuvette. Dry the electrode of the cuvette with a tissue paper
31. Perform a 1.7 kV pulse using an electroporator. The pulse time should be between 4 and 5 ms for optimal electroporation efficiency. Immediately, add 1 mL 37°C prewarmed SOC medium, transfer to a 2 mL cap and incubate for 1 h at 600 rpm
32. To determine the amount of transformants, use 10 µL ($=10^{-2}$ dilution) of the transformation and perform a dilution series down to 10^{-6} dilution. Plate out a 10^{-6} dilution on 2xYT-GAT agar plates and incubate overnight at 37°C
33. Plate out the remaining 990 µL on 2xYT-GAT agar "pizza plate" and incubate overnight at 37°C
34. Calculate the amount of transformants (1×10^6–5×10^8 should be reached to be included into the final library). Control colonies for full-size insert by colonie PCR using the primers MHLacZ-Pro_f and MHgIII_r (Table 4). The Inserts should be about 1,100 bp. Throw away all sublibraries with less than 75% full-size inserts
35. Float-off the colonies on the "pizza plate" with a 40-mL 2xYT medium using a drigalsky spatula. Use 800 µL bacteria solution and 200 µL glycerol for glycerol stocks. Make 5–25 glycerol stocks per sublibrary and store at −80°C
36. When all transformations (about eight) are done, thaw one aliquot of each sublibrary on ice, mix all sublibraries and make new aliquots for storage at −80°C

7.3.6 Library Packaging

1. To package the library, inoculate 400-mL 2xTY-GA in a 1-L Erlenmeyer flask with 1-mL antibody gene library stock. Grow at 250 rpm at 37°C up to an O.D.600 nm~0.5.
2. Infect 25-mL bacteria culture (~1.25 × 1,010 cells) with 2.5 × 1,011 colony-forming units (cfu) of the helper phage M13K07 or Hyperphage (Rondot et al. 2001, Soltes et al. 2007) according to a multiplicity of infection (moi) = 1:20 (see note 10). Incubate 30 min without shaking and the following 30 min with 250 rpm at 37°C.
3. To remove the glucose which represses the lac promoter of pHAL14, and therefore,the scFv::pIII fusion protein expression, harvest the cells by centrifugation for 10 min at 3,200× *g* in 50-mL polypropylene tubes.
4. Resuspend the pellet in 400-mL 2xTY-AK in a 1-L Erlenmeyer flask. Produce scFv-phage overnight at 250 rpm and 30°C.

5. Pellet the bacteria by centrifugation for 10 min at 10,000× *g* in two GS3 centrifuge tubes. If the supernatant is not clear, centrifuge again to remove the remaining bacteria.
6. Precipate the phage from the supernatant by adding 1/5 volume PEG solution in two GS3 tubes. Incubate for 1 h at 4°C with gentle shaking, followed by centrifugation for 1 h at 10,000× *g*.
7. Discard the supernatant, resolve each pellet in a 10-mL phage dilution buffer in SS34 centrifuge tubes and add 1/5 volume PEG solution.
8. Incubate on ice for 20 min and pellet the phage by centrifugation for 30 min at 10,000× *g*.
9. Discard the supernatant and put the open tubes upside down on tissue paper. Let the viscous PEG solution move out completely. Resuspend the phage pellet in a 1-mL phage dilution buffer. Titre the phage preparation. Store the packaged antibody phage library at 4°C.
10. The library packaging should be controlled by 10% SDS-PAGE, Western-Blot and anti-pIII immunostain (mouse anti-pIII 1:2,000, goat anti-mouse IgG AP conjugate 1:10,000). Wildtype pIII has a calculated molecular mass of 42.5 kDa, but it runs at an apparent molecular mass of 65 kDa in SDS-PAGE. Accordingly, the scFv::pIII fusion protein runs at about 95 kDa.

7.4 Notes

1. The animal house should be accredited for NHP housing, and an approval should have been obtained from an ethic committee before beginning the work, according to local regulations
2. if If ELISA titres do not appear to rise after immunization, denaturation of the antigen may have occurred during coating. Full functional proteins (eventually in the presence of co-factor) may be preferred to subunits for coating, to try to prevent that denaturation
3. Low RNA yield might testify the poor quality of the BM sample (constituted of blood rather than BM)
4. Scientists may request vectors of the pComb family from Pr Carlos F. Barbas III (carlos@scripps.edu)
5. When almost no PCR product was obtained before library construction and products were obtained as photographied above, these products can be regarded specifically as coding for antibodies directed against the immunogen; and the probability of the successful isolation of antibody fragments is high

References

Amersdorfer P, Wong C, Smith T, Chen S, Deshpande S, Sheridan R, Marks JD (2002) Genetic and immunological comparison of anti-botulinum type A antibodies from immune and non-immune human phage libraries. Vaccine 20:1640–1648

Andris JS, Miller AB, Abraham SR, Cunningham S, Roubinet F, Blancher A, Capra JD (1997) Variable region gene segment utilization in rhesus monkey hybridomas producing human red blood cell-specific antibodies: predominance of the VH4 family but not VH4–21 (V4–34). Mol Immunol 34:237–253

Barbas CF (2001) Phage display: a laboratory manual, protocol 10.5. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Chassagne S, Laffly E, Drouet E, Herodin F, Lefranc MP, Thullier P (2004) A high-affinity macaque antibody Fab with human-like framework regions obtained from a small phage display immune library. Mol Immunol 41:539–546

Glamann J, Hirsch VM (2000) Characterization of a macaque recombinant monoclonal antibody that binds to a CD4-induced epitope and neutralizes simian immunodeficiency virus. J Virol 74:7158–7163

Kawamura S, Omoto K, Ueda S (1990) Evolutionary hypervariability in the hinge region of the immunoglobulin alpha gene. J Mol Biol 215:201–206

Kawamura S, Saitou N, Ueda S (1992) Concerted evolution of the primate immunoglobulin alpha-gene through gene conversion. J Biol Chem 267:7359–7367

Kirsch MI, Hulseweh B, Nacke C, Rulker T, Schirrmann T, Marschall HJ, Hust M, Dübel S (2008) Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV). BMC Biotechnol 8:66

Laffly E, Danjou L, Condemine F, Vidal D, Drouet E, Lefranc MP, Bottex C, Thullier P (2005) Selection of a macaque Fab with framework regions like those in humans, high affinity, and ability to neutralize the protective antigen (PA) of Bacillus anthracis by binding to the segment of PA between residues 686 and 694. Antimicrob Agents Chemother 49:3414–3420

Meek K, Eversole T, Capra JD (1991) Conservation of the most JH proximal Ig VH gene segment (VHVI) throughout primate evolution. J Immunol 146:2434–2438

Newman RA, Hanna N, Raab RW (1997) US patent 5,693,780, 2 December 1997

Newman RA, Hanna N, Raab RW (1992) European patent 1,266,965 B1, 24 Jun 1992

Pelat T, Hust M, Laffly E, Condemine F, Bottex C, Vidal D, Lefranc MP, Dübel S, Thullier P (2007) High-affinity, human antibody-like antibody fragment (single-chain variable fragment) neutralizing the lethal factor (LF) of Bacillus anthracis by inhibiting protective antigen-LF complex formation. Antimicrob Agents Chemother 51:2758–2764

Rondot S, Koch J, Breitling F, Dübel S (2001) A helper phage to improve single-chain antibody presentation in phage display. Nat Biotechnol 19:75–78

Rosenwasser LJ, Busse WW, Lizambri RG, Olejnik TA, Totoritis MC (2003) Allergic asthma and an anti-CD23 mAb (IDEC-152): results of a phase I, single-dose, dose-escalating clinical trial. J Allergy Clin Immunol 112:563–570

Schopf RE (2001) Idec-114 (Idec). Curr Opin Investig Drugs 2:635–638

Soltes G, Hust M, Ng KK, Bansal A, Field J, Stewart DI, Dübel S, Cha S, Wiersma EJ (2007) On the influence of vector design on antibody phage display. J Biotechnol 127:626–637

Chapter 8
Generation of Rabbit Immune Libraries

Rüdiger Ridder and Hermann Gram

8.1 Introduction

The combinatorial cloning of antibody variable domains and the display of antibody fragments on the surface of filamentous phage by fusion to phage coat proteins provide an alternative strategy for the rapid generation of monoclonal antigen-binding proteins. Various antibody-displaying phage libraries have been described, which are based on the B cell repertoire of rearranged immunoglobulin genes from spleen and bone marrow of previously immunized mice (see Sect. 8.3.1). However, the combinatorial library technique may also be applied for the generation of monoclonal antibody fragments from species that are not easily amenable to conventional methodology based on eukaryotic cell fusion techniques, e.g., humans, rabbits, and chicken. The numerous reports about the isolation of functional antibody fragments from combinatorial libraries prepared from peripheral blood lymphocytes from immunized or nonimmunized human donors exemplify the general applicability of this approach (see Sects. 8.3.2–8.3.4).

Since a long time, rabbits are used for the generation of polyclonal antibodies of excellent quality. However, the routine production of monoclonal antibodies from these animals has been hampered by the lack of efficient strategies for the stable immortalization of antibody-expressing B lymphocytes, although some progress has recently been made in the generation of suitable fusion partners (Raman et al. 1994; Spieker-Polet et al. 1995). Therefore, we and others have successfully used the combinatorial library and phage display technologies to produce monoclonal

R. Ridder (✉)
mtm laboratories AG, Im Neuenheimer Feld 583, 69120 Heidelberg, Germany
e-mail: ridder@mtmlabs.com

H. Gram
Novartis Institutes for Biomedical Research, CHBS, WSJ-386.9.27, Novartis Pharma AG, Forum 1, Basel 4056 Switzerland

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_8,

antibody fragments from rabbits (Ridder et al. 1995; Lang et al. 1996; Foti et al. 1998).

Rabbits show exceptional features with respect to the mechanisms that are utilized for the generation of antibody diversity. Studies of the VH gene rearrangements in rabbit B cells revealed that although there are more than one hundred germline genes, preferentially a single VH gene (VH1) is rearranged in 70–90% of all VHa allotype immunoglobulin expressing B-lymphocytes (Knight and Becker 1990; Raman et al. 1994). Furthermore, the similarity of the individual VH genes is generally more than 80%, indicating the affiliation to a single VH gene family (reviewed in Knight 1992; Knight and Crane 1994). The diversity of the VH antibody repertoire was shown to be mainly generated by somatic gene conversion and hypermutation (reviewed in Mage 1998; Becker and Knight 1990; Knight and Becker 1990; Raman et al. 1994). Similarly, rabbit light chain generation is characterized by the preferential expression of a single C gene (C1), which represents more than 90% of all light chains (Heidmann and Rougeon 1983; Hole et al. 1991; Knight and Crane 1994). Additionally, there is only a small number of functional J-segments present in rearranged VL genes (one functional *b4* and two functional *b9* allotype J-segments, Emorine and Max, 1983; Emorine et al. 1983; Akimenko et al. 1986).

Therefore, the limited number of germline immunoglobulin genes used for antibody generation significantly facilitates the molecular cloning of the rabbit antibody repertoire. In contrast to the high number of degenerative oligonucleotide primers necessary for the PCR amplification of both murine and human immunoglobulin genes (see Sects. 8.3.2–8.3.4), only very few primers are required for the representative cloning of the rearranged immunoglobulin gene repertoire from rabbits (Ridder et al. 1995; Gram et al. 1998). For this reason, immunized rabbits represent rather ideal candidate animals for the generation of monoclonal antibody fragments by the combinatorial library and phage display approach.

8.2 Outline

In this chapter, we focus on the description of a general strategy for the PCR amplification and cloning of cDNAs coding for the variable heavy and light chain domains of rabbits. The combinatorial cloning of these VH- and VL-specific PCR fragments allows the subsequent display of rabbit scFv antibody fragments on the surface of filamentous bacteriophage and thus the affinity selection of phage antibodies with the desired antigen specificity. The general outline for the generation and phage display selection of scFv antibody fragments from rabbits is rather comparable to the protocol described for the production of murine scFv antibodies (Sect. 8.3.1). The essential difference solely exists in the selection of specific primers necessary for the PCR amplification of cDNAs coding for rabbit VH and VL antibody domains (listed in Table 8.1).

The combinatorial cloning approach we used for the generation of rabbit scFv antibody fragments is outlined in Fig. 8.1. In general, any of the various cloning

Table 8.1 Oligonucleotide primers for the amplification of rabbit VL and VH antibody domains

VL	
VL–1	5′-AGC ACC GAG CTC GTG MTG ACC CAG ACT CCA-3′
VL–2	5′-AGC ACC GAG CTC GAT MTG ACC CAG ACT CCA-3′
VL–b4J2	5′-TTT GAC GAC CAC CTC GGT CCC-3′
VL–b9J2	5′-TAG GAT CTC CAG CTC GGT CCC-3′
CL-	5′-CTG CGG TGT KTT ACT GTT CTC G-3′
VH	
VH1	5′-CAG TCG GTG GAG GAG TCC RGG-3′
VH2	5′-CAG TCG GTG AAG GAG TCC GAG-3′
VH3	5′-CAG TCG YTG GAG GAG TCC GGG-3′
VH4	5′-CAG SAG CAG CTG GWG GAG TCC GG-3′
CH1-	5′-GAC TGA YGG AGC CTT AGG TTG C-3′

Wobble positions are indicated according to the IUPAC code: M, A/C; K, G/T; R, A/G; Y, C/T; S, C/G; W, A/T. The *Sac*I restriction endonuclease recognition site used for the cloning of the PCR fragments comprising the VL domain is underlined

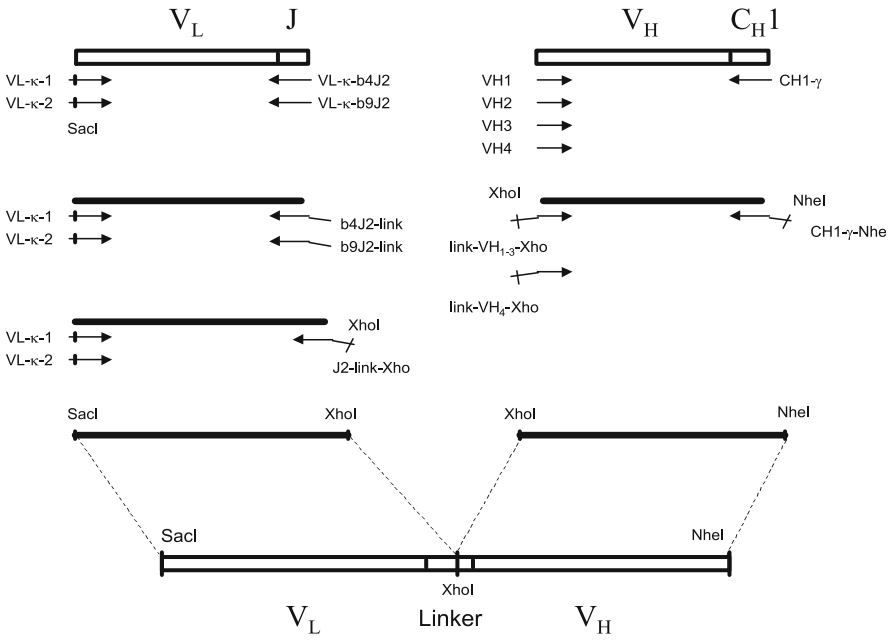


Fig. 8.1 General strategy for the PCR amplification of the expressed VL and VH immunoglobulin gene repertoire from rabbits. Primers used for the subsequent modifying PCR amplifications are listed in Tables 8.1 and 8.2, respectively. Restriction enzymes used for the two-step cloning of the VL and VH domains are indicated

strategies described in the literature might be employed, but may require modifications of the oligonucleotide primers (e.g., by introducing different restriction endonuclease recognition sites) dependent on the used vector and scFv linker sequences.

Additionally, we include primer sequences, which we found suitable for the amplification of the VL and C domains, as well as the VH and CH1 domains for the cloning of rabbit Fab antibodies.

8.3 Materials

8.3.1 Equipment

- Tissue homogenizer for the extraction of RNA from frozen spleen samples (e.g., Ultra Turrax T8, IKA)
- Refrigerated microcentrifuge
- PCR thermocycler.

8.3.2 Reagents

- Rabbit generally used for the production of polyclonal antibodies (e.g., New Zealand white rabbit)
- Adjuvant (e.g., incomplete Freund´s adjuvant)
- Liquid nitrogen (if available) or ethanol/dry ice bath
- *Taq* DNA polymerase and PCR reaction buffer (supplied with the enzyme)
- RNA extraction kit (e.g., Trizol Reagent, Invitrogen Inc.)
- Gel extraction kit (e.g., GENECLEAN II Kit, BIO 101 Inc.)
- PCR product purification kit (e.g., High Pure PCR Product Purification Kit, Roche Diagnostics)
- Oligonucleotide primers for the initial PCR amplification of rabbit VL and VH domains (listed in Table 8.1) as well as for subsequent modifiying PCR reactions for the incorporation of both linker and restriction endonuclease recognition sequences into the PCR fragments (listed in Table 8.2).

Table 8.2 Rabbit VL and VH domain modifying oligonucleotide primers

b4J2-link	5′- acc aga agt aga acc TTT GAC GAC CAC CTC G -3′
b9J2-link	5′- acc aga agt aga acc TAG GAT CTC CAG CTC G -3′
J2-link-Xho	5′- atc cct cga gct tcc cag aac cag aag tag aac cT- 3′
link-VH1-3-Xho	5′- gga agc tcg agg aag gaa aag gcC AGT CGG TGG AGG AG -3′
link-VH4-Xho	5′- gga agc tcg agg aag gaa aag gcC AGS AGC AGC TGG WG -3′
CH1–Nhe	5′- GAG GGC TAG CGA CTG AYG GAG CCT TAG GTT G -3′

Wobble positions are indicated according to the IUPAC code: S, C/G; W, A/T. The *Xho*I restriction endonuclease recognition site introduced into the linker sequence as well as the *Nhe*I site added to the 5′-end of the CH1-specific reverse PCR primer are underlined. Linker sequences are indicated by small letters

8.4 Procedure

8.4.1 Immunization of Rabbits

1. Immunize the rabbit according to standard immunization protocols (typically by subcutaneous injection of 200 μg antigen dissolved in adjuvant, followed by subsequent injections of 100 μg antigen after 4 and 6 weeks).
2. Control for specific antibody titer of the immunized rabbit by performing ELISA assays with serial dilutions (1/1,000–1/20,000) of preimmune control serum and immune serum.

8.4.2 Preparation of B cell fractions

For the preparation of antibody producing B cells, either spleen (Ridder et al. 1995), bone marrow (Lang et al. 1996), or peripheral blood lymphocytes (Foti et al. 1998) may be used. If sufficient serum has been collected, the extraction of RNA from spleen and/or bone marrow from the sacrificed rabbit is recommended.

1. After taking the spleen from the sacrificed rabbit with sterile forceps, put the spleen into a petridish plate, and cut it into 10–12 pieces of about equal size. Transfer each of the pieces into an individual polypropylen tube, and freeze the tubes immediately by placing them into liquid nitrogen or into an ethanol/dry ice bath. Generally, we use 2 ml cryo-tubes to avoid leakiness during freezing and storage.
2. Store the tubes at either −80°C or in liquid nitrogen until use.

8.4.3 RNA Extraction and First Strand cDNA Generation

For total RNA isolation, we generally utilized the acid guanidinium thiocyanate-phenol-chloroform based extraction protocol as described by Chomczynski and Sacchi (1987). However, any commercially available RNA isolation system may be used. Furthermore, mRNA instead of total RNA may be directly isolated from the antibody producing B cells. However, the purification of mRNA is not necessary to obtain material of sufficient quality for the subsequent RT-PCR amplification of the immunoglobulin gene repertoire. In the following, we give some advice concerning critical steps in the preparation of total RNA of adequate quality.

1. Add the guanidinium thiocyanate (GTC) containing RNA extraction buffer directly to a single sample of frozen spleen, thus eliminating any RNAse activity during thawing of the sample in the absence of GTC.

Note: Wear gloves and protection glasses during this and the following steps of the RNA extraction protocol.

2. Homogenize the rabbit spleen sample for about 30 s using a tissue homogenizer. Alternatively, vortex the tube vigourously until the spleen sample is completely disintegrated.
3. Use sufficient amounts of the denaturating RNA extraction buffer. Insufficient quantities of the extraction buffer will result in low RNA yield as well as strong contaminations of the final RNA solution with genomic DNA. Generally, we obtained satisfying results by using about 2 ml of extraction buffer per spleen sample representing one tenth of total rabbit spleen.
4. Control for integrity of RNA by agarose gel electrophoresis. Employing a denaturating sample loading buffer containing formamid, extracted RNA may be separated in 0.8% agarose minigels using standard tris-borate-EDTA buffer (1× TBE).
5. Prepare first strand cDNA by reverse transcription of 3 μg of total RNA using either AMV or M-MLV reverse transcriptase according to the instructions of the manufacturer. For priming of the reverse transcription reaction, either oligo (dT)18, random hexanucleotides pd(N)6, or rabbit immunoglobulin constant domain-specific oligonucleotides CL- (5′-CTG CGG TGT TKT ACT GTT CTC G -3′) and CH1- (5′-GAC TGA YGG AGC CTT AGG TTG C -3′) may be used. We generally obtained good results using 0.2 μg oligo (dT)18 for priming of the reverse transcription reaction of about 3 μg of total RNA.

8.4.4 PCR Amplification of Rabbit VL and VH Antibody Domains

1. For the amplification of rabbit immunoglobulin cDNA, use standard PCR reaction mixtures (total volume: 50 μl) as follows: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTP, 25 pmole of each primer, 2.5 U *Taq* DNA polymerase, and about 1/20 of the first strand cDNA reaction as template. Note: Hot start conditions (e.g., by using TaqStart Antibody, Clontech Laboratories, Palo Alto) significantly increased the yield of PCR products specific for VL and VH antibody domains in our hands.
2. Oligonucleotide primers listed in Table 8.1 are used in individual PCR reactions in the following combinations: VL–1/VL–b4J2, VL–1/VL–b9J2, VL–2/VL–b4J2, and VL–2/VL–b9J2 for the amplification of rabbit VL specific cDNAs; VH1/CH1-, VH2/CH1-, VH3/CH1-, and VH4/CH1- for the amplification of rabbit VH specific cDNAs.
3. After an initial denaturation step for 3 min at 94°C, perform 25–30 cycles of PCR amplification as follows: 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, followed by a final elongation step at 72°C for 7 min.
4. Check 20 μl of the PCR products on 1.2% TAE-buffered agarose gels. PCR products specific for rabbit immunoglobulin variable chain cDNA will result in DNA fragments of 330–340 bp (VL) and of about 400 bp (VH), respectively.

5. Extract the VL and VH specific PCR fragments from the agarose gel by using any commercial gel extraction kit (e.g., Geneclean II, BIO101 Inc.; or QIAquick Geliagen Gel Extraction kit, Qiagen, Hilden, Germany).

8.5 Modification of the VL and VH cDNA Fragments by Subsequent PCR Reactions

For cloning of the antibody variable domain encoding PCR fragments, we generally use a two-step cloning strategy. In contrast to the experimental approach described in Sect. 8.2.2, we usually generate the DNA sequence coding for the scFv linker peptide by 1–2 subsequent rounds of PCR using modifying oligonucleotide primers (listed in Table 8.2). Furthermore, we use a linker initially described by Colcher et al. (1990), which codes for a peptide consisting of 14 amino acids (NH2-gly-ser-thr-ser-gly-ser-gly-lys-ser-ser-glu-gly-lys-gly-COOH). By replacing both serine residues in codon positions 9 and 10 (underlined) with leu-glu residues, a *Xho*I restriction endonuclease recognition site was introduced into the linker sequence. This site was subsequently utilized for joining of the VL and VH encoding cDNA domains.

For the modification of the VL-specific cDNA fragments, two subsequent rounds of PCR reactions are necessary, whereas a single modifying PCR step is sufficient for the VH-specific fragments (see Fig. 8.1).

1. Subject about 1/20 of the purified PCR products obtained after the initial round of VL and VH specific PCRs to the second round of PCR amplifications in individual reactions. Use the primer combinations as shown in Fig. 8.1.
   Note: Primer b4J2-link is employed using the first round VL PCR product generated with reverse primer VL-κ-b4J2, etc., and primer link-VH1-3-Xho is used for the modifying PCR of VH specific products generated with primers VH1, VH2, and VH3, respectively.
2. Perform 25 cycles of PCR amplification using identical conditions as described above. Separate the resulting VL-specific PCR products by agarose gelelectrophoresis, and subsequently extract the DNA fragments from the gel matrix.
3. Perform a second modifying PCR amplification for the VL-specific DNA fragments, using J2-link-Xho as the only reverse primer (see Fig. 8.1).
4. Purify the final VL and VH-specific PCR products using any commercially available PCR product purification kit (e.g., High Pure PCR Product Purification Kit, Roche Diagnostics).
5. Digest the purified PCR products with either restriction endonucleases *Sac*I and *Xho*I (VL) or with *Xho*I and *Nhe*I (VH) for several hours. Separate the cleaved PCR fragments by agarose gel electrophoresis, and subsequently extract the fragments from the gel. The VL and VH fragments are now ready for cloning into a vector suitable for the expression of the rabbit scFv fragments translationally fused to a bacterial leader sequence and to sequences coding for parts of

the minor or major coat proteins of filamentous phage for surface display (e.g., pGEM3-gIII, Ridder et al. 1995).

8.6 Comments

- The restriction enzymes selected for the two-step cloning of the rabbit variable antibody domains (*Sac*I, *Xho*I, and *Nhe*I), which show no cleavage site within the framework sequence of a known rabbit variable domain, may be substituted by other cutting enzymes according to the individual cloning parameters of other plasmid vectors allowing phage display (e.g., by eight-cutters *Sfi*I and *Not*I). Note: it may be necessary to adjust the reading frame at the 3′-end of the scFv fragment when using different cloning sites and phage display vectors.
- For the generation of recombinant Fab fragments from rabbit, the VL and VH forward primers VL–1, VL–2, and VH1 to VH4 may be used in combination with reverse primers specific for the 3′-end of the light chain coding sequence (C–1: 5′- ACA GTC ACC CCT ATT GAA GCT CTG GAC –3′; C–2: 5′- ACA GTT CTT CCT ACT GAA GCT CTG GAC –3′) and for the 3′-end of the first constant domain of the heavy chain (FAB-CH1-Cys230: 5′- GCA TGT CGA GGG TGC AAC GGT CTT GTC CAC TTT G -3′), comprising the cys residue in codon position 230 (underlined, codon position according to Kabat et al. 1991) of the rabbit heavy chain.

References

Akimenko MA, Mariame B, Rougeon F (1986) Evolution of the immunoglobulin light chain locus in the rabbit: evidence for differential gene conversion events. Proc Natl Acad Sci USA 83:5180–5183

Becker RS, Knight KL (1990) Somatic diversification of immunoglobulin heavy chain VDJ genes: evidence for somatic gene conversion in rabbits. Cell 63:987–997

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorofom extraction. Anal Biochem 162:156–159

Colcher D, Bird R, Roselli M, Hardman KD, Johnson S, Pope S, Dodd SW, Pantoliano MW, Milenic DE, Schlom J (1990) In vivo tumor targeting of a recombinant single-chain antigen-binding protein. J Natl Cancer Inst 82:1191–1197

Emorine L, Max EE (1983) Structural analysis of a rabbit immunoglobulin kappa 2 J-C locus reveals multiple deletions. Nucleic Acids Res 11:8877–8890

Emorine L, Dreher K, Kindt TJ, Max EE (1983) Rabbit immunoglobulin genes: structure of a germline *b4* allotype J-C locus and evidence for several *b4*-related sequences in the rabbit genome. Proc Natl Acad Sci USA 80:5709–5713

Foti M, Granucci F, Ricciardi-Castagnoli P, Spreafico A, Ackermann M, Suter M (1998) Rabbit monoclonal Fab derived from a phage display library. J Immunol Methods 213:201–212

Gram H, Schmitz R, Ridder R (1998) Secretion of scFv antibody fragments. Methods Mol Biol 103:179–192

Heidmann O, Rougeon F (1983) Diversity in the rabbit immunoglobulin kappa chain variable regions is amplified by nucleotide deletions and insertions at the V-J junction. Cell 34:767–777

Hole NJ, Young CG, Mage RG (1991) Mapping of the duplicated rabbit immunoglobulin kappa light-chain locus. Eur J Immunol 21:403–407

Kabat TA, Wu TT, Perry HM, Gottesmann KS, Foeller C (1991) Sequences of proteins of immunological interest. US Dept. of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda

Knight KL (1992) Restricted VH gene usage and generation of antibody diversity in rabbit. Annu Rev Immunol 10:593–616

Knight KL, Becker RS (1990) Molecular basis of the allelic inheritance of rabbit immunoglobuline VH allotypes: implications for the generation of antibody diversity. Cell 60:963–970

Knight KL, Crane MA (1994) Generating the antibody repertoire in rabbit. Adv Immunol 56:179

Lang IM, Barbas CFIII, Schleef RR (1996) Recombinant rabbit Fab with binding activity to type-1 plasminogen activator inhibitor derived from a phag-display library against human -granules. Gene 172:295–298

Mage RG (1998) Diversification of rabbit VH genes by gene-conversion-like and hypermutation mechanisms. Immunol Rev 162:49–54

Raman C, Spieker-Polet H, Yam PC, Knight KL (1994) Preferential VH gene usage in rabbit Ig-secreting heterohybridomas. J Immunol 152:3935–3945

Ridder R, Schmitz R, Legay F, Gram H (1995) Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast *Pichia pastoris*. Biotechnology 13:255–260

Spieker-Polet H, Sethupathi P, Yam PC, Knight KL (1995) Rabbit monoclonal antibodies: generating a fusion partner to produce rabbit-rabbit hybridomas. Proc Natl Acad Sci USA 92:9348–9352

Part III
Selection of Antibody Fragments from Combinatorial Libraries

Chapter 9
Immunotube Selections

Roland E. Kontermann

9.1 Introduction

Selection of antibody fragments by "biopanning" on antigen-coated immunotubes is the most commonly used method for the isolation of antibody fragments from phage display libraries (Marks et al. 1991; Harrison et al. 1996; for review see Winter et al. 1994; Hoogenboom et al. 1998). The protocol shown here describes selections from a phagemid library (Vaughan et al. 1996) (see also Chap. 6, Pierre Martineau, this volume) based on phagemid vectors such as pCANTAB6 (McCafferty et al. 1994). In these vectors, the scFv fragment is cloned in front of the gene 3 with an amber stop codon located *C*-terminal of the scFv. For purification and detection, the scFv also contains a Myc-tag and a hexahistidyl-tag at the *C*-terminus preceding the amber stop codon. For the production of phage, the scFv fragment is expressed in fusion with the gene 3 protein propagating the phagemids in suppressor strains such as TG1 (Gibson 1984). The selections are performed by immobilizing the antigen on a plastic surface and subsequent incubation with phage from the antibody library. Unbound phage are eliminated by a washing step and bound phage are eluted and used to infect bacteria. Phage are then rescued by co-infection with helper phage. The resulting phage particles are precipitated from the culture supernatant and are subjected to the next round of selection. Normally, three to four rounds of selections are performed to enrich for specific phage. The success of selection is monitored by polyclonal phage ELISA and monoclonal antibodies are identified by screening of soluble antibody fragments expressed by nonsuppressor strains such as HB2151 (Carter et al. 1981), resulting in the secretion of the antibody fragments into the culture medium (Fig. 9.1).

R.E. Kontermann
Institut für Zellbiologie und Immunologie, Universität Stuttgart, Allmandring 31, Stuttgart 70569, Germany
e-mail: roland.kontermann@izi.uni-stuttgart.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_9,

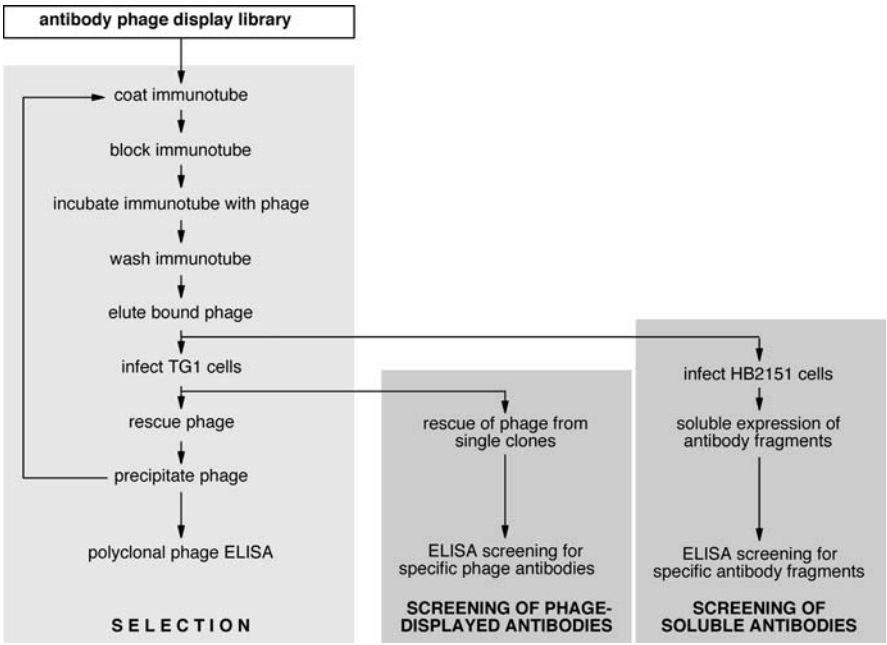


Fig. 9.1 Outline of the selection and screening procedures

9.2 Materials

9.2.1 Selection from Phagemid Libraries

- 5 ml immunotubes (Nunc MaxiSorp™; catalog # 444202)
- Phosphate-buffered saline (PBS)
- 50 mM carbonate buffer pH 9.6
- TG1 (Gibson, 1984)
- HB2151 (Carter et al. 1981)
- Minimal plates: autoclave 15 g agar in 880 ml water. Cool, then add 10 ml 20% glucose, 1 ml 1 M $MgSO_4$, and 1 ml vitamine B1 (2 mg/ml), 100 ml M9 stock (per liter: 58 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl, pH 7.2)
- 2xTY medium: 16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl per liter (autoclaved)
- PBS, 0.1% Tween-20
- 100 mM triethylamine in H_2O (freshly prepared)
- 1 M Tris-HCl pH 7.5
- TYE/amp/glucose plates (9 cm round plates and 24 cm square plates): per liter 2xTY medium containing 100 μg/ml ampicillin and 1% glucose add 15 g agar

- Ampicillin-stock solution (1,000×): 100 mg/ml in H_2O
- Glucose stock solution: 20% glucose in H_2O.

9.2.2 Rescue of Phage with Helper Phage

- 2xTY medium, see 2.1
- helper phage (M13KO7 from Pharmacia or M13 VCS from Stratagene)
- kanamycin-stock solution (1,000×): 25 mg/ml in H_2O
- PEG-NaCl: 20% polyethyleneglycol 6000, 2.5 M NaCl.

9.2.3 Polyclonal Phage ELISA

- Phosphate-buffered saline (PBS), see 2.1
- 96 well microtiter plates for ELISA (Nunc MaxiSorp™; Falcon Microtest III, etc.)
- Horseradish peroxidase-conjugated goat anti-M13 (Pharmacia; catalog # 27-9421-01)
- TMB substrate solution: 100 μg/ml 3′3′,5,5′-tetramethylbenzidine (from a 10 mg/ml stock in DMSO) in 100 mM sodium acetate buffer pH 6.0, add 2 μl 30% H_2O_2 to 10 ml of the solution prior to use.

9.2.4 Screening of Soluble scFv by ELISA

- 2xTY, see 2.1
- 96 well round bottom cell culture plates (e.g., from Greiner)
- Phosphate-buffered saline (PBS), see 2.1
- IPTG stock solution: 1 M isopropyl-β-D-thio-galactopyranoside in 1 ml water
- HRP-conjugated anti-His-tag antibody (Santa Cruz Biotechnology)
- TMB substrate solution: see 2.3.

9.2.5 Screening of Phage-Displayed scFv by ELISA

- 96 well round bottom cell culture plates
- 96 well microtiter plates for ELISA, see 2.3
- Phosphate-buffered saline (PBS), see 2.1.

9.2.6 Characterization of Soluble scFv

- LMB3: 5′- CAG GAA ACA GCT ATG ACC-3′
- fdSeq1: 5′-GAA TTT TCT GTA TGA GG-3′
- See also the indicated chapters.

9.2.7 Preparation of Helper Phage

- Helper phage (M13KO7 from Pharmacia or M13 VCS from Stratagene)
- Top-agar: 2xTY medium containing 7.5 g agar.

9.3 Methods

9.3.1 Selection from Phagemid Libraries

1. Coat immunotubes with 1 ml of antigen in a suitable coating buffer (normally we use PBS or carbonate buffer) at 1–100 μg/ml overnight at 4°C. For standard protocols we use 10 μg/ml.
2. Inoculate an overnight culture of TG1 in 2xTY. Use a fresh TG1 culture grown on a minimal plate for inoculation. Since the genes for production of the pili are encoded by an episome also containing the genes for proline synthesis, growing the strain on a minimal plate ensures production of pili. From round two on, inoculate also an overnight culture of HB2151.
3. Next day, wash the immunotube 2–3 times with PBS and block remaining binding sites with PBS, 2% skimmed milk powder (2% MPBS) for approximately 2 h at RT. Fill tube to the brim (approximately 5 ml).
4. Preincubate phage (e.g., from the library stock or from a previous selection round) for approximately 30 min with 1 ml 2% MPBS at RT.
5. Empty the immunotube, add the preblocked phage, and seal the tube with parafilm. Incubate for 1–2 h at RT with occasional gentle shaking.
6. Per selection, inoculate 11–12 ml of 2xTY with 100 μl of the TG1 overnight culture. Incubate at 37°C until an OD_{600} of 0.4–0.5 is reached (takes approximately 2 h). The OD is very critical. The bacteria should be in an early log-phase to ensure presence of pili.
   Note: the bacteria will not produce pili if grown below 33–34°C. Beginning with round 2, perform the same with HB2151. After having reached an OD_{600} of 0.4–0.5, the bacteria can be kept on ice. However, do not keep them longer than 30 min on ice because the bacteria will start to lose their pili.
7. Wash immunotubes 5–20 times with PBS, 0.1% Tween-20 and subsequently with 5–20 times with PBS. Each washing step is performed by pouring in and

out the wash solution, e.g., using a wash bottle. In the first two rounds, we normally wash only 5–10 times with each solution, so as not to lose rare binders, while in later rounds, stringency can be increased by washing 10–20 times, or even more.

8. Remove the remaining wash buffer, add 1 ml of 100 mM triethylamine and incubate for 8–10 min (longer incubation will destroy the phage!).
9. Transfer solution to a sterile reaction tube containing 500 µl 1 M Tris-HCl pH 7.5 to neutralize the solution.
10. Take 1 ml of neutralized phage and add them to 10 ml of log-phase TG1. Incubate 30 min at 37°C standing and then additional 30 min shaking. In rounds 2–4, take also 10 µl of the neutralized phage and add them to 1 ml of log-phase HB2151 to get single colonies for soluble expression (Sect. 3.4).
11. Make serial dilutions of the TG1 and HB2151 culture (e.g., 1:10, 1:100, 1:1000, etc.) and plate 100 µl from each dilution as well as from the undiluted culture onto a TYE plate containing 100 µg/ml ampicillin, 1% glucose. Incubate overnight at 37°C. The TG1 cultures are used to determine the number of eluted phage. Normally, in the first two rounds, you will get between 10^4–10^6 eluted phage, in later rounds, this number can increase to the number of bacteria used for infection (~10^9) (Fig. 9.2). However, it does not necessarily mean that the selection has failed if the number of eluted phage is not increasing.
12. Take the remaining TG1 culture and spin for 10 min at 2,000 g. Resuspend cell pellet in 1 ml of 2xTY. Plate onto a large 24 × 24 cm TYE plate containing 100 µg/ml ampicillin, 1% glucose. Incubate overnight at 30°C.

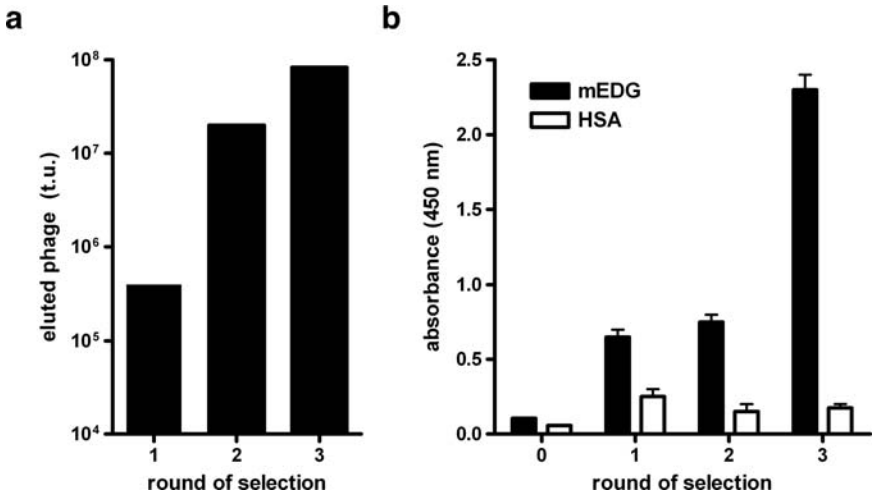


Fig. 9.2 (**a**) Titer of phage eluted from immunotubes coated with recombinant mouse endoglin (mEDG) (Müller et al. 2008) and selected using a synthetic antibody library (Völkel et al. 2004). (**b**) Polyclonal phage ELISA with phage selected on endoglin-coated immunotubes. HSA was included as negative control antigen

9.3.2 Rescue of Phage with Helper Phage

1. Next day, add 10 ml of 2xTY to the large plate and scrap the bacteria from the plate with a glass spreader. Transfer to a 15 ml tube.
2. Make a glycerol stock from 1 ml of the bacteria.
3. Add approximately 50–200 µl of the bacteria to 50 ml of 2xTY, 100 µg/ml ampicillin, 2% glucose to get a starting OD_{600} of 0.1. Incubate shaking at 37°C until an OD_{600} of 0.4–0.5 is reached (1–2 × 10^{10} bacteria). This will take approximately 2 h.
4. Add approximately tenfold excess (10^{11} t.u.) of helper phage (M13 KO7 or VCS M13) to the bacteria. Incubate for 30 min at 37°C standing, and then an additional 30 min shaking.
5. Centrifuge for 10 min at 3,500 rpm and resuspend pellet in 50 ml 2xTY, 100 µg/ml ampicillin, 25 µg/ml kanamycin (**NO glucose !**). The expression of scFv-g3 fusion proteins without induction with IPTG and in the absence of glucose is sufficient for production of phage displaying antibody fragments.
6. Incubate overnight at 30°C.
7. Centrifuge culture for 15 min at 3,500 rpm and transfer 40 ml of the supernatant to a 50 ml tube.
8. Precipitate phage by adding 8 ml (1/5 volume) 20% PEG, 2.5 M NaCl and incubate on ice for 1 h.
9. Centrifuge for 10 min at 3,500 rpm and discard supernatant.
10. Resuspend pellet in 1 ml PBS. Respin to remove remaining bacteria and transfer supernatant to a fresh tube.
11. To determine the phage titer, make serial dilutions of the phage and incubate with 1 ml of log-phase TG1 as described. Plate 100 µl onto a TYE, amp, glucose plate, and incubate at 37°C overnight. Count colonies to calculate phage titer. Phage titer should be around 10^{12}–10^{13} t.u./ml.
12. Repeat selection as described (section 3.1) using 100 µl (10^{11}–10^{12} t.u.) of rescued phage for the next round. We normally perform 3–4 rounds of selection. Further rounds can be performed (and might sometimes be necessary), but this can lead to the loss of diversity of the selected antibodies and the enrichment of those having growth advantages or slow dissociation rates.

9.3.3 Polyclonal Phage ELISA

1. Coat microtiter plate with your antigen at 1–10 µg/ml overnight in a suitable buffer (PBS or carbonate buffer pH 9.6) at 4°C. Use one or more appropriate proteins as negative controls. For each round and antigen, coat two wells, plus two wells as blank.
2. Block remaining binding sites with 2% MPBS for 2 h.
3. Pipette 90 µl of 2% MPS per well and add 10 µl of rescued phage. Incubate for 1 h at RT.

4. Wash plate 6 times with PBS.
5. Add 100 µl of HRP-conjugated anti-M13 antibody diluted 1/5,000 in 2% MPBS. Incubate for 1 h at RT. (If you use an unconjugated anti-M13 antibody, you have to include an additional incubation step with an HRP-conjugated secondary antibody).
6. Wash plate 6 times with PBS.
7. Add 100 µl of TMB/H_2O_2 per well and incubate until blue color has developed. Stop reaction by adding 50 µl of 1 M sulfuric acid. Read plate at 450 nm in a microtiter plate reader (see Fig. 9.2 for a typical result). If you have successfully enriched positive phage, you should see an increase in signal (normally at round 2–3) (Fig. 9.2).

9.3.4 Screening of Soluble scFv by ELISA

1. Pipette 100 µl of 2xTY, 100 µg/ml ampicillin, 1% glucose into each well of a sterile microtiter plate. The high concentration of glucose in the medium ensures that expression of the antibody fragments, which is driven by the lacZ promoter is completely inhibited.
2. Pick single colonies from the HB2151 plate of step 11 (section 3.1) using sterile toothpicks, and transfer them to the microtiter plate (we normally use in initial screens colonies from round 3 and 4). This will be your **master plate**. Leave one or two wells free as blank. Seal plate with parafilm and incubate shaking overnight at 37°C.
3. Transfer an aliquot of the culture to a fresh plate (**induction plate**) containing 125 µl of 2xTY, 100 µg/ml ampicillin, 0.1% glucose. This can be done, for example, with a 96-well transfer device (Sigma). Seal plate with parafilm to avoid cross-contamination.
4. Add 50 µl of 2xTY, 45% glycerol to each well of the master plate, mix well, seal with parafilm, and store plate at −20 or −80°C.
5. Incubate induction plate at 37°C in a shaker until an OD_{600} of 0.8–1 is reached. This takes approximately 2–3 h. The low concentration of glucose will be metabolized by the time IPTG is added to induce expression.
6. Add 25 µl of 2XTY, 100 µg/ml ampicillin, and 6 mM IPTG and incubate overnight at 30°C.
7. Coat a microtiter plate with your antigen as described. Use a second plate with a negative control protein.
8. Next day, block remaining binding sites with 2% MPBS for 2 h at RT.
9. Remove blocking solution and add 50 µl of 4% MPBS to each well.
10. Centrifuge induction plate for 10 min at 3,500 rpm and transfer 50 µl of the supernatant from each well to the corresponding well of the ELISA plate. Incubate for 1 h at RT.
11. Wash 6 times with PBS.

12. Add HRP-conjugated anti-His-tag antibody diluted to 1/1,000 in 2% MPBS and incubate for 1 hr at RT.
13. Wash 6 times with PBS.
14. Add 100 µl TMB/H_2O_2 substrate solution and proceed as described under polyclonal phage ELISA (section 3.3).

9.3.5 Screening of Phage-Displayed scFv by ELISA

As an alternative to screening of soluble scFv, TG1 colonies can also be directly screened for production of phage-displayed antibodies.

1. Pipette 100 µl of 2xTY, 100 µg/ml ampicillin, and 1% glucose into each well of a sterile microtiter plate.
2. Pick single colonies from the 2xTY plate of step 11 (Sect. 3.1) using sterile toothpicks and transfer them to the microtiter plate. Seal plate with parafilm.
3. Grow at 37°C for approximately 1–2 h until bacterial growth becomes visible.
4. Add 10 µl of helper phage diluted to a concentration of approximately 10^{10} t.u./ml (~10^8 t.u./well). Incubate for 30 min at 37°C standing and then an additional 30 min shaking.
5. Centrifuge plate for 10 min at 3,500 rpm. Discard supernatant and resuspend bacterial pellets in 150 µl 2xTY, amp, kan (**NO glucose!**). Seal plate with parafilm.
6. Incubate plate at 37°C overnight.
7. Centrifuge plate for 10 min at 3,500 rpm.
8. Use 10–50 µl of supernatant for ELISA as described in Sect. 3.3.

9.3.6 Characterization of Soluble scFv

1. For further characterization, the scFv genes can be cloned into vectors, allowing soluble expression in TG1, e.g., using vector pAB1 or pAB11 (Chap. 4).
2. For expression and purification of scFv by immobilized metal affinity chromatography (IMAC), see Chap. 22. Purified scFv can then be directly applied to various immunological tests, such as ELISA, immunoblotting experiments, immunocyto-, and histochemistry. In these experiments, bound antibody fragments can be detected via their tag sequences or using protein L or A (Chap. 24).
3. For sequence analysis, grow cultures of positive clones overnight at 37°C in 2XTY, 100 µg/ml ampicillin, 1% glucose, and isolate plasmid DNA by standard procedures. Using phagemid vectors, clones can be sequenced with primers LMB3 and fdSeq1. For sequence analysis, see Chaps 1–3.

9.3.7 Preparation of Helper Phage

1. Melt top agar and cool down to 42°C.
2. Mix 100 µl of serial dilutions of helper phage (VCSM13 or M13KO7) in 2xTY with 100 µl of log-phase TG1 ($OD_{600} = 0.5$) and 3 ml of top agar.
3. Plate onto warm TYE plates and incubate over night at 37°C.
4. pick a single (small) plaque from the lawn and transfer to 3 ml of 2xTY containing 100 µl of a TG1 overnight culture to obtain a good cell density.
5. shake for 3 h at 37°C.
6. Inoculate 500 ml of 2xTY with the grown plaque. Incubate for 1 h shaking at 37°C and then add 25 µg/ml kanamycin. Incubate shaking overnight at 37°C.
7. Transfer culture to a 500 ml centrifuge beaker and centrifuge at 6,000 rpm for 30 min.
8. The supernatant contains the helper phage. Filter through a 0.45 µm Nalgene membrane (use several 150 ml filters instead of one 500 ml). Check an aliquot of the filtrate on TYE plates for remaining bacteria. Altenatively, heat supernatant to 65°C for 15 min and spin down the debris.
9. Freeze phage in liquid nitrogen or dry ice and transfer to −20°C. No glycerol is needed.
10. Titer phage (thawed from the frozen stock) as described above in steps 1–3 to quote actual survival titer of phage (titer should be 10^{11}–10^{12} t.u./ml). If necessary, phage can be concentration by PEG-NaCl precipitation as described.

9.4 Troubleshooting

1. No enrichment of positive phage
   (a) Check the library with another antigen (e.g., BSA) to ensure that the phage library you are using and your selection procedure is working.
   (b) Check TG1 cells used for infection of eluted phage are already infected by plating them on ampicillin and kanamycin plates. If you find colonies on these plates, your TG1 stock is contaminated. Use a fresh stock of TG1.
2. Only few antibodies are enriched after 3–4 rounds
   (a) Reduce washing stringency (i.e., less washing steps) or increase antigen density coated onto immunotubes. Check also immobilization conditions, e.g., by detecting your antigen with available antibodies. Perform additional rounds and check for further enrichment.
3. Selected antibodies have low affinities
   (a) If you are using a naive antibody library of low or moderate diversity (10^6–10^7), no high affinity antibodies might be present in the library. Try

to use a library with greater diversity or generated by a different method (e.g., a semi-synthetic library).

(b) Your protein might possess low antigenicity, i.e., peptides, and haptens quite often produce only antibodies with low or moderate affinity.

4. Immunotube selections are generally applied for the selection of antibody fragments directed against purified proteins or other antigens, which can be immobilized on polystyrol surfaces. For selection of antibodies recognizing small molecules such as peptides or haptens, these molecules can be coupled to a carrier protein (e.g., BSA or keyhole limpet haemocyanin (KLH)). In that case, it is advisable to perform alternating selections using two different carrier proteins to avoid enrichment for antibodies against the carrier. Applications might be limited by impure preparations of antigen, in which case indirect methods for immobilization might be used (e.g., using specific ligands such as antibodies). However, in this case, libraries have to be preadsorbed on the ligand in order to avoid selection of antibodies directed against the ligand. Often, selections are also limited by the amount of antigen available. Selections with antigens coated at 1–10 μg/ml are routinely used with success, although lower concentrations have been applied for immunotube selections. Another problem might arise from the fact that proteins often unfold during immobilization, which might result in the selection of antibodies recognizing (only) epitopes of the denatured protein. In that case, other selection methods, for example, selections on biotinylated antigens bound to streptavidin-coated paramagnetic beads can be applied (Chap. 11, Patrick Chames & Daniel Baty, Chap. 12, Steffen U. Eisenhardt & Karlheinz Peter, Chap. 13, Yu Zhou & James D. Marks, this volume).

References

Carter P, Bedouelle H, Winter G (1981) Improved oligonucleotide site-directed mutagenesis using M13 vectors. Nucleic Acid Res 13:4431–4443

Gibson TJ (1984) Studies on the Epstein-Barr virus genome. PhD. thesis, University of Cambridge

Harrison JL, Williams SC, Winter G, Nissim A (1996) Screening of phage antibody libraries. Methods Enzymol 267:83–109

Hoogenboom HR, de Bruine AP, Hufton SE, Hoet RM, Arends JW, Roovers RC (1998) Antibody phage display technology and its applications. Immunotechnology 4:1–20

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol 222:581–597

McCafferty J, FitzGerald KJ, Earnshaw J, Chiswell DJ, Link J, Smith R, Kenten J (1994) Selection and rapid purification of murine antibody fragments that bind a transition-state analog by phage display. Appl Biochem Biophys 47:157–173

Müller D, Trunk G, Sichelstiel A, Zettlitz KA, Quintanilla M, Kontermann RE (2008) Murine endoglin-specific single-chain Fv fragments for the analysis of vascular targeting strategies in mice. J Immunol Methods 339:90–98

Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, McCafferty J, Hodits RA, Wilton J, Johnson KS (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat Biotechnol 14:309–314

Völkel T, Müller R, Kontermann RE (2004) Isolation of endothelial cell-specific human antibodies from a novel fully synthetic scFv library. Biochem Biophys Res Commun 317:515–521

Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR (1994) Making antibodies by phage display technology. Annu Rev Immunol 12:433–455

Chapter 10
Phage Display and Selection in Microtitre Plates

Michael Hust and Michael Mersmann

10.1 Introduction

The *in vitro* procedure for isolating antibody fragments by their binding activity was called "panning," referring to the gold washers' tool (Parmley and Smith 1988). The antigen is immobilised to a solid surface, such as nitrocellulose, e.g. Hawlisch et al. 2001), magnetic beads, (e.g. Moghaddam et al. 2003), column matrixes, (e.g. Breitling et al. 1991), or most widely on plastic surfaces with high protein binding capacity, such as polystyrene tubes or microtitre plate wells, (e.g. Hust et al. 2002). The antibody phage is incubated with the surface-bound antigen, followed by stringent washing to remove the vast excess of nonbinding antibody phage. Bound antibody phage are then eluted and reamplified by infection of *Escherichia coli*. Here, it is important to understand that successful "panning" relies on a single molecular interaction of the antibody phage with the antigen in a tremendous background of interfering molecules. Consequently, the quality of the surface-coupled antigen and the panning conditions (pH, salt concentrations, competitors, blocking reagents, etc.) determine the quality of the derived antibody. The selection cycle is repeated by infection of the phagemid bearing *E. coli* cells from the former panning round with a helperphage to produce new antibody phage, which can be used for further panning rounds until a significant enrichment of antigen-specific phage is achieved. The number of antigen-specific antibody phage clones should increase with every panning round. Usually two to three panning rounds, rarely up to six, are necessary.

The first step in the evaluation process of potential binders can be done by an ELISA using the polyclonal phage preparations from each panning round against the target antigen and control proteins, e.g., BSA. In the next step, individual

M. Hust (✉) and M. Mersmann
Institut für Biochemie und Biotechnologie, Abteilung Biotechnologie, Technische Universität Braunschweig, Spielmannstr. 7, Braunschweig 38106, Germany
e-mail: m.hust@tu-bs.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_10,

antibody clones are isolated from panning rounds showing a significant enrichment of specific antigen binding in the polyclonal phage ELISA. These antibody clones are recultivated to produce soluble monoclonal antibody fragments in microtitre plates to be analysed by an antigen ELISA. ELISAs with individual monoclonal phage preparations should be omitted to avoid false positives. These false positives are scFv clones, which bind only as scFv-phage or scFv-pIII fusion proteins but not as soluble fragments.

10.2 Outline

The methods describe the selection ("panning") of recombinant antibody fragments from antibody gene libraries by phage display and the screening of the individually selected antibody clones. An overview on the total procedure is given in Fig. 10.1.

10.3 Materials

10.3.1 Coating of Microtitre Wells

- MaxiSorp microtitre plates or stripes (Nunc, Langenselbold)
- PBS pH 7.4 (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4*$2H_2O$, 0.24 g KH_2PO_4 in 1 L)
- Dimethyl sulfoxide (DMSO)
- PBST (PBS+0.1% (v/v) Tween 20).

10.3.2 Panning

- MPBST (2% skim milk in PBST, prepare fresh)
- Panning block solution (1% (w/v) skim milk+1% (w/v) BSA in PBST, prepare fresh)
- 10 μg/mL trypsin in PBS
- *E. coli* XL1-Blue MRF' (Stratagene)
- M13K07 Helperphage (Stratagene)
- 2xYT media pH 7.0 (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl)
- 2xYT-T (2XYT, containing 50 μg/mL tetracycline)
- 2xYT-GA (2XYT, 100 μg/mL ampicillin, 100 mM glucose)
- 2xYT-GA agar plates (2xYT-GA+1,5% (w/v) agar-agar)
- 15 cm Petri dishes
- Glycerol (99.5%).

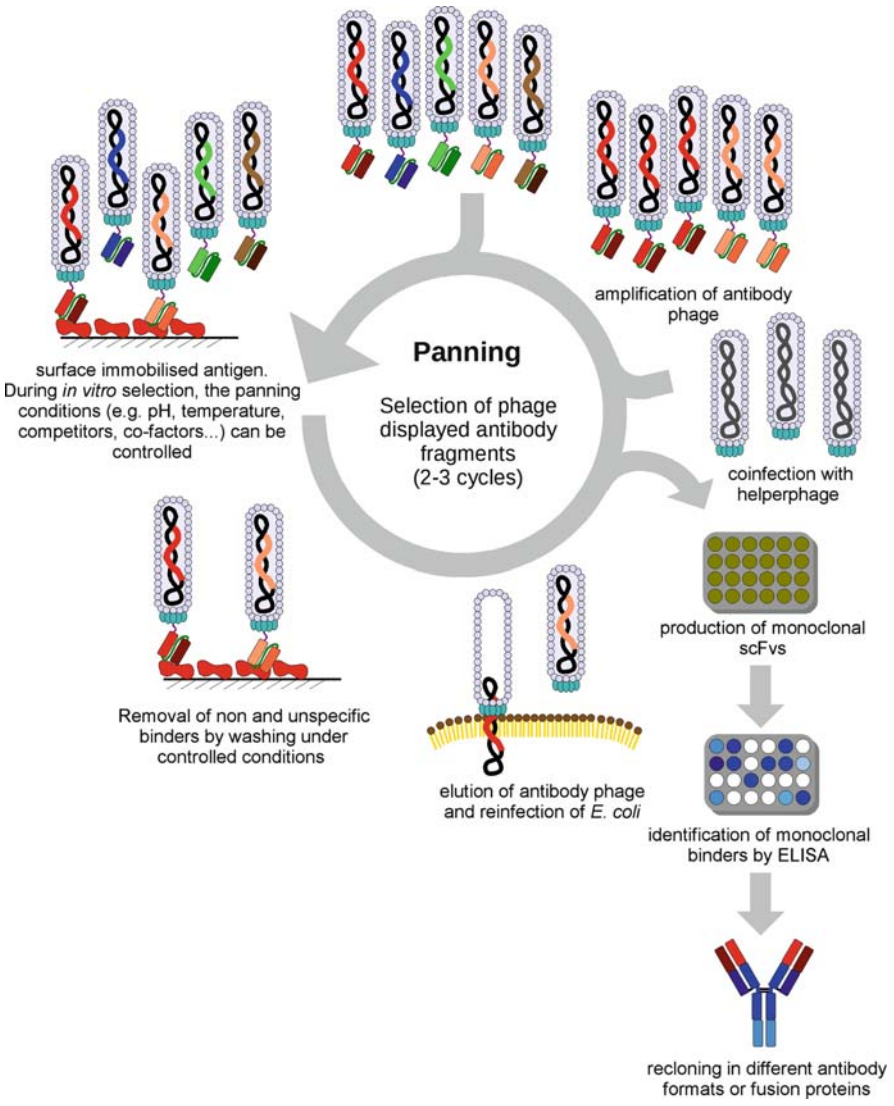


Fig. 10.1 Schematic overview about the panning and screening procedure

10.3.3 Packaging of Phagemids (scFv Phage Production)

- 2xYT-AK (2xYT, containing 100 μg/mL ampicillin, 50 μg/mL kanamycin)
- Polyethylenglycol (PEG) solution (20% (w/v) PEG 6000, 2.5 M NaCl)
- Phage dilution buffer (10 mM TrisHCl, pH 7.5, 20 mM NaCl, 2 mM EDTA).

10.3.4 Phage Titration

– 2xYT-GA agar plates (2xYT-GA+1.5% (w/v) agar-agar).

10.3.5 ELISA of a Polyclonal Antibody Phage Suspension

– Bovine serum albumin (BSA) (prepare a 10 mg/mL stock solution in PBS)
– Anti-M13, horseradish peroxidase (HRP) conjugated monoclonal antibody (GE Healthcare)
– TMB substrate solution A pH 4.1 (10 g citric acid solved in 100 mL water, add 9.73 g potassium citrate add to 1 L water)
– TMB substrate solution B (240 mg tetramethylbenzidine, 10 mL acetone, 90 mL ethanol, 907 μL 30% H_2O_2)
– 1 N H_2SO_4.

10.3.6 Production of Soluble Monoclonal Antibody Fragments in Microtitre Plates

– 96 well U-bottom polypropylene (PP) microtitre plates (Greiner Bio-One, Frickenhausen)
– AeraSeal breathable sealing film (Excel Scientific, Victorville)
– Thermoshaker PST60-HL4 (Lab4You, Berlin)
– Potassium phosphate buffer (2.31% (w/v) (0,17 M) KH_2PO_4 + 12.54% (w/v) (0,72 M) K_2HPO_4)
– Buffered 2xYT pH 7.0 (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 10% (v/v) potassium phosphate buffer)
– Buffered 2xYT-SAI (buffered 2xTY containing 50 mM sucrose + 100 μg/mL ampicillin + 50 μM isopropyl-beta-D-thiogalactopyranoside (IPTG)).

10.3.7 ELISA of Soluble Monoclonal Antibody Fragments

– Mouse α-His-tag monoclonal antibody (α-Penta His, Qiagen, Hilden)
– Mouse α-myc-tag monoclonal antibody (9E10, Sigma)
– Mouse α-pIII monoclonal antibody PSKAN3 (MoBiTec, Göttingen)
– Goat α-Mouse IgG serum, (Fab specific) HRP conjugated (Sigma)
– Oligonucleotide primer MHLacZPro_f : 5′ GGCTCGTATGTTGTGTGG 3′.

10.4 Procedure

10.4.1 Coating of Microtitre Plate Wells

1. (a) Protein antigen: For the first panning round, use 2–10 μg protein/well per panning, for the following rounds use 0.1–1 μg protein/well for more stringent conditions. Dissolve the antigen in 150 μL PBS and incubate in a microtitre plate well overnight at 4°C.
   Note: If the protein does not bind properly to the microtitre plate surface, try bicarbonate buffer (50 mM $NaHCO_3$, pH 9.6).
   (b) Oligopeptide antigen: Use 100–500 ng oligopeptide for each panning round. Dissolve the oligopeptide in 150 μL PBS, transfer into the microtitre plate well and incubate overnight at 4°C.
   Note: More hydrophobic oligopeptides may need to be dissolved in PBS containing 5% DMSO. If biotinylated oligopeptide is used as antigen for panning, dissolve 100 ng Streptavidin in 150 μL PBS and coat overnight at 4°C. Coat two or more wells for each panning, one well is for the panning, the others for the preincubation of the library to remove streptavidin binders! Sometimes, it is necessary to use free streptavidin during panning for competition to remove streptavidin binders. Pour out the wells and wash 3× with PBST. Dissolve 100–500 ng biotinylated oligopeptide in PBS and incubate for 1 h at RT. Alternatively, oligopeptides with a terminal cystein residue can be coupled to BSA and coated overnight at 4°C.
2. Wash the coated microtitre plate wells 3× with PBST using an ELISA washer.
   Note: All washing steps during the panning procedure should be performed with an ELISA washer (e.g. TECAN Columbus Plus) to increase the stringency and reproducibility. To remove antigen wash 3× with PBST ("standard washing protocol" for TECAN washer).

10.4.2 Panning

1. (a) Block the antigen-coated wells with MPBST for 2 h (at least 45 min) at RT. The wells must be completely filled. Wash the blocked antigen-coated wells 3× with PBST.
   Note: To remove blocking solutions wash 3× with PBST.
   (b) *You need to perform this step only in the first panning round but it also can be done in the following rounds!* In parallel, block an additional well (without antigen!) per panning with MPBST for 1 h at RT for preincubation of the antibody gene library. The wells must be completely filled. Wash 3× times with PBST. Incubate 10^{11}–10^{12} antibody phage from the library in 150 μL panning block for 1 h at RT. This step removes unspecific binders, which often

occur from the antibody gene libraries because of incorrect folding of individual antibodies.
Note: The removal of unspecific binders can be enhanced by sequentially repeating the preincubation step.

2. Transfer the preincubated antibody phage library to the blocked wells or dissolve 10^{11} amplified phage from the former panning round solved in 150 µL panning block and fill in blocked wells. Incubate at RT for 2 h for binding of the antibody phage.
3. Remove the unspecifically bound antibody phage by stringent washing. Therefore, wash the wells at least 10× with PBST in the first panning round. In the following panning rounds, increase the number of washing steps (20× in the second panning round, 30× in the third panning round.etc.).
Note: After binding of antibody phage, wash 10× with PBST ("stringent bottom washing protocol" in case of TECAN washer). If no ELISA washer is available, wash manually 10× with PBST and 10× with PBS. For stringent off-rate selection increase the number of washing steps or additionally incubate the microtitre plate in 1 L PBS for several days.
4. Elute bound antibody phage with 200 µL Trypsin solution for 30 min at 37°C.
Note: Phagemids like pSEX81 (Welschof et al. 1997) or pHAL14 (Hust et al. 2007a; Pelat et al. 2007; Kirsch et al. 2008) have coding sequences for a trypsin-specific cleavage site between the antibody fragment gene and the gIII. Trypsin also cleaves within antibody fragments but does not degrade the phage particles, including the pIII that mediates the binding of the phage to the F pili of *E. coli* required for the infection. We observed that proteolytic cleavage of the antibody fragments from the antibody::pIII fusion by trypsin increases not only the elution but also enhances the infection rate of eluted phage particles, especially when using Hyperphage as helperphage (Rondot et al. 2001; Soltes et al. 2007; Hust et al. 2006).
5. Use 10 µL of the eluted phage for titration (see titering).
6. Inoculate 50 mL 2XYT-T with an overnight culture of *E. coli* XL1-Blue MRF' in 100 mL Erlenmeyer flasks and grow at 250 rpm and 37°C.
Note: all given rpms are for shake flasks without baffle.
7. Infect exponentially ($O.D._{600\ nm}$ ~0.5, after 2–3 h) growing 20 mL XL1-Blue MRF' culture with the remaining 190 µL of the eluted phage. Incubate 30 min at 37°C without shaking the following 30 min with moderate shaking (250 rpm).
8. Harvest the infected bacteria by centrifugation for 10 min at 3,200× *g* in 50 mL polypropylene tubes. Resolve the pellet in 250 µL 2xYT-GA and plate the bacteria suspension on 2xYT-GA agar plates (15 cm Petri dish). Grow overnight at 37°C.
Note: The high concentration of glucose is necessary to efficiently repress the lac promoter controlling the antibody::pIII fusion gene on the phagemid. Low glucose concentrations lead to an inefficient repression of the lac promoter and background expression of the antibody::pIII fusion protein. Background antibody expression is a strong selection pressure frequently causing mutations in

the phagemid, especially in the promoter region and the antibody::pIII fusion gene. Bacteria with this kind of mutations in the phagemids proliferate faster than bacteria with non-mutated phagemids. Therefore, the 100 mM glucose must be included in every step of *E. coli* cultivation except during phage production!

9. Harvest the grown colonies by suspending in 2.5 mL 2xYT-GA after scraping with a Drigalski spatula.
10. Use 100 µL of the harvested bacteria for amplification of the eluted phage (Packaging of phagemids).
11. Prepare a glycerol stock of the panning round by adding 250 µL glycerol to 750 mL of the harvested bacteria. Mix and store at −80°C.

10.4.3 Packaging of Phagemids (scFv Phage Production)

1. For the next panning round, the eluted phage must be packaged and reamplified. Inoculate 30 mL 2xYT-GA in a 100 mL Erlenmeyer flask with 100 µL harvested bacteria ($O.D._{600} < 0.1$). Grow at 250 rpm and 37°C up to an $O.D._{600\ nm} \sim 0.5$.
2. Infect 5 mL bacteria culture (~$2.5*10^9$ cells) with $5*10^{10}$ cfu of the helper phage M13K07 (MOI = 1:20). Incubate 30 min without shaking at 37°C and the following 30 min with moderate shaking (250 rpm).
3. To remove the glucose, harvest the cells by centrifugation for 10 min at 3,200× *g* in 50 mL polypropylene tubes.
4. Resuspend the pellet in 30 mL 2xYT-AK and transfer into a 100 mL Erlenmeyer flask. Produce the phage for 12–16 h at 250 rpm and 30°C.
5. Pellet the bacteria by centrifugation for 10 min at 3,200× *g* in 50 mL polypropylene tubes. If the supernatant is not clear, centrifuge again to remove remaining bacteria.
6. Precipitate the phage in the supernatant by adding 1/5 volume PEG solution in 50 mL polypropylene tubes. Incubate for 1 h at 4°C or on ice.
7. Pellet the phage by centrifugation for 1 h at 3,200× *g* and 4°C. Discard the supernatant and put the open tubes upside down on tissue paper. Let the viscous PEG solution run out completely. Resuspend the phage pellet in 500 µL phage dilution buffer. Titrate the phage preparation and use it for the next panning round. Store the prepared phage at 4°C.

10.4.4 Phage Titration

1. Inoculate 5 mL 2xYT-T in a 100 mL Erlenmeyer flask with XL1-Blue MRF' and grow overnight at 37°C and 250 rpm.

2. Inoculate 50 mL 2xYT-T with 500 μL overnight culture and grow at 250 rpm at 37°C up to $O.D._{600}$ ~ 0.5.
   Note: If the bacteria have reached $O.D._{600}$ ~ 0,5 before they are needed, store the culture immediately on ice to maintain the F pili on the *E. coli* cells for several hours. M13K07 helperphage (kan^+) or other scFv-phage (amp^+) can be used as positive control to cheque the infectibility of the *E. coli* cells.
3. Make serial dilutions of the phage suspension in PBS. The number of eluted phage depends on several parameters (e.g. antigen, library, panning round, washing stringency, etc.). In case of a successful enrichment, the titre of eluted phage usually is 10^3–10^5 phage per well after the first panning round and increases one to two orders in magnitude per additional panning round. Phage preparations after reamplification of the eluted phage have a titre of about 10^{12}–10^{14} phage/mL.
   Note: If the antibody gene library is packaged using Hyperphage, the titre of the eluted phage after the second panning may not increase as strongly or even decreases slightly because of the change from oligovalent to monovalent display.
4. Infect 50 μL bacteria with 10 μL phage dilution and incubate 15 min at 37°C.
   Note: Cheque all solutions for phage contamination. To cheque the PBS or PEG solutions, use 10 μL of these solutions for *E. coli* "infection". In parallel, plate out noninfected XL1-Blue MRF' to cheque the bacteria. We recommend to clean the working place each time with virus inactivating solutions (e.g. Barrycidal 36, BIO-HIT, Germany) and to use filter tips for pipetting!
5. You can perform titrations in two different ways:
   (a) Plate the 60 μL infected bacteria on 2xTY-GA agar plates (9 cm petri dishes).
   (b) Pipet 10 μL (in triplicate) on 2xTY-GA agar plates. Here, about 20 titering spots can be placed on one 9 cm petri dish. Dry drops under work bench.
6. Incubate the plates overnight at 37°C.
7. Count the colonies and calculate the cfu or cfu/mL titre according to the dilution.

10.4.5 ELISA of a Polyclonal Antibody Phage Suspension

1. To analyse the enrichment of antigen-specific antibody phage after a panning round, coat microtitre plate wells with 100–1,000 ng antigen per well for each panning round (for method see 3.1). As a control, coat other wells with 100–1,000 ng BSA in 150 μL PBS overnight at 4°C.
2. Wash the coated microtitre plate wells 3× with PBST using an ELISA washer.
   Note: Try to avoid completely dry protein wells as some proteins might change irreversibly under such condition.
3. Block the antigen-coated wells with MPBST for 2 h at RT. The wells must be completely filled.

4. Wash the coated microtitre plate wells 3× with PBST.
5. Resuspend 10^9 and 10^{10} cfu/well antibody phage from each panning round in 150 µL 2%MPBST and incubate them for 1.5 h on the antigen and the BSA control, respectively.
6. Wash the microtitre plate wells 3× with PBST.
7. Incubate each well with 100 µL HRP conjugated anti-M13 antibody 1:5000 diluted in 2%MPBST for 1.5 h.
8. Wash the microtitre plate wells 3× with PBST.
9. Shortly before use, mix 20 parts TMB substrate solution A and 1 part TMB substrate solution B. Add 100 µL of this TMB solution to each well and incubate for 1–15 min.
10. Stop the substrate reaction by adding 100 µL 1 N sulphuric acid. The colour turns from blue to yellow.
11. Measure the extinction at 450 nm using an ELISA reader.

10.4.6 Production of Soluble Monoclonal Antibody Fragments in Microtitre Plates

1. Fill each well of a 96 well U-bottom polypropylene microtitre plate with 150 µL 2XYT-GA.
2. Pick 92 clones with sterile tips from the desired panning round and inoculate each well. Seal the plate with a breathable sealing film.
   Note: Use the polyclonal antibody phage ELISA to select the suitable panning round for picking monoclonals. We recommend picking only 92 clones. Use defined wells for negative and positive controls. We are using the wells H3 and H6 for negative controls – these wells will not be inoculated and not used for the following ELISA with soluble antibodies. We inoculate the wells H9 and H12 with a clone containing a phagemid encoding a known antibody fragment. In ELISA, the wells H9 and H12 are coated with the antigen corresponding to the control antibody fragment in order to cheque scFv production and ELISA.
3. Incubate overnight in a microtitre plate shaker at 37°C and 1,000 rpm.
4. (A) Fill a new 96 well polypropylene microtitre plate with 150 µL 2xYT-GA and add 10 µL of the overnight cultures. Incubate for 2 h at 37°C and 1,000 rpm. (B) Add 30 µL glycerol solution to the remaining 140 µL overnight cultures. Mix by pipetting and store this masterplate at −80°C.
5. Pellet the bacteria in the microtitre plates by centrifugation for 10 min at 3,200× *g* and 4°C. Remove 180 µL glucose containing media by carefully pipetting (do not disturb the pellet).
6. Add 180 µL buffered 2xYT-SAI (containing saccharose, ampicillin and 50 µM IPTG) and incubate overnight at 30°C and 1,000 rpm.
   Note: The appropriate IPTG concentration for induction of antibody or antibody::pIII expression depends on the vector design. A concentration of 50 µM

was well suited for vectors with a Lac promoter like pSEX81 (Welschof et al. 1997), pIT2 (Goletz et al. 2002), pHENIX (Finnern et al. 1997) and pHAL14 (Pelat et al. 2007; Hust et al. 2007b; Kirsch et al. 2008). The method for the production of soluble antibodies works with vectors with (e.g. pHAL14) and without (e.g. pSEX81) an amber stop codon between antibody fragment and gIII. If the vector has no amber stop codon, the antibody::pIII fusion protein will be produced (Mersmann et al. 1998). Buffered culture media and the addition of sucrose enhances the production of many, but not all scFvs. We observed that antibody::pIII fusion proteins and antibody phage sometimes show differences in antigen binding in comparison to soluble antibody fragments, because some antibodies can bind the corresponding antigen only as pIII fusion. Therefore, it is recommended to perform the screening procedure only by using soluble antibody fragment to avoid false positive binders.

7. Pellet the bacteria by centrifugation for 10 min at 3,200× *g* in the microtitre plates. Transfer the antibody fragment containing supernatant to a new polypropylene microtitre plate and store at 4°C.

10.4.7 ELISA of Soluble Monoclonal Antibody Fragments

1. To analyze the antigen specificity of the monoclonal soluble antibody fragments, coat 100–1,000 ng antigen per well overnight at 4°C. Coat 100–1,000 ng BSA for control wells.
2. Wash the coated microtitre plate wells 3× with PBST.
3. Block the antigen-coated wells with MPBST for at least 45 min at RT. The wells must be completely filled.
4. Fill 50 µL MPBST in each well and add 50 µL of antibody solution. Incubate for 1.5 h at RT (or overnight at 4°C).
5. Wash the microtitre plate wells 3× with PBST.
6. Incubate 100 µL mouse 9E10 α-myc tag antibody solution for 1.5 h (appropriate dilution in MPBST).
7. Wash the microtitre plate wells 3× with PBST.
8. Incubate 100 µL goat α-mouse HRP conjugate (1:10000 in MPBST).
9. Wash the microtitre plate wells 3× with PBST.
10. Shortly before use, mix 19 parts TMB substrate solution A and 1 part TMB substrate solution B. Add 100 µL of this TMB solution into each well and incubate for 1–15 min.
11. Stop the colour reaction by adding 100 µL 1 N sulphuric acid. The colour turns from blue to yellow.
12. Measure the extinction at 450 nm using an ELISA reader.
13. Identify positive candidates with a signal (on antigen) 10× over noise (on control protein, e.g. BSA).
    Note: The background (noise) signals should be about $O.D._{450} \sim 0.02$ after 5–30 min TMB incubation time.

14. DNA sequencing of the positive candidates is performed with appropriate oligonucleotide primers (MHLacZ_pro for pHAL14). For performing a colony PCR see chapter 5 - Human antibody gene libraries. The antibody sequences can be analysed by VBASE2 (www.vbase2.org) (Retter et al. 2005).

References

Breitling F, Dübel S, Seehaus T, Klewinghaus I, Little M (1991) A surface expression vector for antibody screening. Gene 104:147–153

Finnern R, Pedrollo E, Fisch I, Wieslander J, Marks JD, Lockwood CM, Ouwehand WH (1997) Human autoimmune anti-proteinase 3 scFv from a phage display library. Clin Exp Immunol 107:269–281

Goletz S, Christensen PA, Kristensen P, Blohm D, Tomlinson I, Winter G, Karsten U (2002) Selection of large diversities of antiidiotypic antibody fragments by phage display. J Mol Biol 315:1087–1097

Hawlisch H, Müller M, Frank R, Bautsch W, Klos A, Köhl J (2001) Site-specific anti-C3a receptor single-chain antibodies selected by differential panning on cellulose sheets. Anal Biochem 293:142–145

Hust M, Maiss E, Jacobsen H, Reinard T (2002) The production of a genus-specific recombinant antibody (scFv) using a recombinant potyvirus protease. J Virol Methods 106:225–233

Hust M, Meysing M, Schirrmann T, Selke M, Meens J, Gerlach G, Dübel S (2006) Enrichment of open reading frames presented on bacteriophage M13 using hyperphage. Biotechniques 41:335–342

Hust M, Jostock T, Menzel C, Voedisch B, Mohr A, Brenneis M, Kirsch MI, Meier D, Dübel S (2007a) Single chain Fab (scFab) fragment. BMC Biotechnol 7:14

Hust M, Toleikis L, Dübel S (2007b) Antibody phage display. In Handbook of therapeutic antibodies. Wiley-VCH, Weinheim

Kirsch M, Hülseweh B, Nacke C, Rülker T, Schirrmann T, Marschall H, Hust M, Dübel S (2008) Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV). BMC Biotechnol 8:66

Mersmann M et al (1998) Monitoring of scFv selected by phage display using detection of scFv-pIII fusion proteins in a microtiter scale assay. J Immunol Methods 220:51–58

Moghaddam A, Borgen T, Stacy J, Kausmally L, Simonsen B, Marvik OJ, Brekke OH, Braunagel M (2003) Identification of scFv antibody fragments that specifically recognise the heroin metabolite 6-monoacetylmorphine but not morphine. J Immunol Methods 280:139–155

Parmley SF, Smith GP (1988) Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene 73:305–318

Pelat T, Hust M, Laffly E, Condemine F, Bottex C, Vidal D, Lefranc M, Dübel S, Thullier P (2007) High-affinity, human antibody-like antibody fragment (single-chain variable fragment) neutralizing the lethal factor (LF) of Bacillus anthracis by inhibiting protective antigen-LF complex formation. Antimicrob Agents Chemother 51:2758–2764

Retter I, Althaus HH, Münch R, Müller W (2005) VBASE2, an integrative V gene database. Nucleic Acids Res 33:D671–D674

Rondot S, Koch J, Breitling F, Dübel S (2001) A helper phage to improve single-chain antibody presentation in phage display. Nat Biotechnol 19:75–78

Soltes G, Hust M, Ng KKY, Bansal A, Field J, Stewart DIH, Dübel S, Cha S, Wiersma EJ (2007) On the influence of vector design on antibody phage display. J Biotechnol 127:626–637

Welschof M, Terness P, Kipriyanov SM, Stanescu D, Breitling F, Dörsam H, Dübel S, Little M, Opelz G (1997) The antigen-binding domain of a human IgG-anti-F(ab')2 autoantibody. Proc Natl Acad Sci USA 94:1902–1907

Chapter 11
Phage Display and Selections on Biotinylated Antigens

Patrick Chames and Daniel Baty

11.1 Introduction

Phage antibody library selections on peptides or proteins are usually carried out using antigens directly coated on a plastic surface (e.g., Petri dishes, microtiter plate well, immunotubes). This straightforward method is easy to perform and has been shown to be very successful for a diverse set of antigens (for review (Winter et al. 1994). However, phage-antibody selections on some proteins, and especially on peptides are not always successful, which is often caused by immobilization-associated features. The main problem observed for selection on peptides is the very poor coating efficiency of some peptides and the altered availability of epitopes on plastic-coated peptides. The direct coating of proteins on plastic is usually more efficient but can also be problematic, because the passive adsorption on plastic at pH 9.6 is a mechanism of protein denaturation. Under these conditions, 95% of adsorbed proteins are nonfunctional (Butler et al. 1992; Davies et al. 1994).This problem is not very important for a classical ELISA because mostly a small fraction of proteins having a native conformation is still detectable. However, this phenomenon can be very troublesome for phage antibody library selections, because phage antibodies binding to epitopes only present in denatured molecules may be selected.

Several methods have been developed to increase peptide coating, including coupling to bigger proteins (Oshima and Atassi 1989), to amino acid linkers binding plastic (Loomans et al. 1998; Pyun et al. 1997), or to multiple antigen peptide (Tam and Zavala 1989). The most successful method had been the indirect coating of biotinylated antigens via streptavidin: biotinylation of the peptide and immobilization via streptavidin improves the sensitivity in ELISA (Ivanov et al. 1992) and allows more efficient selection of anti-peptide phage-antibodies (de Haard et al. 1999; Henderikx et al. 1998).

P. Chames (✉) and D. Baty
INSERM – U624, 163 avenue de Luminy, 13288 Marseille Cedex 09, France
e-mail: patrick.chames@inserm.fr

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_11,

In the case of phage library selection against proteins, the indirect coating via streptavidin results in higher density coating, more uniform distribution of antigens on the well surface, and above all, 60–70% of active molecules (Butler et al. 1992; Davies et al. 1994). Most importantly, however, the use of biotinylated peptide or protein allows the use of paramagnetic streptavidin-coated microbeads to capture the biotinylated antigens with the phage bound to them. The interaction between the phage particle and the antigen therefore takes place in solution; antigen-bound phage is retrieved via a short incubation with the beads. This technique permits precise control of the antigen concentration and the time of exposure of the antigen to the phage-antibody library, two parameters that are very useful in affinity selection, for example, during affinity maturation protocols (Hawkins et al. 1992; Schier et al. 1996). This interaction between antigen and phage antibody in solution leaves a maximum of epitopes available for binding, and avoids the selection of scFv fragments with low affinity but with a high tendency to form dimers (Schier et al. 1996). The latter will be preferentially selected on antigen-coated surfaces because of their avid binding.

This chapter contains protocols for chemical or enzymatic biotinylation, as well as phage library selection in solution and sensitive ELISA procedures for using indirectly-coated biotinylated antigen. The advantages and drawbacks of each method are discussed.

11.2 Biotinylation of Antigens

11.2.1 Biotinylation of Proteins/Peptides with NHS-ss-Biotin

11.2.1.1 Purpose

Chemical biotinylation is the most common way to obtain a biotinylated antigen. There are many commercially available reagents that can be used for biotinylation using a variety of chemistries. For most of our biotinylations, we prefer to use the chemical reagent NHS-SS-Biotin (Sulfo-Succinimidyl-2-(biotinamido)ethyl-1, 3-dithiopropionate MW = 606.70). This molecule is a unique biotin analog with an extended spacer arm of 24.3 Å in length, capable of reacting with primary amine groups (lysines and NH_2 termini). The long chain reduces steric hindrances associated with binding of biotinylated molecules to avidin or streptavidin and should not interfere with the structure of the protein/peptide involved.

The presence of the S–S linker in NHS-S-S-Biotin enables the use of a reducing agent (DTT, DTE, β-mercaptoethanol) to separate the antigen and all phage-antibodies bound to it from the beads. This feature allows a more specific elution, which is very useful when undesired streptavidin binders are preferentially selected from a phage antibody repertoire. The following method is modified from (Hnatowich et al., 1987) and the Pierce instruction manual.

11.2.1.2 Materials

- 50 mM Sodiumbicarbonate pH = 8.5:4.2 g $NaHCO_3$ adjust pH with 6 M NaOH to pH 8.5, adjust to 1 l with H_2O, filter-sterilize, store at RT
- NHS-SS-Biotin (Pierce 21331, Rockford, Illinois)
- Peptide/Protein of interest
- The best molar ratio of biotin to the protein has to be determined empirically. Try different molar ratios if possible. NHS-SS-Biotin MW = 606.70 NHS-LC-Biotin MW = 556.58
- Alternatively, if no dialysis is used: Centricon 30 or Centricon 10 (Amicon, 4306, 4304; Millipore, Billerica, MA)
- Dialysis tubing (tubing with MW-cut-off of 1,000–50,000 kD can be found at Cellu.Sep, Waterloo, Belgium)
- 5* PBS pH = 7.4: 43.8 g NaCl (750 mM), 7.1 g Na_2HPO_4 (40 mM), 1.08 g KH_2PO_4 (7.8 mM), adjust volume to 1 l. Before the test, add 800 ml H_2O to 200 ml of 5* PBS and check the pH (7.4)

Precautions

Avoid buffers containing amines (such as Tris or glycine) since these compete with peptide/protein the biotinylation reaction. Also, reducing agents should not be included in the conjugation step to prevent cleavage of the disulphide bond within NHS-SS-Biotin.

11.2.1.3 Procedure

1. Dissolve 1–10 mg/ml of the peptide/protein of interest in 50 mM $NaHCO_3$ (pH = 8.5). If the peptide/protein is already in another solvent, dialyse against 50 mM $NaHCO_3$ (2–3 x for at least 4 h in 1 l)
2. Calculate the required amount of NHS-SS-Biotin taking into account a Molar ratio Biotin:Protein = 5:1–20:1. Although this amount depends on the number of lysines present within the protein, usually a ratio of 5:1 works fine. When enough protein is available, it is advised to test different ratios of Protein: Biotin. Ideally 1–2 biotinylated residues per molecule are present. Overbiotinylation often results in nonfunctional protein (aggregation, etc.)
3. Dissolve the required amount of NHS-SS-Biotin in distilled water and immediately add this to your protein sample, or alternatively, when using larger amounts of protein, add NHS-SS-Biotin directly to the protein solution
4. Place the tube on ice for 2 h or 30 min at room temperature
5. Add 1 M Tris (pH = 7.5) to a final concentration of 50 mM, and incubate 1 h on ice to block free biotin
6. To remove free biotin, dialyse against the biotinylated protein in PBS at 4°C (3× for at least 4 h in 1 L PBS) overnight or alternatively: do steps 7 to 9. For

small peptides (<20 amino acids), alternative separation protocols (e.g. affinity chromatography, HPLC) should be followed
7. Alternatively for 6: Spin at 3,000 rpm a pretreated ultra-filtration device (e.g. Centricon 10 or 30 for 15–30 min) to concentrate the sample
8. Dilute sample in PBS to dilute out free biotin left after concentration
9. Repeat step 6 and 7 two more times
10. Add sodium azide to a final concentration of 0.1%
11. Store in small aliquots at −20°C or at 4°C. (Storage conditions should be tested for each type of protein)

11.2.2 Enzymatic Biotinylation of Recombinant Proteins

11.2.2.1 Purpose

Escherichia coli possesses a cytoplasmic enzyme, birA, which is capable of specifically recognizing a sequence of 13 amino acids and adding a biotin on a unique lysine present on this sequence (Schatz 1993). If this sequence is fused as a tag to the N or C-terminal part of a protein, the resulting fusion will be biotinylated as well. The main advantage of this system is that the protein by itself remains fully intact. Conversely, the chemical biotinylation randomly modifies any accessible lysine. An overbiotinylation often leads to inactivation of the protein of interest, especially if a lysine is present in the active site of the protein. The use of a low ratio biotin/protein aimed to reduce this problem may lead to poor yield of biotinylation. The enzymatic biotinylation avoids this drawback, leading not only to a 100% active protein, but also to a high yield of biotinylation (typically 85–95%).

The specific biotinylation via the tag presents another important advantage: it allows an ideal orientation of the protein during the selection or the ELISA analysis. In both instances, the tag will be bound to streptavidin and will thus be directed toward the solid surface (beads or plastic); the rest of the molecule is perfectly oriented and available for interaction with the phage-antibody. This also allows a very uniform presentation of the antigen, whereas chemical biotinylation will lead to a number of antigens having the epitope of interest directed toward streptavidin, and thus not being available for phage-antibody binding.

The main drawbacks of this method are that it cannot be applied on nonrecombinant proteins, and that the link between biotin and the antigen cannot be broken using DTT (see selection protocol 2).

Enzymatic biotinylation can be done in vivo if the antigen is produced in the cytoplasm of *E. coli*. In this case, the only requirement is to overexpress birA and add free biotin to the culture medium. Surprisingly, biotinylation is also very efficient on intracellularly expressed proteins that form inclusion bodies. However, if the antigen has to be produced in the periplasm of *E. coli*, the biotinylation yield is very poor (0.1–1%) (Chames et al., unpublished). In this case, and when the antigen is produced in another expression system, the biotinylation of the tag can

still be performed in vitro, on the purified protein, using purified commercially available birA. Both protocols are described here.

11.2.3 In Vivo Enzymatic Biotinylation

11.2.3.1 Materials

- Plasmid pBirCm (Avidity, Denvers, USA, www.avidity.com)
- d-Biotin, SIGMA (B4501)
- 2xTY, Ampicillin, Chloramphenicol, IPTG

11.2.3.2 Procedure

1. Using PCR and classical molecular biology protocols, reclone the gene of interest fused at its 5′ or 3′ end with the sequence coding for the biotinylation tag (see Fig.11.1)
2. Once a recombinant clone is obtained, co-transform *E. coli* with the recombinant plasmid coding for ampicillin resistance, and pbirACm, a plasmid coding for Chloramphenicol resistance, compatible with pUC-based vectors and used to overexpress birA. Transformed bacteria have to be plated on plate containing 100 μg/ml ampicillin and 15 μg/ml chloramphenicol
3. Dilute 10 ml of an overnight culture of one of the double-transfectants in 1L of 2xTY containing 100 μg/ml ampicillin and 15 μg/ml chloramphenicol. Incubate at 37°C with shaking until OD_{600} of 0.5
4. Add 1 mM IPTG to induce birA and the protein of interest expression, 50 μM biotin from a 500 mM stock in DMSO and incubate 3 h at 37°C (if the gene of interest is not under the control of tac or lac promoter, add the required inducer as well). The production can alternatively be done at 30°C
5. Spin the culture for 20 min at 4,000 rpm
6. Resuspend the pellet in 10 ml of sonication buffer 20 mM Tris–Hcl pH 7.5, 100 mM NaCl
7. Lyse the cells by sonication. Keep the solution on ice during the whole process
8. Purify the protein by affinity chromatography. If the protein possesses a tag, use IMAC procedures. An interesting alternative is to purify the protein using avidin monomers (allowing soft elution condition unlike tetramers) fused to sepharose (Softlink soft release avidin resin, Promega, V2011). This method not only

	G	S	L	H	H	I	L	D	A	Q	**K**	M	V	W	N	H	R
GGA TCC	CTG	CAT	CAT	ATT	CTG	GAT	GCA	CAG	**AAA**	ATG	GTG	TGG	AAT	CAT	CGT		
BamHI																	

Fig. 11.1 Sequence specifically biotinylated by the *E. coli* enzyme birA. The modified lysine is shown in bold

purifies the biotinylated fraction of the recombinant protein but will also purify the only endogenously biotinylated protein of E coli (although present in very low amount in *E. coli*, in the μg/l range)

11.2.4 In Vitro Enzymatic Biotinylation

11.2.4.1 Materials

- birA enzyme and buffers (**biomix A and B**) (Avidity, Denvers, USA, www.avidity.com)
- Centricon (Millipore, Billerica, MA)

11.2.4.2 Procedure

1. Using PCR and classical molecular biology protocols, reclone the gene of interest fused at its 5′ or 3′ end with the sequence coding for the biotinylation tag (see Fig. 11.1, protocol 1.3)
2. Produce and purify the recombinant protein using the appropriate method
3. Use ultracentrifugation devices (Centricon, Amicon) to exchange the buffer for Tris–Hcl 20 mM pH 7.5 and concentrate the protein to 40 μM (the birA enzyme is inhibited by NaCl and glycerol)
4. Mix one volume of recombinant protein at 40 μM with 0.125 volume of Biomix A and Biomix B plus 2.5 μl of birA for each 10 nmol of protein, in less than 500 μl
5. Incubate for 1 h at 30°C
6. Remove the excess of biotin by gel filtration (for example, on Superdex S75 column, Pharmacia), collect fractions of 500 μl, pool fractions containing the protein and estimate the protein concentration by UV measurement at 280 nm

11.2.5 Determination of Biotinylation Efficiency

11.2.5.1 Purpose

It is important to determine the percentage of protein that has actually been biotinylated. If the antigen has to be used for selection in solution, the nonbiotinylated part of the preparation will be very detrimental, blocking specific phages and impairing their binding to the biotinylated fraction. Hence, this nonbiotinylated fraction must represent less than 10–15%. The following protocol permits to determine this percentage. This protocol is also used to determine the amount of biotinylated peptide captured by a certain amount of magnetic beads. Extrapolation of the results can be used for determining the concentration of antigen and amount of beads to be used during phage library selection.

11.2.5.2 Materials

- Streptavidin dynabeads (Dynal, M280, Oslo, Norway), magnetic separation device (Dynal)
- Biotinylated peptide/protein, (5 dilutions between 5–50nM in PBS, in 200 μl)
- PBS: see protocol 1.1 of this chapter
- T-PBS: 1 ml Tween 20 in 1 l PBS
- 0.05 M Tris-buffer pH = 7.6: 0.6 g Tris/100 ml H_2O. Adjust pH to 7.6 with HCl
- SDS-page gel (12%, 10% or 7.5 dependent on the size of the peptide/protein)
- Coomassie bleu 0.1% in 40 % methanol, 10% acetic acid, 50% H_2O
- Destainer: 40% methanol, 10% acetic acid, 50% H_2O
- Nonreducing protein sample buffer: 30% glycerol, 0.025% bromophenol blue, 0.05 M Tris pH 6.8 in H_2O
- End-over-end rotator
- UV spectrophotometer (280 nm), quarts cuvettes

11.2.5.3 Procedure

1. Resuspend Dynabeads by gentle shaking
2. Transfer 10 μl per peptide/protein dilution (e.g. 50 μl for 5 dilutions of peptide/protein) to a tube that fits into the magnetic separator and add an excess of PBS, shake gently
3. Put the tube in the separation device for 2 min and pipette off the PBS.
4. Add 0.5 ml T-PBS and incubate for 60 min
5. Remove T-PBS and resuspend the beads in 50 μl T-PBS
6. Transfer 10 μl of beads to appropriate tubes
7. Add 100 μl of peptide/protein dilution to each tube
8. Incubate for 30 min at room temperature in an end-over end rotator
9. Remove 100 μl of incubated peptide/protein fractions by means of the magnet and store them (fractions 1)
10. Wash Dynabeads 5× with T-PBS and finally add 100 μl SDS nonreducing protein sample-buffer (fractions 3) if protein measurements will be done by SDS page, incubate for10 min
11. Load two volumes (e.g. 10 μl and 50 μl) of each sample on SDS-PAGE (use nonreducing sample buffer). (Unbiotinylated protein = fraction 0. Input biotinylated protein = fraction 1. Supernatant = fraction 2. Dynabeads = fraction 3)
12. Perform SDS-page electrophoresis
13. Stain with Coomassie blue, destain
14. Compare protein bands
15. Alternatively, measure UV_{280} absorption of fraction 0 and fraction 1
16. Determine the percentage of biotinylation by comparing the fractions of the lowest concentrations used (protein should be seen in fraction 0 and fraction 3) and check the maximum amount of protein able to bind 10 μl

Streptavidin-Dynabeads. Extrapolate this amount to phage-selection conditions (e.g., a maximum of 30 nM can be bound at about 100% to 10 µl of beads: for 500 mM used during the selections, 166 µl of magnetic beads should be used)

17. To determine the number of biotin molecules per protein/peptide, the HABA method can be used (see Pierce, http:/www.piercenet.com)

11.2.6 Troubleshooting

11.2.6.1 NHS-ss-Biotin Biotinylation

- For very low amounts of protein/peptide: keep them as concentrated as possible, because hydrolysis of the NHS-ester does compete for reaction with the amine, and use a high amount of NHS-ester (without exceeding a 30 molar excess) (Pierce, personal communication).

11.2.6.2 Enzymatic Biotinylation

- Failure to obtain good yields of biotinylation using these techniques can, most of the time, be explained by a degradation of the biotinylation tag due to the presence of protease co-purified with the protein of interest. This problem can occur during biotinylation for the in vitro protocol during the 1h incubation at 30°C. To avoid this, one can add a cocktail of protease inhibitor to the reaction mix. However, EDTA, a common metalloprotease inhibitor, must not be added (magnesium is needed for enzymatic activity). Use, for example, Complete™ EDTA-free (Roche Diagnostics).
- For in vivo biotinylation, tag degradation can occur during storage. In this case, a complete protease inhibitor cocktail can be used (e.g. Complete™, Roche Diagnostics).
- For in vitro biotinylation, a common problem is a loss of activity of the purified birA. The enzyme is delivered frozen and can be kept several months at –70°C. However, several cycles of freeze and thaw rapidly inactivate the enzyme. After the first thawing of the enzyme, it is thus strongly recommended to store the remaining enzyme in small frozen aliquots.

11.3 Selection of Phage-Antibodies Using Biotinylated peptide/Protein

11.3.1 Purpose

The aim of this procedure is to select phage antibodies directed to a biotinylated antigen. The principle is to incubate the phage antibody repertoire in solution

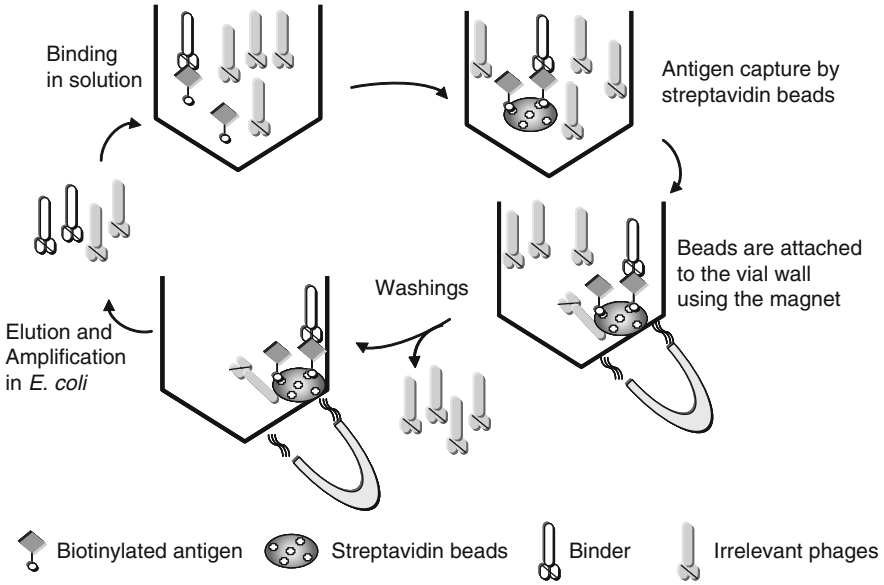


Fig. 11.2 Principle of page antibody selection on paramagnetic beads. This cycle can be repeated 1–4 times to increase the proportion of specific binders

with the biotinylated antigen. Once specific phages are bound to the antigen, paramagnetic beads coupled to streptavidin are added into the solution. The biotinylated antigens with bound phages are captured and the whole complex is drawn out from the suspension by applying a magnet on the side of the tube. Beads are washed several times and specific phages are eluted from the beads (see Fig. 11.2).

11.3.1.1 Materials

- DMSO
- Skimmed dry milk (marvel)
- Dynabeads M280 (Dynal, Oslo, Norway)
- 5*PBS and PBS see protocol 1.1
- 2% M-PBS: 2 g dried skimmed milk (marvel) in100 ml PBS
- 4% M-PBS: 4 g dried skimmed milk powder (marvel, Sainsbury, UK)/100 ml PBS.
- MT-PBS:: 2% Marvel, 2% Tween 20 in PBS.
- MT-PBS(DMSO): MT-PBS 5% DMSO (for peptides difficult to disolve)
- 100 mM TEA: 140 µl triethylamine in10 ml water (pH 12)
- 1 M Tris–HCl, pH = 7.4
- Rotator

Precautions

Check whether the antigen is water soluble in the selection buffers used. If the antigen (peptide) is too hydrophobic, you have to find alternative buffer conditions in which it remains in solution and use these conditions for the selection. We have, for example, successfully used 5% DMSO in all solutions.

11.3.2 Procedure

1. Mix equal volumes of a phage library and 4% M-PBS. During the 1st selection, the number of phage particles should be at least 100 times higher than the library size
2. Incubate on a rotator at room temperature for 60 min
3. While preincubating the phage, wash 100 µl (typically 200 µl for the first round) streptavidin-dynabeads per peptide in a tube, fitting in the magnetic separation device, as described in protocol 1.5. The minimal required amount of beads for selection can be calculated as described in protocol 1.5 (step 16)
4. Remove PBS and resuspend beads in 2% M-PBS
5. Equilibrate on rotator at room temperature for 1–2 h
6. Add biotinylated antigen (100–500 nM; see protocol 1.5) diluted in PBS (DMSO) directly into equilibrated phage mix
7. Incubate on rotator at RT for 30 min to 1 h
8. Draw equilibrated beads (see 5.) to one side with magnet and remove M-PBS
9. Resuspend Dynabeads in 250 µl per antigen in MT-PBS (DMSO)
10. Add Dynabeads to phage-antigen mix and incubate on a rotator at room temperature for 15 min
11. Place tubes in the magnetic separator and wait until all beads are bound to the magnetic site (20 s)
12. Tip rack upside down and back again, with caps closed. This will wash down the beads from the cap
13. Leave tubes in the rack for 2 min
14. Aspirate the tubes carefully, leaving the beads on the side of the tube
15. Wash the beads carefully six times with 1 ml MT-PBS
16. Transfer beads to a new eppendorf tube
17. Wash the beads six times with 1 ml MT-PBS
18. Transfer the beads to a new eppendorf tube
19. Wash the beads two times with 1 ml PBS
20. Transfer to a new tube
21. Elute page from beads with 1 ml 100 mM TEA, or in case of an -SS-biotin labeled protein/peptide, use 200 µl 10 mM DTT, rotate 5 min at room temperature

22. In case of elution with TEA: transfer the solution to an eppendorf tube containing 0.1 ml Tris–HCl, 1 M, pH = 7.4 and mix by inversion. It is necessary to neutralize the phage eluate immediately after elution. In case of DTT elution, transfer solution to new tube
23. Titrate in and output by infection of bacterial (TG1) cells
24. Re-infect bacterial cells (TG1 cells) with selected phages for another round of selection
25. Store the remaining beads or eluate at 4°C as a backup

11.3.2.1 Troubleshooting

- If a significant proportion of the peptide/protein is not labeled, one can incubate the antigen with the streptavidin beads, taking into account the molarity of the biotinylated peptide/protein, and wash away the nonbiotinylated peptide. The beads are then used directly for the selection.
- For the selection of high affinity antibodies, it is advised to select with a decreasing antigen concentration. For example, with: 100 nM biotinylated peptide during the first round, with 20 nM for the second round, with 5 nM for the third round, and with 1 nM for the 4th round.
- If streptavidin binders are preferentially selected (which may be the case when using nonimmunized or synthetic antibody libraries) the following steps can be undertaken:
  - If the peptide/protein is biotinylated with NHS-L-C-biotin, it is advised to deplete the library by incubating for 1 h (from round 2 on and later) with 100 µl streptavidin-Dynabeads before adding the biotinylated antigen to the depleted library.
  - If the peptide is biotinylated with NHS–SS-biotin, 10 mM DTT should be used to elute the antigen-binding phage specifically.

11.4 (*Inhibition*) ELISA with Indirectly Coated Biotinylated antigen

11.4.1 Purpose

This very sensitive ELISA aimed to screen monoclonal phage uses the same biotinylated antigen already used during the selection step. The indirect coating via streptavidin ensures maintenance of the native structure of the antigen. A precoating of the plastic surface with biotinylated BSA is used to circumvent the low adsorption properties of streptavidin (see Fig. 11.3).

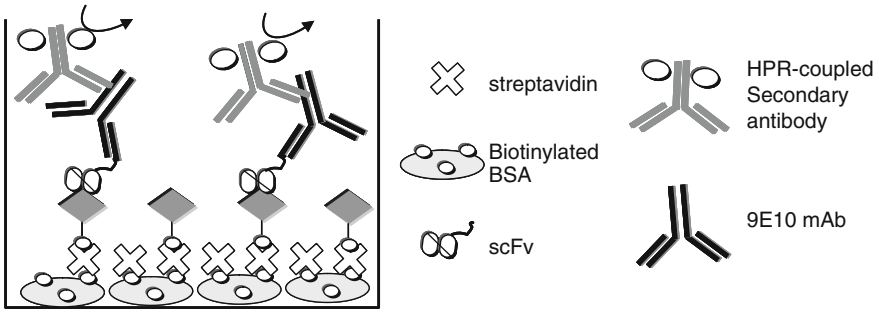


Fig. 11.3 Scheme of an ELISA experiment performed using biotinylated antigen and soluble antibody fragments. The first layer of biotinylated BSA is used to circumvent the low adsorption properties of streptavidin

11.4.2 Materials

- Supernatant of induced fractions coming from colonies picked from the selections with Dynabeads
- Biotinylated antigen of interest, at a concentration of 1–5 μg/ml M-PBS (DMSO). For inhibition ELISA the concentration should be 1 μg/ml
- *For inhibition ELISA (IE) Non-biotinylated antigen at a concentration of 1 mg/ml in 2% M-PBS. Steps involved in inhibition ELISA are printed in italics*
- PBS, PBS (DMSO), M-PBS, T-PBS see protocol 2
- T-PBS: 2% Tween 20 in PBS
- Biotinylated BSA, stock solution: 2 mg/ml PBS, working solution: per microtiter plate add 10 μl of stock solution to 10 ml PBS
- PBS/0.5% gelatine: 0.5 g gelatin/100 ml PBS. Store at 4°C. Prewarm this solution before use (60°C or microwave) to dissolve the gelatine. Use at room temperature
- Streptavidin solution: stock solution: 1 mg/ml H_2O, working solution: Add per plate 100 μl of the stock solution to 10 ml PBS/0.5% gelatine
- 2% M-PBS: see protocol 2
- 4% M-PBS: see protocol 2
- 9E10 monoclonal antibody (Santa Cruz Biotechnology): used for Myc-tagged antibodies, dilute with 2% M-PBS at a dilution advised by the supplier
- Rabbit Anti Mouse PerOxidase (RAMPO) working solution: dilute the antigen at a concentration advised by the supplier in 2% M-PBS
- Ten times Tetra-Methyl-Benzydine-buffer (TMB): dissolve 37.4 g NaAcetate. $3H_2O$ in 230 ml H_2O. Adjust pH with saturated citric acid (92.5 g citric acid/50 ml H2O) and adjust volume to 250 ml
- TMB stock: Dissolve 10 mg of TMB in 1 ml DMSO
- TMB-staining solution: Mix 1 ml ten times TMB with 9 ml of H_2O per microtiterplate. Add, per plate, 100 μl of the TMB to 10 ml of TMB-buffer. Add 1 μl of 30% hydrogen peroxidase. Make this solution fresh and keep it in the dark

- 2N H2SO4: 53.2 ml of concentrated H_2SO_4/liter H_2O
- 96-well flat-bottomed ELISA microtiter plates (two plates to screen 96 colonies) (Falcon 3912)
- *Only for IE:+ microtiter plates with low coating efficiency (2/96 colonies)*
- Microtiter plate reader (for OD_{450} measurements)

11.4.3 Procedure

1. Add 100 µl of biotinylated BSA to each well of the microtiter plate. For 96 induced fractions, two plates should be coated (negative control and positive plate)
2. Incubate for 1 h at 37°C or overnight at 4°C
3. Discard the solution of step 2. Wash plates three times for 5 min with T-PBS by submersing the plate into the buffer and removing the air bubbles by rubbing the plate
4. Add 100 µl/well streptavidin to negative and positive plates
5. Incubate for 1 h at room temperature while shaking
6. Wash plates as described in 3
7. Add 100 µl of biotinylated antigen to each well of the positive plate and add 100 µl 2% M-PBS to the wells of the negative control plate. (*For IE: add the biotinylated antigen to both plates*)
8. Incubate for 1 h at room temperature
9. Wash plate three times with T-PBS (DMSO) as described in 3
10. Block with 2% M-PBS/DMSO. Use 120 µl/well, incubate for at least 30 min at room temperature
11. Empty wells, add 50 µl/well of 4% M-PBS (DMSO) to all wells of both plates *(for IE: use two noncoated plates with low coating efficiency)*
12. Add 50 µl/well of culture supernatant containing soluble antibody fragment
13. *Only for IE: add 10* µl*/well of M-PBS to the positive plate, add 10* µl*/well of nonbiotinylated antigen to the negative plate. Mix by pipetting and incubate for 30 min. Transfer 100* µl *to microtiter plates coated with biotinylated antigen*
14. Mix by pipetting and incubate for 1.5 h at room temperature
15. Wash three times with T-PBS as described in 3
16. Add 100 µl/well of 9E10 solution to all wells and incubate for1 h RT
17. Wash as in 3
18. Add 100 µl/well of RAMPO solution to all wells and incubate for 1 h at room temperature
19. Wash as in 3
20. Stain with TMB by adding100 µl/well of TMB staining solution. Incubate for 10–30 min in the dark
21. Add 50 µl /well 2N H_2SO_4
22. Read plate at 450 nm

23. If the optical density of a clone on the positive plate is higher than two times the optical density of the same clone on the negative plate, it can be considered positive and should be tested further

References

Butler JE, Ni L, Nessler R, Joshi KS, Suter M, Rosenberg B, Chang J, Brown WR, Cantarero LA (1992) The physical and functional behavior of capture antibodies adsorbed on polystyrene. J Immunol Methods 150:77–90

Davies J, Dawkes AC, Haymes AG, Roberts CJ, Sunderland RF, Wilkins MJ, Davies MC, Tendler SJ, Jackson DE, Edwards JC (1994) A scanning tunnelling microscopy comparison of passive antibody adsorption and biotinylated antibody linkage to streptavidin on microtiter wells. J Immunol Methods 167:263–269

de Haard HJ, van Neer N, Reurs A, Hufton SE, Roovers RC, Henderikx P, de Bruïne AP, Arends JW, Hoogenboom HR (1999) A large non-immunized human fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J Biol Chem 274:18218–18230

Hawkins RE, Russell SJ, Winter G (1992) Selection of phage antibodies by binding affinity. Mimicking affinity maturation. J Mol Biol 226:889–896

Henderikx P, Kandilogiannaki M, Petrarca C, von Mensdorff-Pouilly S, Hilgers JH, Krambovitis E, Arends JW, Hoogenboom HR (1998) Human single-chain Fv antibodies to MUC1 core peptide selected from phage display libraries recognize unique epitopes and predominantly bind adenocarcinoma. Cancer Res 58:4324–4332

Hnatowich DJ, Virzi F, Rusckowski M (1987) Investigations of avidin and biotin for imaging applications. J Nucl Med 28:1294–1302

Ivanov VS, Suvorova ZK, Tchikin LD, Kozhich AT, Ivanov VT (1992) Effective method for synthetic peptide immobilization that increases the sensitivity and specificity of ELISA procedures. J Immunol Methods 153:229–233

Loomans EE, Gribnau TC, Bloemers HP, Schielen WJ (1998) Adsorption studies of tritium-labeled peptides on polystyrene surfaces. J Immunol Methods 221:131–139

Oshima M, Atassi MZ (1989) Comparison of peptide-coating conditions in solid phase plate assays for detection of anti-peptide antibodies. Immunol Invest 18:841–851

Pyun JC, Cheong MY, Park SH, Kim HY, Park JS (1997) Modification of short peptides using epsilon-aminocaproic acid for improved coating efficiency in indirect enzyme-linked immunosorbent assays (ELISA). J Immunol Methods 208:141–149

Schatz PJ (1993) Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in Escherichia coli. Biotechnology N Y 11:1138–1143

Schier R, Bye J, Apell G, McCall A, Adams GP, Malmqvist M, Weiner LM, Marks JD (1996) Isolation of high-affinity monomeric human anti-c-erbB-2 single-chain Fv using affinity-driven selection. J Mol Biol 255:28–43

Tam JP, Zavala F (1989) Multiple antigen peptide. A novel approach to increase detection sensitivity of synthetic peptides in solid-phase immunoassays. J Immunol Methods 124:53–61

Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR (1994) Making antibody by phage display technology. Ann Rev Immunol 12:433–455

Chapter 12
Phage Display and Subtractive Selection on Cells

Steffen U. Eisenhardt and Karlheinz Peter

12.1 Introduction

Antibodies were the first proteins to be successfully displayed on the surface of phage (McCafferty et al. 1990), and today, phage display has found its most powerful application in the isolation of recombinant antibodies from antibody libraries with unique specificities (Barbas et al. 1991; Clackson et al. 1991; Griffiths 1993; Hoogenboom et al. 1998). The selection of recombinant antibodies from antibody libraries has great potential for the generation of antibodies for diagnostic and therapeutic use. The advantage of the in vitro selection process of phage display lies in the possibility of specifically designing the selection matrix, and therefore obtaining more specific antibodies than with the classical hybridoma technology, thus resulting in a smaller variety of more specific antibodies. This reduces the amount of laborious work that needs to be invested into the characterization of different clones. However, this requires a thorough design of the panning protocol in order to obtain specific antibodies and to make full use of the advantages of the in vitro selection process. Therefore, the choice of the appropriate display and panning strategy is of major importance.

Various display methods have been described for the selection of recombinant antibodies from antibody libraries such as bacterial surface display (Fuchs et al.

S.U. Eisenhardt (✉)
Department of Plastic and Hand Surgery, University of Freiburg Medical Centre, Hugstetter Strasse 55, Freiburg 79106, Germany
e-mail: s.eisenhardt@gmx.net

K. Peter
Baker Heart Research Institute, 6492 St Kilda Road Central, Melbourne, VIC 8008, Australia

Conflict of interest: The authors declare no conflict of interest. The protocol described has been published in parts in Eisenhardt et al. *Nature Protocols* 2007;2:3063–3073.

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_12,

1991), ribosomal display (Zahnd et al. 2007), puromycin display (Roberts and Szostak 1997), or yeast surface display (Boder and Wittrup 1997). However, to date, phage display based on the M13 pIII peptide phage display technology (Smith 1985) is the most widely used method. Very often, the choice of the preferred display system is solely dependent on personal preferences. However, they all depend on suitable selection or "panning" strategies.

The main advantage of the traditional phage display over the classical hybridoma strategy is that the selection can be carried out on isolated, highly purified epitopes. These can be presented to the phage library as immobilized targets on solid surfaces, such as 96-well polystyrole microtiter plates (Barbas et al. 1991), polystyrole tubes (Hust et al. 2002), nitrocellulose (Hawlisch et al. 2001), or magnetic beads (Moghaddam et al. 2003). Other protocols describe the artificial immobilization of target epitopes (Clackson et al. 1991), epitopes on intact mammalian cells (Giordano et al. 2001), or even in vivo epitopes (Arap et al. 2002) for screening purposes. When deciding on the ideal protocol for panning purposes, it should be taken into consideration that conformational changes can occur within the epitope upon immobilization, or that functionally important epitopes may not be exposed or functionally regulated by stimuli when immobilized. These limitations can be avoided by the use of an in vivo phage display system (Robert et al. 2006). However, this gives away the advantages of the traditional phage display, and therefore has the same limitations as hybridoma strategies such as the unspecific binding to the abundance of antigens in the circulation.

The protocol described in this chapter provides detailed descriptions of a panning strategy of a scFv-library on cell surface receptors in cell suspension and is based on the M13 pIII phage display system. We provide a subtractive strategy, which is based on an initial depletion step against unwanted epitopes followed by a selection step for the target epitope. In this protocol cells can be kept in physiological buffer systems, thus conformational changes can be induced by activation of cells and antibodies can be selected for specificity to various activation states of cell surface epitopes, thus allowing for the selection of conformation-specific antibodies against intact, functionally active, complex, multimeric epitopes on cell membrane molecules (Eisenhardt et al. 2007a; Schwarz et al. 2004, 2006).

The protocol has been performed previously with a single-chain antibody (scFv) phage library (Dorsam et al. 1997; Little et al. 1999), and allows for the selection of highly specific scFv antibody clones that are able to discriminate between various conformational states of cell surface receptors, e.g., this panning strategy is suitable for the selection of scFvs against adhesion receptors of the integrin family (Eisenhardt et al. 2007b; Schwarz et al. 2004, 2006), which are known to undergo a rapid shape change upon activation (Hynes 1992; Takagi and Springer 2002), resulting in a high affinity state for their ligands (Fig. 12.2).

Due to the subtractive, cell-suspension based concept, complex, multimeric, fully functional cell membrane epitopes without alteration of structure due to purification and immobilization can be targeted. The functional properties of cells and receptors remain untouched, therefore, physiological changes can be induced

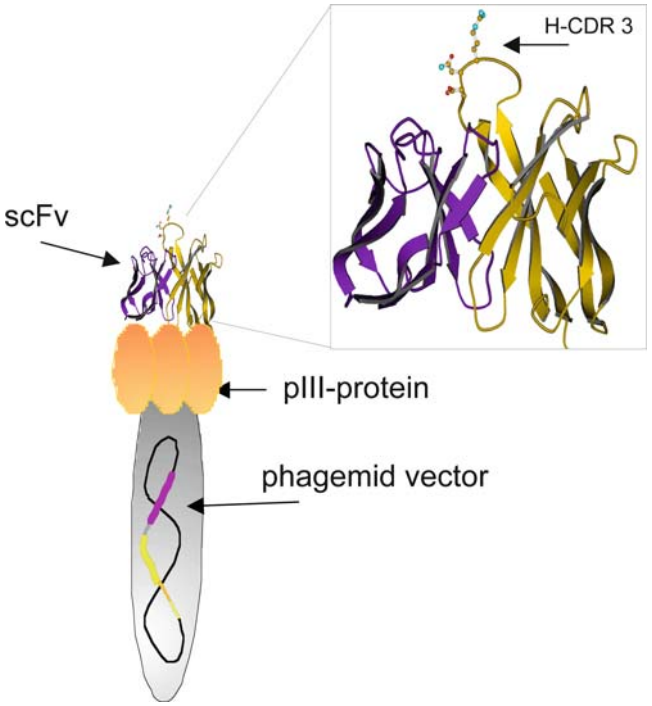


Fig. 12.1 Single-chain antibody display on pIII of the M13 phage. The phagemid encodes for single-chain antibodies with the antibody heavy chain fused to the antibody light chain via a linker peptide. Each scFv is His(6)-tagged for purification and detection. The scFv is displayed as a pIII-fusion protein on the surface of the M13 phage. The antibody heavy chain is depicted in pink, and the light chain in yellow. Enlarged is the 3D-structure of a typical scFv clone. The model was created with Rasmol-pdb viewer (University of Massachusetts). Reproduced from Eisenhardt et al. (2007a, b)

and phage can be specifically depleted or selected for neoepitopes that get exposed after stimulation or other physiological alterations if required. Furthermore, this subtractive strategy avoids the selection of phage that bind to the plethora of cell surface epitopes that are not of interest. It is highly effective in reducing the number of recovered nonspecific clones, in reducing the number of washes required in the selection steps, and ultimately the number of potential ligands that are lost during the repeated washing steps. The latter is a typical drawback of classical panning strategies. However, the protocol can also be performed without the initial depletion step. The affinity maturation process of the panning rounds can be easily screened by restriction digest and the binding of selected clones can be screened by either ELISA or flow cytometry (FACS).

When choosing the right cell type or cell line for the panning process, several facts have to be considered:

1. The multiple step panning approach allows tailoring of specificity by changing the selection matrix between the panning rounds in order to obtain maximal

specificity and tailoring of affinity by variation of elution conditions and by competitive elution steps (Eisenhardt et al. 2007a, b). In our hands, the combination of native and recombinant cell lines proved to be ideal to reduce binding to other neoepitopes that are exposed on the membranes after cell activation. If a recombinant cell line is not available, all panning rounds can be performed on native cells, but a higher diversity of the resulting antibodies has to be expected.
2. Competitive elution steps can improve the properties of the selected antibodies when panning for a blocking antibody or an antibody that is competing with a known ligand of the receptor. We used this approach for the selection of an anti-GPIIb/IIIa activation-specific antibody to achieve fibrinogen blocking properties for a potential therapeutic use (Schwarz et al. 2004).
3. The subtractive panning strategy is a powerful tool to obtain antibodies with a high specificity. However, in the planning of the selection strategy, it should be taken into account that some cross reactivity is useful, e.g., if antibodies for a potential therapeutic application are selected, a cross reactivity with mice epitopes can facilitate the further testing of the antibody in mouse models. In this case, a panning round on mouse-specific epitopes should be considered.

The protocol described in this chapter is unique in that it is specifically designed for cell surface epitopes rather than soluble proteins, and it does not depend on the existing "guiding" antibodies like otherwise described selection methods (Osbourn et al. 1998a,b; Parsons et al. 1996). The protocol is widely transferable and could be used for alternative approaches such as the BRASIL-protocol as described by Giordano et al (Giordano et al. 2001). We recommend the use of phagemid libraries as used in this protocol, as the low valency of phagemid-based display systems reduces background binding and increases the affinity of the selected clones (Levitan 1998). However, our protocol is transferable to other commercially available scFv phage libraries (de Wildt et al. 2000), and to other library display systems.

12.2 Materials

12.2.1 Reagents

- 0.1 M Glycine pH 2.2 (Astral Scientific, cat.no. 0167)
- 0.1 M PIPES pH 6.6 (Sigma, cat. no. P1851)
- 0.4 M Sucrose (Merck, cat. no. 102747E)
- 10× Cellfix (Becton Dickinson, cat. no. 340181)
- 10× PBS without Ca^{2+}/Mg^{2+} (Cambrex, cat. no. 17-515F)
- 1 M IPTG (Invitrogen, cat. no. 15529-019)
- 1× PBS with Ca^{2+}/Mg^{2+} (Invitrogen, cat. no. 14040-133)
- 2 M Tris HCl pH 8 (Sigma, cat. no. T6666)
- Acetic acid (Sigma, cat. no. A6283)

- ADP (Moelab, cat.no. 0203011)
- Agarose (Sigma, cat. no. A9414)
- Alkaline Phosphatase (Roche, cat. no. 713023)
- Ampicillin (Sigma, cat. no. A0166)
- Anti-His(6) antibody HRP (Roche, cat. no. 1965085)
- BCA Protein Assay Kit (Pierce, cat. no. 23227)
- Blue/Orange Loading Dye 6x (Promega, cat. no. G1881)
- BSA (Sigma, cat. no. A7906)
- Bug Buster Mastermix (Merck, cat. no. 71456.4)
- $CaCl_2$ (Sigma, cat. no. C3306)
- ddH_2O
- DNA Ladder 1 kb (Promega, cat. no. G5711)
- EDTA (Sigma, cat. no. E6758)
- Epifibatide (Essex Pharma)
- Ethanol (Biolab, cat. no. BSPEL975.2.5)
- Ethidiumbromide (Astral Scientific, cat. no. AMX328). EthBr is carcinogenic Wear appropriate protection and handle with care
- fMLP (Sigma, cat. no. F3506)
- Glucose (Riedel de Haen, cat. no. 16325)
- Glycerol (Sigma, cat. no. G5516)
- Helper-Phage M13K07 (GE Healthcare, cat. no. 27-1542-01)
- HEPES (Sigma, cat. no. H7523)
- Human scFv phage library (Affimed) or other commercially available scFv library (e.g., Tomlinson I + J, MRC geneservice)
- Kanamycin (Invitrogen, cat. no. 55-0204)
- KCl (Merck, cat. no. 1.04936.0500)
- LB agar (Sigma, cat.no. L3027)
- Liquid nitrogen. Liquid nitrogen causes burns. Wear appropriate protection and handle with care
- LPS (Sigma, cat. no. L4391)
- Luria Broth Media (Sigma, cat. no. L3522)
- Manganese (Sigma, cat. no. 244589)
- $MgCl_2$ (Sigma, cat. no. M2670)
- Mouse IgG1 Isotype Control (Beckman Coulter, cat. no. IM0639)
- NaCl (Biolab, cat. no. BSPSL944.5)
- $NaHCO_3$ (Merck, cat. no. 1.0632905)
- Ni-NTA Agarose (Qiagen, cat. no. 30210)
- PEG 6000 (Sigma, cat. no. 81260)
- Penta-His Alexa Fluor 488 (Qiagen, cat. no. 35310)
- pexHAM vector (Affimed)(Le Gall et al. 1999)
- pHOG-21 Vector (Affimed)(Kipriyanov et al. 1997)
- PMA (Sigma, cat. no. P1585)
- QIA quick Gel Extraction Kit (Qiagen, cat. no. 28704)
- QIAprep Spin Plasmid Preparation Kit (Qiagen, cat. no. 27106)
- Rapid DNA Ligation Kit (Roche, cat. no. 1635379)

- Restriction endonucleases (all from New England Biolabs): BstN I (cat. no. R0168L), Rsa I (cat. no. R0167S)
- Tetracycline (Sigma, cat. no. T7660)
- Theophylline (Sigma, cat. no. T1633)
- Trizma Base (Sigma, cat. no. T1503)
- E. coli XL-1 blue (Stratagene, cat. no. 200249) Grow in LB media containing 20 μg/ml tetracycline

12.2.2 Reagent Setup

3% (wt/vol) *agarose gel* 300 ml 1xTAE buffer, 3 g agarose. Heat in the microwave until the agarose is completely dissolved, and then add 30 μl ethidiumbromide. Ethidiumbromide is carcinogenic. Always handle with care. We recommend using double layers of gloves and avoiding direct contact with the gels, or using safer alternatives such as GelRedTM or SYBR Green.

50 × TAE buffer 2 M Tris, 5 mM EDTA pH 8, 5.5% Acetic acid.

Agar plate 25 g LB agar, 50 mM Glucose, 100 μg/ml Ampicillin, 20 μg/ml Tetracycline. Autoclave LB agar, then add Glucose, Ampicillin, and Tetracycline and pour the plates.

Elution buffer pH 8.0 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, adjust pH.

HEPES modified Tyrode's buffer, pH 6.5 10 mM HEPES, 12 nM $NaHCO_3$, 138 mM NaCl, 2.9 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 g/l Glucose, 1 g/l BSA, adjust pH.

Hyperosmotic Shock Solution pH 8 20% Sucrose, 1 mM EDTA, 50 mM Trizma Base.

LBGAT agar plate LB agar, 50 mM Glucose, 100 μg/ml Ampicillin, 20 μg/ml Tetracycline. Autoclave LB agar, then add Glucose, Ampicillin, and Tetracycline and pour the plates.

LBGAT media LB medium, 50 mM Glucose, 100 μg/ml Ampicillin, 20 μg/ml Tetracycline, autoclave LB medium, then add Glucose, Ampicillin, and Tetracycline.

LBTc LB medium, 20 μg/ml Tetracycline, autoclave LB medium, let medium cool down, then add Tetracycline.

Lysis buffer pH 8.0 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, adjust pH with 1 M NaOH to raise pH, or with 1 M HCl to lower pH.

Modified Tyrode_s buffer, pH 7.4 150 mM NaCl, 12 mM $NaHCO_3$, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mg/ml BSA, 1 mg/ml glucose, adjust pH.

PDB (Phage dilution buffer) pH 7.5 10 mN Tris HCl, 20 mM NaCl, 2 mM EDTA, adjust pH and autoclave.

Transformation Buffer 25 ml 1 M $CaCl_2$, 150 ml 50% Glycerol, 0.1 M PIPES pH 6.6, 275 ml H_2O, autoclave.

Wash buffer pH 8.0 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, adjust pH.

12.3 Methods

12.3.1 Depletion of Non-specific Phage

1. Thaw frozen aliquots of human scFv phage library (e.g., 1 ml of phage in phage dilution buffer) on ice. The number of phage should exceed the complexity of the used library by 1,000-fold.
2. Isolate cells that do not express the target epitope or that express the target receptor in its resting conformation. For cells that are easily activated by preparative steps, such as platelets, activation can be suppressed by addition of inhibitors (e.g., theophylline in the case of platelets) to the buffer. For leukocytes, preparative steps that include plastic adhesion should be avoided, since leukocyte adhesion can cause cell activation. Magnetic separation methods are preferable. Alternatively, a recombinant cell line, expressing the target receptor can be used.
   The purity of the cell lines should be monitored by flow cytometry. For cells originating from whole blood preparation, it should be $\geq$90%. As this protocol does not use purified epitopes, the preparation and the purity of the cells is crucial for the selection of specific antibodies. In particular, this is the case for the selection step. In the depletion step, contamination with other cells is less crucial or might even be beneficial, as phage binding to the contaminating epitopes will be discarded.
3. Centrifuge harvested or isolated cells for 10 min at 140 *g* and resuspend pellet to a concentration of 2×10^6 in 1 ml modified Tyrode's buffer.
4. Add phage library and incubate for 2 h at room temperature (20–23°C) with gentle rotation on a tube rotator. If a rotator is unavailable, keep cells in suspension by periodic inversion. Phage internalization can occur during incubation of the phage library with the cell line at room temperature. This can be avoided by performing step 4 at 4°C. However, lowering the temperature to 4°C can lead to receptor clustering, unspecific cell activation (in particular with platelets), and may also inhibit the expression of function (e.g., cell activation)-specific epitopes. Thus, for targeting of function-specific epitopes, we recommend performing the incubation step at room temperature.
5. Pellet cells at 140 g for 20 min and transfer supernatant to fresh Falcon tube.
   Always use filtered pipette tips when manipulating phage to avoid contamination.

12.3.2 Selection of Activation-Specific Binding Phage

1. Prepare selection cell line that bears the target epitope or the target receptor in its activated conformation. For the conformation-specific panning against platelet integrins, we used 20 μM ADP, for a monocyte panning 100 ng/ml PMA. If you

are working with recombinant cell lines expressing integrins, conformational changes can be achieved by mutations or incubation with Mn.
2. Pellet 2×10^6 cells and resuspend in the supernatant from step 5, which contains the unbound phage from the depletion step. Mix by gentle inversion. The low pH of the selection buffer reduces the nonspecific binding of insert-free phage. The cell number in the selection rounds influences the result. Low numbers of cells increase the selection pressure, and thus result in low numbers of high affinity clones, whereas high cell numbers reduce the selection pressure.
3. Incubate for 30 min at room temperature with gentle rotation to allow binding of phage to recognition patterns that were not available in the depletion step.
4. Pellet cells for 10 min at 140 g and take off supernatant that contains the unbound phage. The unbound phage can be discarded.
5. Wash cells by resuspending pelleted cells in 50 ml HEPES buffer.
6. Centrifuge at 200 *g* for 20 min and resuspend pellet in 1 ml HEPES buffer and transfer to a 1.5 ml Eppendorf tube. Repeat washing (steps 10 and 11) with lower volumes (e.g., 1 ml) twice.
7. Elute phage by resuspending the pellet in 1 ml glycine elution buffer (pH = 2.2) and incubating for 15 min at room temperature with gentle inversion every other minute.
8. Neutralize by addition of 100 µl 2 M Tris pH = 8, and transfer into a new 1.5 ml Eppendorf tube. Transfer to step 3.2.4. quickly as neutralized phage could rebind to the cells that are still present in the tube.
9. Spin in a tabletop centrifuge for 10 min at 18,000 *g* and transfer supernatant into a new 1.5 ml Eppendorf tube.
   The phage elute can be kept at 4°C overnight if convenient.

12.3.3 Reinfection of XL-1 Blue E. coli with the Selected Phage

1. Incubate XL1-blue *E.coli* in LBTc (10 µl XL1 blue in 5 ml) with shaking at 200 rpm at 37°C for approximately 5 h until the cells reach mid-log phase ($OD_{600} = 0.8$). Monitor the growth of the cells by taking multiple OD measurements after an incubation period of 4 h.
2. Mix 10 ml of log phase XL1-blue E.coli with the phage eluate from step 3.2.8.
3. Incubate for 30 min at 37°C without shaking. Gently invert every 10 min.
4. Centrifuge at 5,500 *g* for 10 min and resuspend pellet in 400 µl LBGAT-media. The phage carry an ampicillin resistance marker. Only successfully infected bacteria will grow.
   The resistance marker can vary in different vector systems and phage libraries. Resistance markers have to be carefully checked before adding suitable antibiotics.

5. Plate bacterial suspension onto a 14.5 cm LBGAT-agar plate and grow overnight at 37°C.
6. Count the number of colonies of reinfected XL-1 blue on the LBGAT plates. This can be done by imaging and analysis with contrast-based counting programs, such as Image Pro Plus. Expect numbers of 2,000–5,000 colonies after the first round.

12.3.4 Repacking of Phage with M13K07 Helper Phage for the Following Panning Rounds

1. Pipette 5–10 ml of LBGAT media on the plates and carefully dilute the bacterial colonies by mixing them with the media using a spatula.
2. Take off the resuspended bacteria by pipetting and transfer to a 15 ml Falcon tube. Take off 10 µl and add to 990 µl of LBGAT and measure the OD_{600} of this 1:100 dilution.
3. Inoculate 20 ml LBGAT media with the bacteria at an OD_{600} of 0.1.
   Example: If you measured an OD_{600} of 0.09 for your 1:100 dilution, your OD_{600} of your bacterial suspension is 9, and to achieve an OD_{600} of 0.1, you need to prepare a 1:90 dilution, which equals 222 µl on 20 ml.
4. The rest of the bacterial suspension can be kept as glycerol stocks. This allows going back to previous panning rounds if one panning round failed or did not result in the amplification of a clone with the sought-after characteristics. Add sterile glycerol to a final concentration of 15% to the bacteria from step 3.4.2. and store at −80°C.
   The panning can be stopped here, if convenient. Bacterial stocks from step 3.4.2. can be kept for several years at −80°C. When proceeding with the panning, continue with step 3.4.3.
5. Incubate the 20 ml LBGAT inoculated with the XL-1 blue carrying phage with shaking at 200 rpm at 37°C for 1 h.
6. Add M13K07 helper phage in a MOI (Multiplicity of Infection: phage/bacteria) of 10–20. You can estimate the number of bacteria. An OD600 of 1 equals 1×10^9 bacteria/ml. The culture was started at an OD of 0.1, which corresponds to 1×10^8 bacteria/ml (see step 3.4.3.). In the 20 ml suspension, 4×10^9 bacteria can be expected as the number has doubled within 1 h of cultivation. 4×10^{10} phage should be added (MOI = 10). 40 ml of the phage suspension at a phage titre of 10^{12} phage/ml should be added.
7. Mix carefully by gentle inversion and incubate at 37°C for 30 min with gentle inversion every 10 min, but without shaking.
8. Incubate a further 30 min with shaking in a bacterial shaker at 200 rpm and 37°C.
   For successful phage infection, bacteria have to be grown at temperatures >34°C, as this is essential for pili formation.

9. Pellet bacteria by centrifugation for 5 min at 5,500 *g*.
10. Discard supernatant and resuspend in 20 ml LB media containing ampicillin and kanamycin (50 μg/ml) without glucose and tetracyclin. The M13K07 helper phage carries a kanamycin resistance marker that allows the identification of successfully co-infected bacterial clones.
11. Incubate overnight with shaking in a bacterial shaker at 200 rpm and 30°C.

12.3.5 PEG-Precipitation of Phage

1. Centrifuge bacterial culture at 5,500 *g* for 10 min.
2. Precipitate phage particles by transferring the supernatant to a new tube containing 1/5 volume of PEG/NaCl. Mix carefully by inversion, do not vortex.
3. Incubate for 2 h on ice.
4. Centrifuge for 1 h at 5,500 *g* at 4°C and discard the supernatant.
5. Resuspend pellet in 1 ml phage dilution buffer and transfer to 1.5 ml Eppendorf tube.
6. Centrifuge for 5 min at 14,000 *g* in a tabletop centrifuge, take off supernatant, and store at 4°C for the next panning round.
   The stock is stable at 4°C for up to 6 months. For long-term storage, sterile glycerol can be added to a final concentration of 15% and phage stored at −80°C.
7. For the next panning round, the number of phage should be determined. Inoculate 5 ml LBTc-media with 100 μl of thawed XL1-blue stock. Incubate log-phase bacteria with the phage dilution and infect as described in step 3.3.3. Make stepwise dilutions (for example, 10^3–10^{10}) and spread transduced bacteria on several LBGAT agar plates, or subdivide on 14.5 cm LBGAT agar plates in multiple sections and spread different dilutions in these sections. A LBGAT plate with kanamycin can serve as control.
8. Incubate overnight in a bacterial incubator and count colonies the next day. The number of colonies multiplied with the dilution factor will give an estimate of how many phage are in the dilution after the panning round. Expect 10^{11}–10^{13}. Always change pipette tips after each dilution step to avoid false high results and use filtered pipette tips.

12.3.6 Random Pattern Restriction Analysis

1. When the number of colonies after reinfection (see step 3.3.6.) is amplified two to fourfold, the panning should be stopped and the colonies should be screened for diversity.
   Pick 10–20 clones with sterile pipette tips from the LBGAT plates, drop the tips into 5 ml of LBGAT media and grow them overnight at 37°C with shaking at

220 rpm. The picked colonies should be marked and numbered with a permanent marker at the backside of the plate and the plates kept at 4°C.
2. Isolate DNA of the picked clones with a commercial mini-prep kit (Quiagen) and follow the manufacturer's instructions. Determine concentration. Approximately 0.3 μg/μl can be expected. If preferred, all picked clones can be sequenced and the diversity can be analyzed.
3. Digest 10–20 DNA preparations with BSTN-1 or RSA-1 for random pattern restriction analysis. Prepare reaction solution as described in the manufacturers' protocol.
4. Add 2 μl loading dye per tube and run on a 3% agarose gel in TAE buffer. The time of the run depends on the gel electrophoresis system used. We recommend loading a marker to assess the progress of the run. However, the marker is not necessary for the analysis of the results.
5. Stain DNA-gels with ethidium bromide and analyze restriction patterns.
   At this stage, glycerol stocks should be made of each different clone. The numbered clones can be picked from the agar plate (from step 3.6.1.), grown overnight, and glycerol stocks prepared as described in Step 24. Bacterial stocks of the picked clones can be kept for several years at −80°C.

12.3.7 Small Scale Preparation

1. All clones that differ in their restriction patterns should be screened for their binding characteristics. For testing, a small preparation of the antibodies can be performed. The scFvs are expressed as periplasmic proteins, and for screening purposes, it is not necessary to purify them from other periplasmic proteins. With this method, 40–50 clones can be screened at once. Thaw glycerol stocks or pick clones from the agar plate from step 3.6.1. Inoculate 5 ml LBGA media and grow overnight at 37°C with shaking and 220 rpm.
2. Inoculate 5 ml of LBGAT media with 100 μl of the preculture and grow at 37°C with shaking at 220 rpm for approximately 5 h until the cells reach mid-log phase ($OD_{600} = 0.8$). Monitor the growth of the cells by taking multiple OD measurements after an incubation period of 4 h.
3. Centrifuge at 1,800 *g* for 10 min at room temperature.
4. Discard supernatant and resuspend pellet in 5 ml LB-media with 0.4 M sucrose, 50 μg/ml ampicillin, and 0.1 mM IPTG and grow overnight at 24°C with shaking at 220 rpm. The pexham vector contains a TAG amber-stop codon between the scFv sequence and the pIII sequence. XL-1 blue is a suppressor strain, and thus does not stop translation at the amber codon. The translation stops after the pIII protein and the scFv are expressed and purified as scFv-pIII fusion proteins for initial testing of binding.
5. Centrifuge bacteria at 5,000 *g* for 10 min at 4°C.
6. Resuspend the pellet in 1/20 of the starting volume in ice-cold hyperosmotic shock solution containing 50 mM Tris, 20% saccharose, and 1 mM EDTA at

pH 8 (e.g., if you inoculated 5 ml of LB media in step 46 with the bacteria, use 250 µl of buffer). Transfer to 1.5 ml Eppendorf tube and incubate for 1 h on ice.
7. Centrifuge for 30 min at 30,000 *g* at 4°C. (Place tabletop centrifuge in a cool room).
8. Take the periplasma supernatant and transfer to a Slide-A-Lyzer MINI Dialysis Unit. Dialyse for up to 12 h against PBS. Change buffer several times. The dialysis is necessary to remove IPTG from the samples.
9. Analyze periplasma product for the expression of the scFv by SDS-PAGE and Western blotting and staining with anti-His(6)-Tag-HRP. Western blotting is highly suitable for the screening of the scFv-expression, since it allows assessing the correct size of the bands and the purity of the preparation.
   The expression and purification conditions can vary between different vector and expression systems. If you are using a commercially available library, please refer to the data sheet for the suggested culture, induction, and purification conditions.

12.3.8 Screening for Binding Characteristics

1. Purify target cells (e.g., platelets or monocytes; whole blood preparations are a time saving option as well) or harvest cell lines expressing the epitope of interest and adjust to 6×10^6/ml. Prepare one sample with a cell line that does not express the target epitope, or one nonactivated and one activated sample in case of conformation-specificity. The same stimulant that was used in the panning rounds to induce conformational changes should be used for the initial screening. However, once a successfully binding clone has been identified, other stimulants can be used to confirm the activations-specificity, e.g., we used PMA for the initial panning on activated monocytes and confirmed activation-specificity with LPS and fMLP.
2. Incubate both samples with 30 µl of periplasma product for 15 min at room temperature.
3. Add 1 µl of anti-His-tag-AlexaFluor 488 conjugate and incubate for 15 min. Also prepare adequate negative controls. For screening purposes, incubating the cells with 1 µl of anti-His-tag-AlexaFluor 488 conjugate alone, without primary antibody or isotype matched control antibody is sufficient.
4. Add 200 µl of Cellfix and proceed to flow cytometric analysis. Figure 12.3c. shows a typical example of the flow-cytometric analysis of selected clones after phage display.

Screening for binding can also be performed by ELISA. However, immobilization of the target epitope can lead to conformational changes, and thus alter the results.

The DNA of the clones that show the sought-after binding characteristics can now be purified and cloned into a suitable vector for the preferred expression and

purification system. We prefer expression in the pHOG-21 vector and TG-1 E-coli (Eisenhardt et al. 2007a,b). For advice and protocols on other expression systems, please refer to the relevant chapters in this book.

Troubleshooting advice can be found in Table 12.1.

Table 12.1 Troubleshooting advice

Step	Problem	Solution
3.6.5.	No identical clones in the re-striction-pattern analysis.	No amplification of specific phage. More panning rounds are needed. Modify panning conditions (e.g., decrease cell number, perform additional washing steps, and use competitive elution). Modern phage libraries have a complexity of up to 10^{11} unique clones. A greater complexity increases the probability of the selection of a highly specific clone. However, for the screening of high complexity libraries, it is necessary that at least in the first panning round, the large number of phage have to be matched by a large number of antigens. Thus, low copies of the target receptor on the surface of the selection and depletion cell line can reduce the success of depletion and selection. We recommend the use of a recombinant cell line for depletion and selection, if available. However, reducing the antigen-concentration in the course of the panning can be a useful tool to enrich high affinity binders among the phage selected in the previous rounds.
3.6.5.	All picked clones show identical restriction patterns.	Proceed with small-scale purification and test for positive binding. A very strong binder may have been amplified. If the amplified clone does not show the sought-after binding characteristics, screen the clones of the previous panning round for diversity and binding.
3.8.4.	No binding clones in screening of the periplasmic product.	The screening of the periplasmic product is a crude method to obtain preliminary information on the binding characteristic of individual clones. As the amount of purified scFv can be very low when expressed in pexHAM, negative binding in this assay does not generally rule out a positive clone. In case of low numbers (one to two different clones in ten picked clones), we recommend proceeding with the cloning of the DNA into the preferred expression vector and purification in a superior expression system. Also consider the potential loss of protein during dialysis or an insufficient dialysis with remaining EDTA in the buffer, which can inhibit activation and binding. In the case of low protein expression (see Western blot) the volume of the periplasmic product needs to be increased (e.g., 50 µl/sample).

12.4 Results

Cell suspension-based display strategies are ideal for subtractive panning strategies as these can take full advantage of the physiological conditions under which the panning is performed. Furthermore, they prevent the selection of clones that bind nonspecifically to cell surface epitopes.

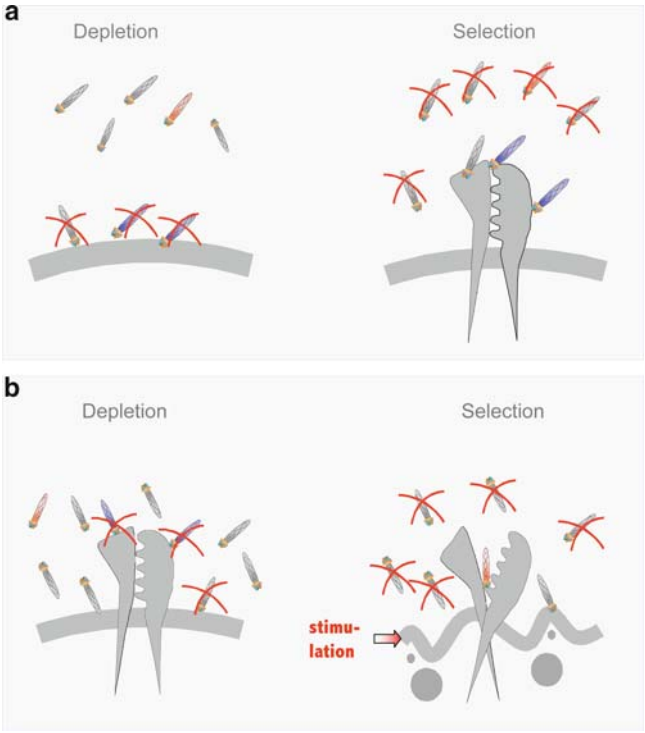


Fig. 12.2 Schematic drawing of the subtractive panning (depletion/selection) strategy on cells for single-chain antibodies. (**a**) Nonconformation-specific panning for epitopes expressed on the selection cell line, but not on the depletion cell line. (**b**) Panning for conformation-specific antibodies. The depletion step is performed on nonactivated cells, whereas phage are selected on activated cells that expose the target epitope upon activation with a stimulating agent. Each panning round starts with a depletion step, in which phage are incubated either with cells that are not expressing the target epitope (a), or in the case of panning for conformation-specific antibodies, with nonactivated cells (b). Phage encoding for scFv-antibodies that are binding to these nonexpressing (a) or nonactivated (b) cells are centrifuged with the cells and discarded. The supernatant containing the phage, which are not binding to the nonactivated cells and not to other surface epitopes, will be transferred to the selection round. Phage that are transferred from the depletion step are added to cells expressing the target epitope (a) or activated cells expressing receptors in their activated conformation (b). Cells are centrifuged down with the binding phage. The supernatant, containing phage that bind neither to nonepitope expressing cells in the depletion step, nor to activated or epitope-bearing cells in the selection step, is discarded. Phage that did not bind to the nonactivated cells or nonexpressing in the depletion step, but to the target epitope-expressing or activated cells in the selection step, are bound to the pelleted cells and will be eluted and transferred to the next panning round

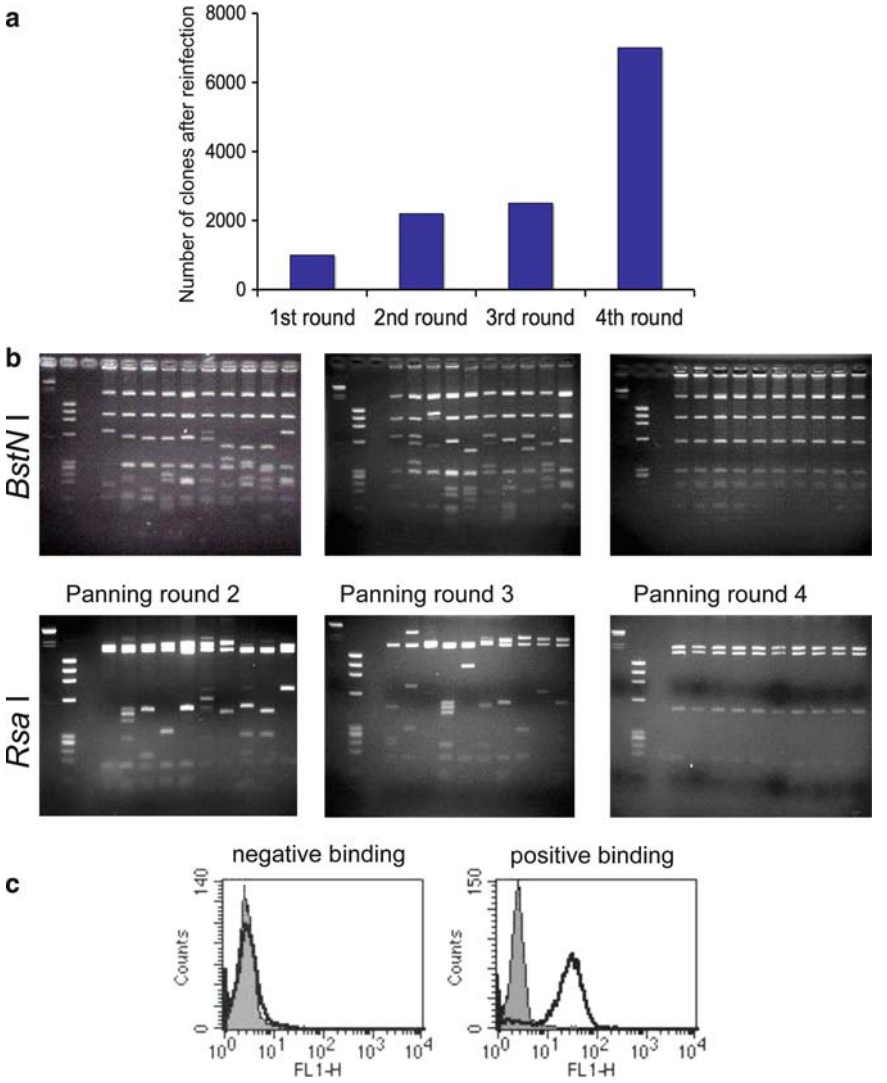


Fig. 12.3 Monitoring the course of the panning process. (**a**) Numbers of recovered phage after each panning round. After each round of panning, the rescued phage are used for infection of log-phase XL-1 blue *E.coli* bacteria and the numbers of colonies are counted. This helps to monitor the success of the selection process. After the first panning rounds, the number of colonies will increase when a few epitope-specific clones have been selected as these specific clones are not lost in depletion or selection steps, and thus amplified over the course of panning. The panning should be interrupted at this stage and the recovered phage should be screened for diversity by random pattern restriction digest or sequencing. (**b**) Fingerprinting of scFv clones by BstN I and Rsa I digest. The diversity of the scFv clones can be evaluated by digestion with the restriction enzymes. Phagemid DNA of ten randomly picked natural clones of panning round 2, 3, and 4 was purified and digested with the restriction enzymes BstN I (*upper panel*) and Rsa I (*lower panel*) and separated in agarose-gel-electrophoresis and stained with ethidium bromide. The DNA

The choice of the selection cell line is of major importance for the success of this protocol as every panning protocol relies on a suitable selection matrix. If the panning is performed as a subtractive strategy for the selection of conformation-specific antibodies as schematically depicted in Fig. 12.2b, it is important that conformational changes in the target epitope can be induced by a known stimulant and that the cells can be isolated to a sufficient purity. Changing to another cell line expressing the same receptors can be useful to reduce background binding.

If a conformation-specific antibody is not required or wanted, the depletion step can be performed with a cell line of the same background as the selection cell line but without expression of the target epitope, e.g., CHO-cells expressing the target epitope for the selection step and nontransfected CHO-cells for the depletion step as depicted in Fig. 12.2a. However, phage display can be performed with the selection step only. In this case, start with the selection step # 3.2.1.

We recommend starting with the least specific cell line for the initial depletion and selection step, e.g., native cells purified from whole blood in the first round, and then switching to the more controlled environment of recombinant cell lines. This strategy reduces binding to nontargeted membrane epitopes.

The number of colonies after reinfection is a parameter to screen the process of in vitro affinity maturation. The number of colonies on the agar plates after reinfection will drop after the first panning round, when the majority of nonspecifically binding clones are lost in the first depletion step. Over the following rounds, the numbers will plateau. Once specific binders have been selected and are transferred to the next panning round, they will not be lost in the depletion step and the number of colonies after reinfection will dramatically increase (three to fivefold). This is a good indicator of the amplification of a few clones. Typical results for the number of colonies over the course of panning are shown in Fig. 12.3a.

The restriction digest of a few picked clones will reveal different patterns, and thus identify identical clones. Two to four different patterns out of ten picked clones can be expected. These should be screened for binding. We found a sufficient number of clones after four panning rounds with the sought-after binding characteristics (Eisenhardt et al. 2007a, b; Schwarz et al. 2004, 2006). A typical result of the random pattern restriction analysis with two different restriction enzymes over the course of a four-round panning procedure is depicted in Fig. 12.3b. After small-scale preparation, the selected clones can then be tested for binding to the target epitope. Flow cytometry is highly suitable for the analysis of the binding to the cell

Fig. 12.3 (continued) markers Lambda DNA/Hind III and PhiX174DNA/Hae III shown in lane 1 and 2. The restriction patterns of the clones differ after panning round 2 and 3. After panning round 4, all investigated clones show an identical restriction pattern demonstrating the amplification of one clone. Shown is the panning for an anti-Mac-1 integrin antibody as described previously (Eisenhardt et al. 2007a, b). (**c**) Screening for binding clones in flow cytometry. scFv clones can be screened by flow cytometry. A secondary anti-His(6) tag antibody, either FITC or PE labeled is used for detection of binding. The histogram on the left shows a negative binding result, the histogram on the right, a binding scFv (*black curves*) as compared to an isotype matched control (*light gray*)

surface epitopes. Figure 12.3c shows a typical example of one binding and one nonbinding clone that were selected in a phage display with the method described above. In our hands, one to two among ten picked different clones showed the sought-after binding characteristics.

References

Arap W, Kolonin MG, Trepel M, Lahdenranta J, Cardo-Vila M, Giordano RJ et al (2002) Steps toward mapping the human vasculature by phage display. Nat Med 8(2):121–127

Barbas CF 3 rd, Kang AS, Lerner RA, Benkovic SJ (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci USA 88(18): 7978–7982

Boder ET, Wittrup KD (1997) Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol 15(6):553–557

Clackson T, Hoogenboom HR, Griffiths AD, Winter G (1991) Making antibody fragments using phage display libraries. Nature 352(6336):624–628

de Wildt RM, Mundy CR, Gorick BD, Tomlinson IM (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. Nat Biotechnol 18(9):989–994

Dorsam H, Rohrbach P, Kurschner T, Kipriyanov S, Renner S, Braunagel M et al (1997) Antibodies to steroids from a small human naive IgM library. FEBS Lett 414(1):7–13

Eisenhardt SU, Schwarz M, Bassler N, Peter K (2007a) Subtractive single-chain antibody (scFv) phage-display: tailoring phage-display for high specificity against function-specific conformations of cell membrane molecules. Nat Protoc 2(12):3063–3073

Eisenhardt SU, Schwarz M, Schallner N, Soosairajah J, Bassler N, Huang D et al (2007b) Generation of activation-specific human anti-alphaMbeta2 single-chain antibodies as potential diagnostic tools and therapeutic agents. Blood 109(8):3521–3528

Fuchs P, Breitling F, Dübel S, Seehaus T, Little M (1991) Targeting recombinant antibodies to the surface of Escherichia coli: fusion to a peptidoglycan associated lipoprotein. Biotechnology (N Y) 9(12):1369–1372

Giordano RJ, Cardo-Vila M, Lahdenranta J, Pasqualini R, Arap W (2001) Biopanning and rapid analysis of selective interactive ligands. Nat Med 7(11):1249–1253

Griffiths AD (1993) Production of human antibodies using bacteriophage. Curr Opin Immunol 5(2):263–267

Hawlisch H, Muller M, Frank R, Bautsch W, Klos A, Kohl J (2001) Site-specific anti-C3a receptor single-chain antibodies selected by differential panning on cellulose sheets. Anal Biochem 293(1):142–145

Hoogenboom HR, de Bruine AP, Hufton SE, Hoet RM, Arends JW, Roovers RC (1998) Antibody phage display technology and its applications. Immunotechnology 4(1):1–20

Hust M, Maiss E, Jacobsen HJ, Reinard T (2002) The production of a genus-specific recombinant antibody (scFv) using a recombinant potyvirus protease. J Virol Methods 106(2):225–233

Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69(1):11–25

Kipriyanov SM, Moldenhauer G, Little M (1997) High level production of soluble single chain antibodies in small-scale Escherichia coli cultures. J Immunol Methods 200(1–2):69–77

Le Gall F, Kipriyanov SM, Moldenhauer G, Little M (1999) Di-, tri- and tetrameric single chain Fv antibody fragments against human CD19: effect of valency on cell binding. FEBS Lett 453(1–2):164–168

Levitan B (1998) Stochastic modeling and optimization of phage display. J Mol Biol 277(4):893–916

Little M, Welschof M, Braunagel M, Hermes I, Christ C, Keller A et al (1999) Generation of a large complex antibody library from multiple donors. J Immunol Methods 231(1–2):3–9

McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348(6301):552–554

Moghaddam A, Borgen T, Stacy J, Kausmally L, Simonsen B, Marvik OJ et al (2003) Identification of scFv antibody fragments that specifically recognise the heroin metabolite 6-monoacetylmorphine but not morphine. J Immunol Methods 280(1–2):139–155

Osbourn JK, Derbyshire EJ, Vaughan TJ, Field AW, Johnson KS (1998a) Pathfinder selection: in situ isolation of novel antibodies. Immunotechnology 3(4):293–302

Osbourn JK, Earnshaw JC, Johnson KS, Parmentier M, Timmermans V, McCafferty J (1998b) Directed selection of MIP-1 alpha neutralizing CCR5 antibodies from a phage display human antibody library. Nat Biotechnol 16(8):778–781

Parsons HL, Earnshaw JC, Wilton J, Johnson KS, Schueler PA, Mahoney W et al (1996) Directing phage selections towards specific epitopes. Protein Eng 9(11):1043–1049

Robert R, Jacobin-Valat MJ, Daret D, Miraux S, Nurden AT, Franconi JM et al (2006) Identification of human scFvs targeting atherosclerotic lesions: selection by single round in vivo phage display. J Biol Chem 281(52):40135–40143

Roberts RW, Szostak JW (1997) RNA-peptide fusions for the in vitro selection of peptides and proteins. Proc Natl Acad Sci USA 94(23):12297–12302

Schwarz M, Rottgen P, Takada Y, Le Gall F, Knackmuss S, Bassler N et al (2004) Single-chain antibodies for the conformation-specific blockade of activated platelet integrin alphaIIbbeta3 designed by subtractive selection from naive human phage libraries. FASEB J 18(14):1704–1706

Schwarz M, Meade G, Stoll P, Ylanne J, Bassler N, Chen YC et al (2006) Conformation-specific blockade of the integrin GPIIb/IIIa: a novel antiplatelet strategy that selectively targets activated platelets. Circ Res 99(1):25–33

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228(4705):1315–1317

Takagi J, Springer TA (2002) Integrin activation and structural rearrangement. Immunol Rev 186:141–163

Zahnd C, Amstutz P, Pluckthun A (2007) Ribosome display: selecting and evolving proteins in vitro that specifically bind to a target. Nat Methods 4(3):269–279

Chapter 13
Selection of Phage Antibody Libraries for Binding and Internalization into Mammalian Cells

Yu Zhou and James D. Marks

13.1 Introduction

Large nonimmune human (naïve) antibody gene diversity libraries displayed on filamentous phage, have proven a reliable source of human antibodies to any purified protein antigen (Marks et al. 1991; Sheets et al. 1998). Recently, it has also been proven possible to directly select peptides and antibody fragments binding cell-surface receptors from filamentous phage libraries by incubation of phage libraries with a target cell line (Marks et al. 1993; Cai and Garen 1995; de Kruif et al. 1995; Andersen et al. 1996; Barry et al. 1996). This has led to a marked increase in the number of potential cell targeting molecules. However, the isolation of cell type-specific antibodies from naïve libraries has been difficult because selections often result in binders to common cell surface antigens (Hoogenboom et al. 1999).

The ability of bacteriophage to undergo receptor-mediated endocytosis (Hart et al. 1994; Barry et al. 1996) indicated that phage libraries might be selected not only for cell binding but also for internalization into mammalian cells. Such an approach would be particularly useful for generating ligands, which could deliver drugs or toxins into a cell for therapeutic applications. Recently, a methodology was developed (Becerril et al. 1999) which allows isolation of specifically internalized phage, while excluding phage merely bound to the cell surface. In the model system studied, not only could phage be selected on the basis of endocytosis, but enrichment ratios were significantly greater when phage were recovered from within the cell rather than from the cell surface.

Y. Zhou and J.D. Marks (✉)
Departments of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, CA, USA
e-mail: marksj@anesthesia.ucsf.edu

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_13,

This strategy of selection for internalization was employed to select scFv from a large naïve library (Sheets et al. 1998) capable of undergoing endocytosis into breast tumor cells upon receptor binding (Poul et al. 2000). Upon analysis of a large number of different antibodies isolated in this way, distinct cell surface antigens recognized by the scFvs were identified. Several of the scFvs recognized the ErbB-2 growth factor receptor and one scFv bound the transferrin receptor. Interestingly, both the ErbB-2 and the transferrin receptor are rapidly internalized and are specific markers for a number of cancers. The phage antibodies and the native scFv were both rapidly endocytosed into cells expressing the appropriate receptor (Poul et al. 2000). It is likely that selection on other cell types will identify cell-specific markers, because endocytosed receptors are more likely to be associated with specific cell functions (such as growth factor and nutrient transport receptors on cancer cells or Fc receptors on cells of the immune system).

Internalizing antibodies (or fragments of antibodies) are also required for many targeted therapies, such as targeted drugs (toxins, RNases, radioisotopes), targeted liposome therapy (e.g., for delivery of chemotherapeutics), or for targeted delivery of genes (especially of nonviral vectors). It is our experience that not all antibodies that bind internalizing receptors are rapidly internalized. Rather, antibodies to certain epitopes are internalized significantly better than antibodies to other epitopes.

The strategy described below has aided us in isolating a number of cancer cell-specific antibodies. In addition to the HER2 antibody described above, we have also used the protocol to isolate antibodies to EGF receptors stably expressed on CHO cells (Heitner et al. 2001). These antibody fragments are rapidly internalized into cells overexpressing the target receptor and can be used to construct receptor-targeted drugs, such as anti-HER2 and anti-EGFR immunoliposomes (Nielsen et al. 2002; Zhou et al. 2007). We have also generated panels of antibodies to prostate and breast cancer cell surface proteins by selecting phage and phagemid antibody libraries on tumor cell lines (Liu et al. 2004; Goenaga et al. 2007). All of the isolated antibodies have proven to be rapidly internalized into cells expressing the target antigens, something we did not observe with antibodies to the same receptors selected on recombinant antigen using traditional panning strategies (Poul et al. unpublished data). The protocols used for this type of selection are outlined below.

13.2 Materials

– Cells for depletion of nonspecific cell surface antigens. For example, we use luminal subtype breast cancer cell lines, MDAMB453 and SUM52PE, to deplete prior to selection for basal subtype breast cancer-specific antibodies. Cells are grown in tissue culture flasks (Costar, T75) to a density of approximately 3–5 million cells per flask (approximately 80% confluent)

- Cells for specific selection of phage antibody libraries. For example, basal subtype breast cancer cell lines (MDAMB231, SUM159PT, BT549) Cells are grown in tissue culture flasks (Costar, T75) to a density of approximately 3–5 million cells per flask (approximately 80% confluent)
- Cell culture media (cell type-specific)
- Fetal bovine serum (FBS) (Equitech) for growing cell lines
- Phosphate buffered saline (PBS)
- Cell stripping buffer 1: 100 mM glycine/150 mM NaCl, pH 2.5
- Cell stripping buffer 2: 100 mM glycine/500 mM NaCl, pH 2.5
- Cell stripping buffer 3: 50 mM glycine/150 mM NaCl/ 200 mM urea/ 2 mg/mL polyvinylpyrrolydone (PVP), pH 2.8
- Cell stripping buffer 4: 50 mM glycine/500 mM NaCl/ 200 mM urea/ 2 mg/mL polyvinylpyrrolydone (PVP), pH 2.8
- 0.05% Trypsin-EDTA (Gibco, Cat. 25300)
- 100 mM triethylamine (TEA)
- 1 M Tris-HCl, pH 7.4
- Exponentially growing *E. coli* TG1 (OD_{600} nm approximately 0.5)
- 2× TY media
- 2× TY media containing 50 μg/mL tetracycline
- Sterile 96-well round bottom microtiter plates made for bacterial culture, e.g., Nunc 62162 (VWR Scientific)
- 96-pin transfer device (Nunc)
- 100 and 150 mm TYE plates containing 50 μg/mL tetracycline (TYE/tet)
- 50% glycerol
- PEG/NaCl solution: 20% (w/v) polyethylene glycol 6000, 2.5 M NaCl
- Purified fd-phage antibody library
- Flow cytometry buffer (PBS supplemented with 1 mM MgCl2, 0.1 mM $CaCl_2$, and 1% FBS)
- Sterile 96 well V-bottom plates (Becton-Dickinson)
- Paraformaldehyde
- Biotin conjugated rabbit anti-fd bacteriophage (Sigma-Aldrich)
- Streptavidin-Phycoerythrin (PE) (Invitrogen-Biosources)
- FACS LSRII (BD Biosciences)
- Sulfo-NHS-LC-biotin (Pierce)
- Cell lysis buffer: 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP 40, 1 mM EDTA, 1 mM Vanadate, 1% protease inhibitor cocktail for mammalian cells (Sigma, Cat. P8340)
- Immunoprecipitation (IP) buffer: 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP40
- Poly-Prep chromatography column (Bio-Rad, Cat. 731-1550)
- Protein A-agarose (Sigma-Aldrich)
- Ni-NTA-agarose (Qiagen)
- Pre-poured SDS-PAGE gels (8–16%) (Invitrogen)
- HRP-conjugated streptavidin (Pierce)
- Siliconized tube (PGC Scientific)

- NH_4HCO_3 (HPLC grade, Fisher)
- Acetonitrile (HPLC grade, Fisher)
- Dithiothreitol (DTT) (Sigma-Aldrich)
- Iodoacetamide (Sigma-Aldrich)
- Trypsin (Mass Spectrometry grade, Promega)
- Trifluoroacetic Acid (Fisher)
- Speed Vac (Savant)
- Nco I (New England Biolabs)
- Not I (New England Biolabs)
- GeneClean Turbo (MP Biomedicals, LLC, Cat. 1102-600)
- T4 DNA Ligase (New England Biolabs)
- Plasmid vector pSYN1 as described (Schier et al. 1995)
- 100 mm TYE plates containing 100 μg/mL Ampicillin and 1% glucose (TYE/Amp/Glu)
- *E. coli* TG1 chemically competent cells (Zymo Research)
- 2 × TY media containing 100 μg/mL Ampicillin and 2% glucose
- 2 × TY media containing 100 μg/mL Ampicillin and 0.1% glucose
- 1 M IPTG
- PPB buffer (200 g/L Sucrose, 1 mM EDTA, 30 mM Tris-HCl, pH 8.0)
- 5 mM MgSO4
- DNase I (Boerhinger Mannheim)
- Protease inhibitor cocktail for bacterial (Sigma, Cat. P8465)
- Dialysis tubing (Spectrum Laboratories, Inc., MWCO: 6–8 kDa, Cat. 132665)
- 10 mM Immidazole in PBS, pH 7.4
- 250 mM Immidazole in PBS, pH 7.4
- PD-10 column (Pharmacia)
- Relevant tumor cell lines (ATCC) and appropriate culture media
- Ni-NTA liposomes as described (Nielsen et al. 2006)
- $NiSO_4$
- 0.01 M NaOH
- FL600 microfluorimeter (BIOTEK)

13.3 Protocols

13.3.1 Selection of Tumor Cell Specific Internalizing Antibodies from Phage Antibody Libraries

For the selections described below, we utilize a naïve human scFv phage antibody library, constructed in the true phage vector fd (O'Connell et al. 2002). This vector results in multicopy phage display, which may increase the selection efficiency (Becerril et al. 1999). Alternatively, a phagemid display antibody library may be

used (Sheets et al. 1998), in which case the method of phage preparation would differ (steps 12–14).

1. Culture the target tumor cells in T75 flasks to 80–90% confluence, which normally takes about 3 days.
2. Change media 1 h before selection.
3. Remove the culture media and add 1 mL of 10^{12} phage antibodies diluted in 3 mL cold culture media to the target tumor cells and incubate for 2 h at 4°C with occasional rocking. After incubating at 4°C, the cells are incubated for 30 min at 37°C in a 5% CO_2 gassed incubator to allow internalization to occur. Rock the flasks several times during incubation to keep the phage evenly distributed.
4. After the 37°C incubation, aspirate the supernatant and wash cells three times in 10 mL of cold PBS.
5. Wash three times to remove uninternalized phage by adding 4 mL of cell stripping buffer to the cells for 5 min per wash. After each wash, the stripping buffer is collected and neutralized by adding 1 mL of 1 M Tris-HCl, pH 7.4. Save the stripping and neutralization buffers for phage titration.
6. Wash the cells in 10 mL of complete culture media twice at RT for 1 min per wash.
7. Add 5 mL of trypsin/EDTA solution to the cells and incubate at 37°C until the cells start detaching from the flask. Add 10 mL of the culture medium to the flask, triturate, and transfer cells to a 15 mL centrifuge tube. Pellet cells by centrifugation at 300 *g* for 5 min. Discard the supernatant.
8. Wash cells twice by resuspending in 10 mL of complete culture media and then pelleting the cells by centrifugation at 300 *g* for 5 min. Discard the supernatant.
9. Lyse the cell pellet by resuspending in 1 mL of fresh 100 mM TEA solution to the cell pellet, pipetting to mix, and incubating for 2 min at room temperature or 10 min on ice.
10. Neutralize the cell lysate by adding 0.5 mL of 1 M Tris-HCl, pH 7.4 and mixing.
11. Add half of the neutralized cell lysate (0.75 mL) to 10 mL of exponentially growing *Escherichia coli* TG1 (OD_{600}~0.5) and incubate the bacterial culture without shaking at 37°C for 30 min.
12. Titer the phage in the cell lysate and in the stripping buffer washes by plating 10 μL, 1 μL, and 0.1 μL of each sample, in a total volume of 10 μL, on 100 mm TYE/tet plates.
13. Centrifuge the remainder of the bacterial culture at 3,000 *g* for 15 min, resuspend in 0.5 mL of 2 × TY, plate on two 150 mm TYE/tet plates, and incubate overnight at 30°C.
14. The next day, add 5 mL of 2 × TY/tet media to each plate and scrape to harvest the bacteria. Make glycerol stocks by mixing 1.4 ml of bacteria and 0.6 ml of 50% glycerol. Store glycerol stocks at −80°C. Prepare phage particles for the next round of selection by inoculating the glycerol stocks in 500 ml of 2 × TY/tet media, to an initial $OD600_{nm}$ of 0.01–0.05. Grow the culture at 30°C with shaking (300 rpm) for 12–18 h.

15. Centrifuge the bacteria at 6,000× *g* in 500 mL centrifuge tubes in a GS3 rotor for 30 min.
16. Transfer the supernatant to new 500 mL centrifugre tubes and precipitate the phage by adding 1/10–1/5 volume of PEG/NaCl solution and leave on ice for 1 h. Phage should be visible as a clouding of the supernatant.
17. Pellet the phage in 500 mL bottles by centrifuging in a GS3 rotor at 3,000 *g* for 15 min at 4°C. Discard the supernatant. Centrifuge the "dry" pellet again for 30 s to bring down the last drops of supernatant, and remove the liquid. Resuspend the pellet in 1/10 volume of PBS.
18. Centrifuge the bottles at 15,000 *g* in a SA-600 rotor for 15 min to pellet bacterial debris and transfer the supernatant to a new tube.
19. Repeat steps 16–18 to further purify the phage, resuspending in a final volume 1/50 of the original culture volume.
20. Titer the purified phage by following steps 11 and 12 (above).
21. Repeat the selection process, but this time incorporate a "depletion" step prior to the selection step (step 3, above). For depletion, incubate 10^{12} phage antibodies diluted in 3 mL of cold culture media with an appropriate cell line grown to 90% confluence in T75 flask or use 10^7 cells in suspension. After incubation, aspirate the culture media and the depleted phage antibodies to a culture of the target cells as described in step 3 to initiate the next round of positive selection. Typically, only two to three rounds of selection are required.

13.3.2 Profiling the Specificity of Phage Antibodies on Tumor Cells Using Flow Cytometry

This method is used to identify monoclonal phage antibodies of the desired specificity after two to three rounds of selection. For initial screening of phage antibodies, binding is measured to the cells used for positive selection and the cells used for depletion. After identification of phage that preferentially bind the selecting cell line, positive phage can be further screened for binding to a larger panel of cell lines.

13.3.2.1 Preparation of fd scFv Phage Antibodies in 96-Well Microtiter Plates

1. Pick individual bacterial colonies using sterile toothpicks, dip into 96-well microtiter plates containing 100 μL of 2 × TY/tet media/well, and grow at 30°C overnight with shaking (300 rpm).
2. Add 100 μL of 2 × TY/tet containing 30% glycerol to each well and store the plate at −80°C. This is designated as the master plate.
3. Inoculate about 2 μL of the bacterial culture from the master plate into a new 96-well plate containing 150 μL of 2 × TY/tet/well using a 96-well sterile

transfer device (96-pin duplicator), and grow the culture with shaking (300 rpm) at 30°C for 12–18 h.
4. Centrifuge the plate at 2,000 *g* for 10 min and use 50 μL of supernatant for flow cytometric analysis.

13.3.2.2 Quantitating Cell Binding by Flow Cytometry in 96-Well Microtiter Plates

1. Grow depleting and selecting tumor cell lines to 80–90% confluence.
2. Detach the adherent cells from the flask by trypsinizing the cells in 3 mL of 0.05% trypsin.
3. Transfer the cells to a 50 mL Falcon tube, pellet cells by centrifugation at 300 *g* for 5 min. Discard the supernatant.
4. Wash cells once by resuspending in 10 mL of flow cytometry buffer, followed by centrifugation, resuspend in 2 mL of flow cytometry buffer.
5. Count the cells using a hematocytometer.
6. Aliquot 50 μL of the cell suspension containing 5×10^4 cells into 96-well V-bottom plates.
7. Add 50 μL of phage supernatant from 13.3.2.1 into each well.
8. Incubate overnight at 4°C with rocking.
9. Wash cells twice with 200 μL of flow cytometry buffer.
10. Resuspend cells in 100 μL of flow cytometry buffer containing 1 μg/ml of biotin conjugated rabbit anti-fd bacteriophage. Incubate for 1 h at 4°C.
11. Wash cells once with 200 μL of flow cytometry buffer.
12. Resuspend cells in 100 μL of flow cytometry buffer containing 1 μg/ml of streptavdin-PE. Incubate for 30 min at 4°C.
13. Wash cells twice with 200 μL of flow cytometry buffer and resuspended in PBS containing 1% paraformaldehyde.
14. Measure cell fluorescence in a FACS LSRII flow cytometer or other comparable cytometer using the PE channel.

13.3.3 Identification of the Cognate Antigen Recognized by Tumor Specific scFv by Using Mass Spectrometry

This method utilizes purified native scFv expressed in *E.coli* to immunoprecipitate antigen from cell lysates followed by mass spectrometry to identify the antigen.

13.3.3.1 Preparation of Biotinylated Cell Lysates

1. Use the tumor cell line that gives the highest mean fluorescent intensity when phage antibody binding is measured by flow cytometry. Grow these cells to 90% confluence in 150 mm culture dish.

2. Wash cell twice with PBS, then add 5 mL of sulfo-NHS-LC-biotin (0.1 mg/mL in PBS) to each dish and incubate for 20 min at 4°C with rocking.
3. Remove biotinylation solution by aspiration and wash the cells twice with 20 mL of cold PBS containing 50 mM glycine.
4. Add 3 mL of lysis buffer to each dish and incubate for 1 h at 4°C with rocking.
5. Harvest the cell lysates by scraping the dish with a plastic scraper.
6. Pellet any remaining cells in the cell lysate by centrifugation for 10 min at 4°C.
7. The cleared cell lysates can be used for immunoprecipiation right away or stored at −80°C.

13.3.3.2 Immunoprecipitation with scFv Antibodies

1. Express and purify hexahistidine tagged native scFv by following protocol 13.3.4.1 below.
2. Set up a reaction to deplete the cell lysates of any proteins binding nonspecifically to scFv. Incubate 10 mL of the biotinylated cell lysates and 300 μg of an irrelevant scFv antibody for 2 h at 4°C with rocking.
3. Wash 200 μL of either Ni-NTA-agarose or protein A-agarose (for scFv binding protein A) three times in IP buffer, resuspended in an equal volume of IP buffer, and add to the depleting reaction (step 2, above) and incubate for 1 h at 4°C with rocking.
4. Set an unpacked Biorad column in a rack, pour the preclearing immunoprecipitation reaction into the column, and let it run by gravity while collecting the precleared cell lysates flowing through.
5. Add 200 μg of the relevant scFv antibody to the precleared cell lysates and incubate for 2 h at 4°C with rocking, followed by incubating with 200 μL of washed Ni-NTA-agarose or protein A-agarose for 1 h at 4°C with rocking.
6. Set an unpacked Biorad column in a rack, pour the immunoprecipitation reaction into the column, and let it run by gravity while collecting the cell lysate flowing through, which can be used for immunoprecipitation with other scFv antibodies.
7. Wash the column with 5 mL of IP buffer five times.
8. After the final wash, add 1 mL of IP buffer to the column and transfer the slurry into a 1.5 mL eppendorf tube.
9. Spin down the slurry at top speed for 1 min and remove the supernatant completely.
10. Add 50 μL of 2.5-fold SDS loading buffer (nonreducing) and boil the beads for 8 min at 94°C.
11. Centrifuge the tube containing the boiled beads to pellet the beads at top speed for 5 min, transfer the supernatant to a new microtube, and store the supernatant at −20°C.

12. Resolve the immunoprecipitates on a 8–16% SDS-PAGE (Invitrogen) in duplicate. One gel should be transferred to a PVDF membrane and proteins stanied with HRP-conjugated streptavidin (Pierce). This will stain only proteins that were on the cell surface and labeled with the sulfo-NHS-LC-biotin. The other gel should be stained with Coomassie R250. A dominant band should be observed on the HRP-streptavidin stained gel, which is the antigen recognized by the scFv. The two gels should be aligned and the corresponding band on the Coomassie R250 gel excised for mass spectrometry evaluation.

13.3.3.3 In-gel Digestion and Mass Spectrometry Analysis

1. The excised protein gel slice is diced into small pieces (~1 mm^2), placed in 0.65 mL siliconized tubes (PGC Scientific), and destained in 150–250 μL of 25 mM NH_4HCO_3/50% ACN vortexing for 10 min, which can be repeated several times until gels have been stripped of stain.
2. After removing the supernatant, add 100% acetonitrile to cover the gel pieces, and wait until the gel pieces shrink and turn white. Dry the gel pieces in a Speed Vac with no heating.
3. Add 40 μL of 10 mM DTT solution to the dried gels, vortex, spin briefly, and incubate 45 min at 56°C to reduce the target protein.
4. After removing the supernatant, add 40 μL of 55 mM iodoacetamide solution to the gel pieces, vortex, spin briefly, and incubate in the dark for 30 min at room temperature to alkylate the reduced –SH group.
5. Remove the supernatant, wash the gel pieces with 100 μL of 25 mM NH_4CO_3, vortex for 15 min, and spin briefly. Repeat step 2 to dry the gel pieces.
6. Estimate the volume of the dried gel pieces, add about 3× volume of 12.5 ng/μL trypsin solution to cover the gel pieces, rehydrate the gel pieces on ice for 15–30 min until the trypsin solution has been absorbed, add 25 mM NH_4CO_3 as needed to cover the gel pieces, spin briefly, and incubate for 4 h to overnight at 37°C to digest the target protein.
7. Remove samples from heat, spin briefly on a microcentrifuge, and transfer the solution into a clean 1.5 mL siliconized tube. Add 30 μL of 50% ACN/5% TFA to the gel pieces, vortex for 20–30 min, and spin briefly. Transfer supernatant to the previously extracted solution, and repeat this step once more. Concentrate the extractions in a Speed Vac to approximately 10 μL. The peptide extracts can be stored at −80°C or −20°C until mass spectrometric analysis.
8. Analyze the tryptic peptide digests by LC MS/MS using a qTOP mass spectrometer (QSTAR XL, Applied Biosystems/PE Sciex), as described (Liu et al. 2002).
9. Identify the protein by searching protein databases using the Protein Prospector search engine and the peptides identified by mass spectrometry (http://prospector.ucsf.edu).

13.3.4 Characterization of the Internalizing Potential of Tumor-Specific Antibodies using Chelated Ligand Internalization Assay (CLIA)

13.3.4.1 Preparation of Hexahistidine-Tagged Single Chain Fv (scFv) Antibodies

1. Digest the scFv-fd DNA with *Nco* I and *Not* I restriction enzymes in the manufacturer's NEB 3 buffer and 1× BSA solution, under conditions recommended by the manufacturer for double digests.
2. Purify the digested scFv DNA on a 0.8% agarose gel. Extract the scFv fragment from the gel using GeneClean Turbo Kit.
3. Digest the pSYN1 vector DNA (Schier et al. 1995) with the *Nco* I and *Not* I restriction enzymes in the manufacturer's NEB 3 buffer and 1× BSA solution, under conditions recommended by the manufacturer.
4. Purify the digested vector DNA on a 0.8% agarose gel. Extract the vector fragment from the gel using GeneClean Turbo Kit.
5. Ligate the digested scFv DNA and the digested vector DNA in a 20 μL reaction volume of T4 DNA ligase reaction buffer under conditions recommended by the manufacturer.
6. Transform the ligated DNA into chemically competent *E. coli* TG1 cells under conditions recommended by the manufacturer. After transformation, dilute the cells in 2 × TY media, plate different amounts of cells on 100 mm TYE/Amp/Glu plates, and incubate overnight at 30°C.
7. A single colony of *E. coli* TG1 bearing pSYN1-scFv is inoculated into 5 mL of 2 × TY media with 100 μg/mL ampicillin and 2% glucose and grown overnight at 30°C with shaking at 250 rpm.
8. Inoculate 500 mL of 2 × TY media containing 100 μg/mL ampicillin and 0.1% glucose in a 2 L flask with a 5 mL of the overnight culture and grow at 37°C to an OD_{600} of 0.9.
9. Induce scFv expression by adding isopropyl-b-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM.
10. Grow the culture for 4 h at 30°C with shaking at 250 rpm.
11. Harvest the culture by centrifugation for 20 min at 4,000 × *g*. Pour off the supernatant and resuspend the pellets in 12.5 mL of periplasmic extraction buffer (PPB), containing protease inhibitor cocktail and DNase I (100 μg/mL) and incubate on ice for 30 min.
12. Centrifuge the PPB extracted cell at 5,000 × *g* for 20 min and transfer the supernatant to a high-speed centrifuge tube. Resuspend the pellets in 12.5 mL of osmotic shock buffer (5 mM MgSO4) and incubate for 20 min on ice.
13. Centrifuge the osmotic shock fraction at 5,000 × *g* for 20 min and combine the supernatant with the PPB extract supernantant.

14. Centrifuge the combined PPB and osmotic shock fractions in high speed centrifuge tubes (Nalgene 3119-0050) at 32,000 *g* for 30 min at 4°C to remove any remaining cellular debris.
15. Load the cleared periplasmic preparation from step 14 into dialysis tubing (6–8 kDa MWCO (Spectrum Laboratories, Inc.)) and dialyze in 4 L PBS for 3 h with two changes at 4°C.
16. Purify the hexahistidine tagged scFv by immobilized metal affinity chromatography (IMAC) under conditions recommended by the manufacturer.
17. Change the buffer after IMAC purification by desalting on a PD 10 column (Pharmacia) equilibrated with PBS and following the protocol recommended by the manufacturer.
18. Determine the scFv concentration spectrophotometrically at A280 using an extinction coefficient $\varepsilon = 1.4$.

13.3.4.2 Preparation of Hexa-Histidine-Tagged Single Chain Fv (scFv) Antibodies in Microtiter Plate

1. Pick individual bacterial colonies generated by protocol 13.3.4.1 using sterile toothpicks, dip into 96-well microtiter plates containing 100 μL of 2 × TY/Amp/2% Glucose media/well, and grow at 30°C overnight with shaking (250 rpm).
2. Add 100 μL of 2 × TY/Amp/2% Glucose containing 30% glycerol to each well and store the plate at −80°C. This is designated as the master plate.
3. Inoculate about 2 μL of the bacterial culture from the master plate into a new 96-well plate containing 150 μL of 2 × TY/Amp/0.1% Glucose per well using a 96-well sterile transfer device (96-pin duplicator), and grow the culture with shaking (250 rpm) at 37°C to an OD600 ≈ 1.
4. Add IPTG solution to each well to a final concentration of 0.5 mM to induce scFv expression, and grow the culture overnight at 30°C with shaking at 250 rpm.
5. Pellet cells by centrifuging the plates at 1,000× *g* for 10 min and use 30 μL of supernatant containing scFv in the internalization assay (protocol 13.3.4.3, below).

13.3.4.3 Chelated Ligand Internalization Assay (CLIA)

1. Human breast cancer cells (or the relevant target cell lines) are grown to 80–90% confluence in the media recommended by American Type Culture Collection (ATCC) with 10% FBS and harvested by trypsinization using standard techniques.
2. Cells (10,000) are seeded in 96-well plates and incubated overnight at 37°C.
3. Ni-NTA liposomes (1 mM) are incubated with 20 μg/mL of hexahistidine tagged scFv in 100 μL tissue culture media supplemented with 0.08 mM NiSO4 for 10 min at room temperature.

4. For analysis of scFv expressed in 96 well microtiter plates where the scFv has not been purified, incubate 30 μL of supernatant from each well with 1 mM Ni-NTA liposomes in 100 μL tissue culture media supplemented with 0.08 mM NiSO4 for 10 min at room temperature. This can be done in 96 well microtiter plates.
5. After incubation, scFv-liposomes mixtures are added to cancer cells and incubated for 4 h at 37°C, followed by washing with 250 mM phophate buffered immidazole (pH 7.4).
6. After incubation, lyse the cells in 0.01 M NaOH (50 μL) and read the fluorescence in a FL600 microfluorimeter (BIOTEK) (or equivalent device) using bandpass filters at 460/30 nm for excitation and 530/20 nm for emission.

References

Andersen PS, Stryhn A, Hansen BE, Fugger L, Engberg J, Buus S (1996) A recombinant antibody with the antigen-specific, major histocompatability complex-restricted specificity of T-cells. Proc Natl Acad Sci USA 93(2):1820–1824

Barry MA, Dower WJ, Johnston SA (1996) Toward cell-targeting gene therapy vectors: selection of cell-binding peptides from random peptide-presenting phage libraries. Nat Med 2(3): 299–305

Becerril B, Poul MA, Marks JD (1999) Toward selection of internalizing antibodies from phage libraries. Biochem Biophys Res Commun 255(2):386–393

Cai X, Garen A (1995) Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. Proc Natl Acad Sci USA 92(14):6537–6541

de Kruif J, Terstappen L, Boel E, Logtenberg T (1995) Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc Natl Acad Sci USA 92(6):3938–3942

Goenaga AL, Zhou Y, Legay C, Bougherara H, Huang L, Liu B, Drummond DC, Kirpotin DB, Auclair C, Marks JD, Poul MA (2007) Identification and characterization of tumor antigens by using antibody phage display and intrabody strategies. Mol Immunol 44(15):3777–3788

Hart SL, Knight AM, Harbottle RP, Mistry A, Hunger H-D, Cutler DF, Williamson R, Coutelle C (1994) Cell binding and internalization by filamentous phage displaying a cyclic arg-gly-asp-containing peptide. J Biol Chem 269:12468–12474

Heitner T, Moor A, Garrison JL, Marks C, Hasan T, Marks JD (2001) Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library. J Immunol Methods 248(1–2):17–30

Hoogenboom HR, Lutgerink JT, Pelsers MM, Rousch MJ, Coote J, Van Neer N, De Bruine A, Van Nieuwenhoven FA, Glatz JF, Arends JW (1999) Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. Eur J Biochem 260(3):774–784

Liu B, Huang L, Sihlbom C, Burlingame A, Marks JD (2002) Towards proteome-wide production of monoclonal antibody by phage display. J Mol Biol 315(5):1063–1073

Liu B, Conrad F, Cooperberg MR, Kirpotin DB, Marks JD (2004) Mapping tumor epitope space by direct selection of single-chain Fv antibody libraries on prostate cancer cells. Cancer Res 64(2):704–710

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G (1991) By-passing immunization: Human antibodies from V-gene libraries displayed on phage. J Mol Biol 222(3):581–597

Marks JD, Ouwehand WH, Bye JM, Finnern R, Gorick BD, Voak D, Thorpe S, Hughes-Jones NC, Winter G (1993) Human antibody fragments specific for blood group antigens from a phage display library. Biotechnology 11(10):1145–1149

Nielsen UB, Kirpotin DB, Pickering EM, Hong K, Park JW, Refaat Shalaby M, Shao Y, Benz CC, Marks JD (2002) Therapeutic efficacy of anti-ErbB2 immunoliposomes targeted by a phage antibody selected for cellular endocytosis. Biochim Biophys Acta 1591(1–3):109–118

Nielsen UB, Kirpotin DB, Pickering EM, Drummond DC, Marks JD (2006) A novel assay for monitoring internalization of nanocarrier coupled antibodies. BMC Immunol 7:24

O'Connell D, Becerril B, Roy-Burman A, Daws M, Marks JD (2002) Phage versus phagemid libraries for generation of human monoclonal antibodies. J Mol Biol 321(1):49–56

Poul MA, Becerril B, Nielsen UB, Morisson P, Marks JD (2000) Selection of tumor-specific internalizing human antibodies from phage libraries. J Mol Biol 301(5):1149–1161

Schier R, Marks JD, Wolf EJ, Apell G, Wong C, McCartney JE, Bookman MA, Huston JS, Houston LL, Weiner LM, Adams GP (1995) *In vitro* and *in vivo* characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. Immunotechnology 1(1):73–81

Sheets MD, Amersdorfer P, Finnern R, Sargent P, Lindquist E, Schier R, Hemingsen G, Wong C, Gerhart JC, Marks JD, Lindqvist E (1998) Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. Proc Natl Acad Sci USA 95(11):6157–6162

Zhou Y, Drummond DC, Zou H, Hayes ME, Adams GP, Kirpotin DB, Marks JD (2007) Impact of Single-chain Fv Antibody Fragment Affinity on Nanoparticle Targeting of Epidermal Growth Factor Receptor-expressing Tumor Cells. J Mol Biol 371(4):934–947

Chapter 14
Improving Phage Display Throughput by Using Hyperphage, Miniaturized Titration and pVIII (g8p) ELISA

Frank Breitling, Olaf Broders, Saskia Helmsing, Michael Hust, and Stefan Dübel

14.1 Introduction

Since its invention in the early 1990s, phage display has revolutionized the generation and engineering of monoclonal antibodies *(for review, see* 1). Without the need for laboratory animals or hybridomas, it was now possible to create antibodies binding to almost any antigen of choice. All this is accomplished in a system that completely bypasses our body's immune system (see chapter 9–13 of this book).

Antibody phage display is done by fusing antigen binding antibody fragments to the phage minor coat protein pIII. Incorporation of this fusion protein into the mature phage coat results in the presentation of the antibody on the phage surface, while the genetic material of the fusion resides within the phage particle. This physical linkage between the antibody gene and its product allows the enrichment of antigen-specific phage antibodies by employing immobilized or labeled antigen. While nonadherent phage will be removed by washing, phage that display the relevant antibody will be retained on an antigen-coated surface. Bound phage can then be recovered from the surface, re-infected into bacteria, and thus amplified for further enrichment. Each re-grown colony represents a single molecular interaction event, thus allowing an enormous sensitivity. By using large combinatorial antibody fragment repertoires (10^8–10^{10} independent clones), antigen-specific antibodies to almost any chosen antigen can be selected. These highly specific antibodies can then be recloned into various expression vectors and/or be further modified to optimize their diagnostic or therapeutic capabilities.

O. Broders, S. Helmsing, M. Hust, and S. Dübel (✉)
Institute of Biochemistry and Biotechnology, Technische Universität Braunschweig, Spielmannstr. 7, Braunschweig38106Germany
e-mail: s.duebel@tu-bs.de

F. Breitling
Karlsruhe Institute of Technology, Helmholtzplatz 1, 76344 Eggenstein-Leopoldshafen, Germany

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_14,

A breakthrough to enhance the performance of antibody phage display was the development of Hyperphage technology (2). By using this method, antigen binding activity was increased approximately 400-fold by enforcing oligovalent antibody display as well as display on every phage particle. The use of Hyperphage for packaging a universal human scFv library improved the specific enrichment factor: after two rounds of panning, more than 50% of the isolated antibody clones bound to the antigen, compared to 3% when conventional M13KO7 helper phage was used (2). Thus, Hyperphage are particularly useful in stoichiometrical situations where the chances of a single phage capable of locating the wanted antigen are known to be low. Consequently, the larger the library (in respect of repertoire diversity), the more it benefits from the use of Hyperphage. The unique production system of Hyperphage, based on a genomically integrated phage pIII gene, delivers superior performance when compared to other "polyvalent helper" phage (3). It is important to understand that after the first (maximal the second) panning round using Hyperphage, the diversity of the eluted antibody repertoire can be expected to be reduced to a number allowing to use "monovalent" display. Used in this way, the Hyperphage-based initial selection(s) benefits from the increased chance (by about two to three orders of magnitude) to enrich any possible binder present in the repertiore, whereas the subsequent selections using M13KO7 avoid the avidity effect of polyvalent display, thus allowing to obtain binders with the highest affinities. The very efficient enrichment in the first step usually reduces the number of necessary panning rounds to 2–3 (2).

14.2 Outline

14.2.1 Concept of the Hyperphage

An overview of various phage display systems can be found in (1). The approach described in this protocol is applicable to all phagemid-based display systems on filamentous phage (M13/f1/fd), which employ full-length pIII. It has been widely applied to antibody display as well as scaffolds.

In the most commonly used phagemid-based systems (4–6) and their descendants, only a small percentage of the total phage population carries an antibody fragment on its surface, typically a few or even less than 1% (Fig. 14.1 *left lane*). This problem arises from the presence of two copies of the gene III, which encodes the pIII gene product (g3p). One copy resides on the antibody expression phagemid, and is fused to the antibody gene. However, the phagemid lacks the other structural genes required for phage assembly. To provide these, infection with a helperphage is employed. This helperphage, however, brings in a second pIII gene. This is a wildtype pIII gene and cannot easily be deleted from the helperphage genome, since functional pIII is an essential surface protein for infection, by providing F-pilus binding. This wildtype pIII protein (g3p) is favored during assembly of the phage particle, resulting in a very minor fraction of phage carrying any antibody::pIII

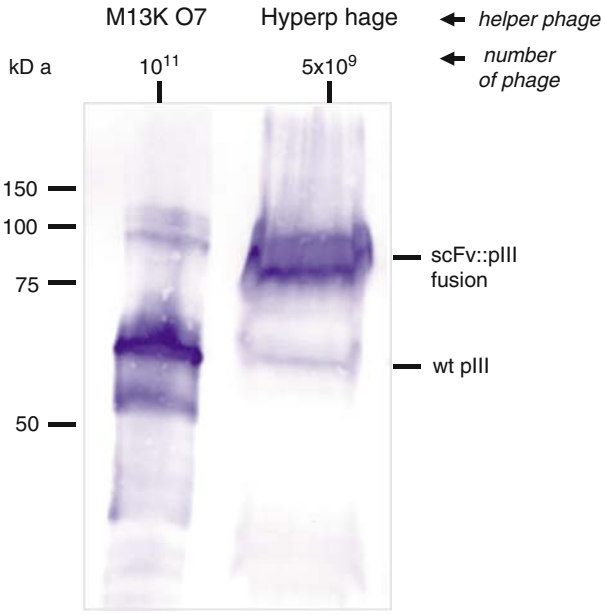


Fig. 14.1 Comparison of phage antibody preparations obtained after packaging with M13KO7 or Hyperphage. A universal/naive scFv antibody fragment library (HAL4, see (1)) was either packaged with M13KO7 or Hyperphage. Phage particles (determined as cfu) were applied per lane and pIII and scFv::pIII fusion proteins were detected with an anti-pIII rabbit serum on the corresponding immunoblot

fusion protein at all. The problem was finally overcome by avoiding the delivery of a wildtype pIII gene during the phage antibody packaging (Fig. 14.2). To achieve this, Hyperphage was constructed to contain a deletion in the pIII gene within their delivered helper genome, but with wildtype pIII protein phenotype, thus capable of infecting F^+ *Escherichia coli* cells with high efficiency. During phagemid packaging to create an antibody expression phage library preparation, this renders the phagemid encoded antibody-pIII fusion as the only source of pIII in phage assembly. This results in both a dramatic increase of the fraction of phage carrying antibodies at all and the number of antibodies displayed per phage (Fig. 14.1 *right lane*), the latter providing a significant increase of the apparent affinity by the avidity effect. Both effects combine to increase the chances of a binder genetically present in the repertoire binding to the panning antigen, being of particular importance for highly diverse libraries, where any individual binder is only represented by a few hundred molecules or less (as in a typical panning using 10^{12} cfu per panning well of a library with a diversity of 10^{10}).

Most reported approaches to generate a respective helperphage combined a pIII deleted helperphage genome and a pIII supplementing plasmid. These approaches, however, were impeded by the occurrence of packaging of the pIII supplementing plasmid into helper phage particles even though the plasmid lacks signals for the phage assembly machinery. These “wrongly packaged” plasmids then contaminate

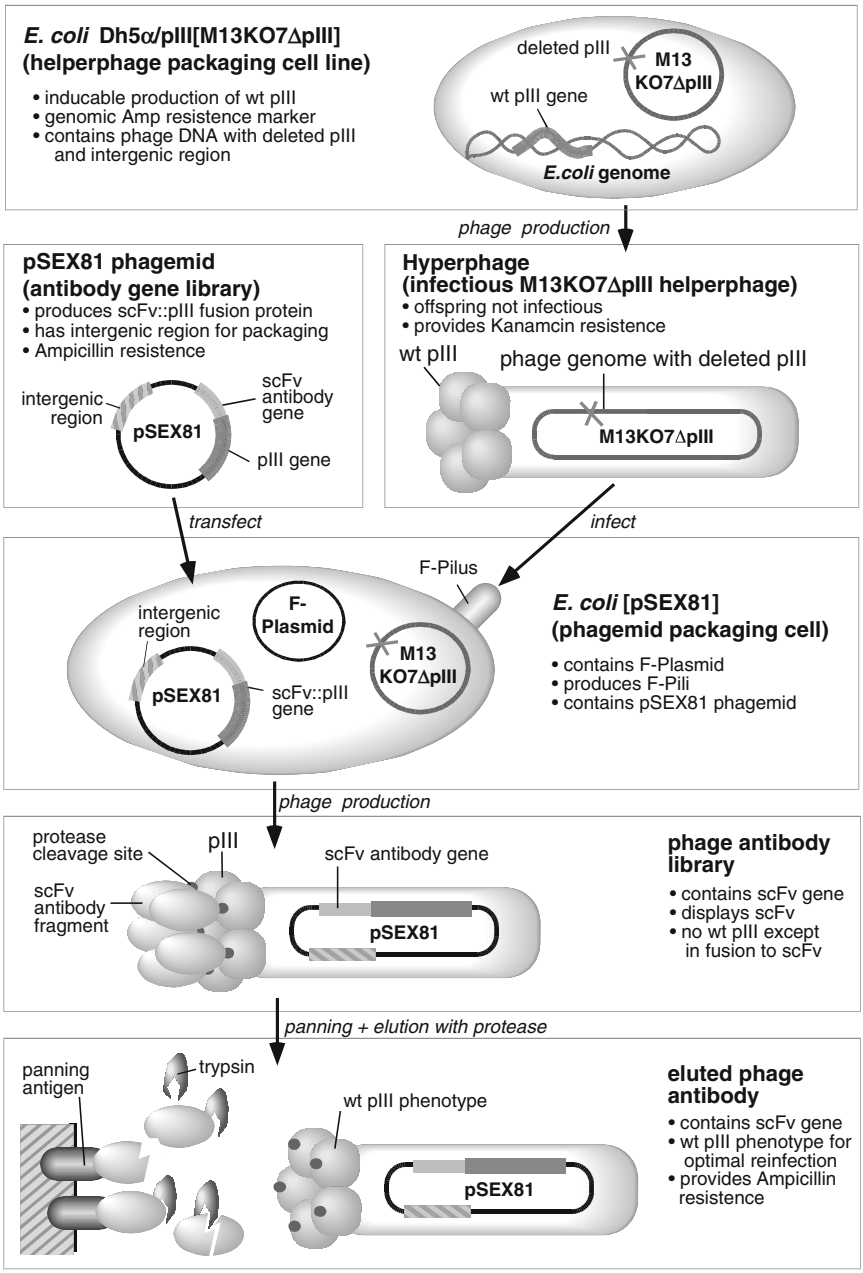


Fig. 14.2 The concept of hyperphage: a gene pIII-deleted helper phage with wild-type infection phenotype. Note that elution during panning can be done with proteases (e.g., trypsin) when using the pSEX81 phagemid encoding a trypsin cleavage site between the pIII and the antibody fragment (*lowest panel*). This allows the use of physiological pH throughout the panning procedure while ensuring elution even of very high affinity binders. Further, it restores a pIII protein phenotype very similar to wildtype by removing the antibody fragments, thus maximizing reinfection yields

the phage with significant amounts of wildtype pIII genes and protein, thereby decreasing the quality of the resulting phage-antibodies. To avoid the presence of a plasmid during helper phage, Hyperphage are produced without a supporting plasmid, by employing an *E. coli* packaging cell line with a copy of the gene 3 integrated into the bacterial genome (see Ref. 2 and Fig. 14.2). A genomically integrated pIII gene is expressed under the control of a strong, but tightly repressable synthetic promoter allowing an inducable expression of the pIII protein during helper phage generation.

The following protocol describes a typical phagemid "packaging" and two convenient titration methods for its control, Nitrocellulose Plating (8) and Phage Titration ELISA, the latter allowing to substitute plate-based titration in favor of a automated, microtitre-based method, employing a monoclonal antibody to the phage major coat protein (9). These methods can help to improve panning throughput, being of particular importance for the current renaissance of antibody phage display to generate protein binding agents for research (10).

14.3 Materials

14.3.1 Packaging of a Single-Chain Fv Antibody Fragment Library Employing Hyperphage (M13K07ΔpIII)

- 2X YT medium (1 L): 16 g peptone, 10 g yeast, 5 g NaCl. Bring to 1 L with bidestilled water. Adjust pH to 7.0 if necessary. Autoclave and store at room temperature for up to several weeks
- 2 M glucose. Filter to sterilize
- 100 mg/mL ampicillin and 10 mg/mL kanamycin. Filter to sterilize
- Phage dilution buffer: 10 mM Tris–HCl (pH 7.5), 20 mM NaCl, 2 mM EDTA
- PEG/NaCl: 16.7% (w/v) PEG-6000, 3.3 mM NaCl. Autoclave and store at 4°C
- 450 mL sterile plastic rotor flasks and conventional Erlenmeyer flasks
- Hyperphage stock (Progen Biotechnik GmbH, Heidelberg, Germany)
- F^+ bacteria transfected with an antibody expression phagemid library (e.g., see chapters 4–8 of this book)

14.3.2 Miniaturized Titration on Nitrocellulose Filters to Determine the Number of Infective Particles (cfu Calculation)

- Round nictrocellulose filters (BA 85, 0.45 mm, ∅ 82 mm, Schleicher & Schuell, Dassel, Germany)

- Luria Bertani agar plates (use 20 g peptone, 10 g yeast, 20 g NaCl and 15 g agar for 1 L, adjust pH to 7.0 and autoclave) supplemented with 100 mM glucose and 50 μg/mL kanamycin
- *E. coli* plating bacteria
- Phage dilution buffer: see 4.3.1

14.3.3 Phage ELISA for the Estimation of Total Phage Particle Number

- MaxiSorp ELISA plates (Nunc, Naperville, USA)
- 100 mM $NaHCO_3$ (pH 8.6)
- 1M H_2SO_4
- 2% (w/v) skim milk/PBS/0.05% (v/v) Tween (SERVA, Heidelberg, Germany). Always prepare freshly prior to use, or store frozen until use. Do not store at 4° for more than 2 h
- Antibody B62-FE2::HRP (mouse monoclonal antibody enzyme conjugate) to filamentous phage major coat protein (pVIII) (Progen Biotechnik GmbH, Heidelberg, Germany)
- Developer solution for ELISA: Mix 4.5 mL H_2O, 0.5 mL NaAc (1 M, pH 6), 12.5 μL TMB substrate (Promega, Madison, USA) and 6 μL 30% (v/v) H_2O_2. Prepare freshly before use. Alternatively use TMB substrate from Progen Biotechnik GmbH (Heidelberg, Germany)

14.4 Protocols

14.4.1 Packaging of a Single-Chain Fv Antibody Fragment Library Employing Hyperphage (M13K07ΔpIII)

1. Grow an overnight culture of bacteria transfected with an antibody expression phagemid library (for details on phagemid vectors please see *(1)*) in 150 ml 2XYT medium supplemented with 100 μg/mL ampicillin and 100 mM glucose (*see Note 1*)
2. Supplement 500 mL of fresh 2XYT medium with ampicillin and glucose and incoculate with 1/100 volume of the overnight culture. Let the bacteria grow to an OD_{600} of 0.5
3. Infect the bacteria with Hyperphage at a multiplicity of infection (MOI) of 20 and incubate the culture at 37°C for 15–20 min without shaking
4. Shake for 45 min with 250 rpm/37°C (*see Note 2*)
5. Pellet the bacteria in 250 mL centrifuge tubes at 1,500–2,000× *g* for 10 min at 4°C

6. Resuspend the pelleted bacteria in 500 mL of 2XYT medium supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin but without glucose (*see Note 3*)
7. Shake overnight with 230 rpm at 37°C for antibody-phage production
8. Pellet the bacteria with 3,200× g for 20 min at 4°C and recover the supernatant
9. Precipitate the produced phage particles with 1/5 volume of PEG/NaCl for > 5 h on ice
10. Pellet the antibody phage by centrifugation with 10,000× *g* at 4°C for 1 h. Discard the supernatant. Remove all traces of medium carefully
11. Resuspend the white phage pellet in 1/100 of the initial culture volume (5 mL) of phage dilution buffer and aliquot into 1.5 mL Eppendorf tubes
12. Remove bacterial debris by two times centrifugation with 10,000× *g* for 5 min at 4°C in a table-top centrifuge
13. Titrations may be done by two methods (see *Note 4*): colony forming units (cfu) calculation (on conventional plates or on nitrocellulose, see Protocol 14.4.2 or *(8)*, or pVIII ELISA (see Protocol 14.4.3)

14.4.1.1 Notes

Note 1: Glucose at this step of the protocol is required in order to prevent expression of the antibody:pIII fusion protein during the initial amplification of the library. In pSEX or pHAL-based phagemid libraries, successfully transfected clones can be selected through the ampicillin resistance encoded on the phagemid.

Note 2: During this step, successfully infected cells will aquire a kanamycin resistance provided by the helperphage genome.

Note 3: Glucose removal leads to scFv expression by activation of the promoter driving scFv::pIII transcription. Only applicable for compatible vectors.

Note 4: The obtained cfu are not necessarily identical to the amount of phage particles determined by pVIII quantitative ELISA since typically, a fraction of the produced particles is not infective and phage containing a phagemid are shorter than wt phage (i.e., carry less copies of pVIII). We have, however, observed that the ratio of cfu to particle is close to constant for a given combination of phage and phagemid vector whenever this combination is used, so that the assays can substitute each other in routine applications once this ratio has carefully been established.

14.4.2 Titration on Nitrocellulose Filters to Determine the Number of Infective Particles (cfu Calculation)

Usually, phage titration is done by infecting *E. coli* plating bacteria with dilution series of phage (7). Approximately 16 h after embedding the infected bacteria in

top-agar, plaques can be counted. Since these plaques are transient, a more convenient method is to plate infected bacteria on agar plates and select for the antibiotics resistance gene provided by the phage genome. The resulting colonies can be identified and counted easily. To save material, the miniaturized method presented below uses plating of multiple samples on a nitrocellulose filter (8). It trades some accuracy for a significant saving of material, and reduces workload when many pannings are done in parallel.

1. Mark 16 fields on round nictrocellulose filters (Schleicher & Schuell) with a ballpen and place onto Luria Broth agar plates containing 50 μg/mL kanamycin
2. Infect 2 ml *E. coli* at an OD_{600} of 0.6 with 100 μL of serial dilutions of phage (depending on expected titer, start with 10^{-2}–10^{-9}, but use at least three different dilutions) in phage dilution buffer for 20 min at 37°C
3. Pipet 10 μL aliquots of each infection onto the nitrocellulose filters into the middle of each field and incubate overnight at 27°C (*see Note 1*)
4. Count colonies and calculate the titer of the initial phage suspension (*see Note 2*)

14.4.2.1 Notes

Note 1: Higher temperatures may be used if the developing colonies are too small the next morning. Standard (37°C) incubation usually yields colonies too large to be counted anymore because of extensive overlapping. The optimal temperature depends on the used *E. coli* strain as well.
Note 2: In case the retrieved colonies are too small to be counted after the overnight incubation, put the plates at 37°C for 1–2 h to increase colony size.

14.4.3 Phage ELISA for the Estimation of Total Particle Number

Counting the number of phage particles can be done by using an electron microscope, a method not applicable as a routine method. A surrogate parameter for particle number can be determined by ELISA using an antibody specifically recognizing the pVIII phage outer surface protein (9). This ELISA estimates the particle number in comparison to a dilution series of a phage suspension of a known titre (standardization curve). The number of phage particles determined for a packaged antibody library may not be exactly identical to the number of cfu, but usually the ratio of these two parameters is constant for samples made with the same helper phage, vector, and protocol. See note 4 of Protocol 14.4.1.

1. Coat MaxiSorp ELISA plates with serial dilutions of a reference phage of known titre and your new phage in parallel (e.g., 10^{-1}–5×10^{-4} dilutions in 100 mM $NaHCO_3$, pH 8.6). Apply 100 μL of each dilution per well and coat 2–3 h at room temperature or overnight at 4°C (*see Note 1*)

2. Block with 2% (w/v) skim milk/PBS/0.05% (v/v) Tween. Apply 200 µL per well and incubate for 1–2 h at room temperature
3. Wash five times with PBS/0.05% (v/v) Tween. Apply 200 µL per well (*see Note 2*)
4. Apply 100 µL of the mouse monoclonal antibody B62-FE2::HRP in 2% (w/v) milk/PBS/0.05% (v/v) Tween according to the manufacturer. Incubate for 1 h at room temperature
5. Wash five times with 200 µL PBS/0.05% (v/v) Tween
6. Prepare the developer solution consisting of 4.5 mL H_2O, 0.5 mL NaAc (1 M, pH 6), 12.5 µL TMB substrate (Promega, Madison, USA) and 6 µL 30% (v/v) H_2O_2. Apply 100 µL per well (*see Note 3*)
7. To stop the color development, add 50 µL of 1 M H_2SO_4 to each well and measure the absorption at OD_{450} with an ELISA reader. Calculate the total number of phage particles by comparison with the calibration curve of your reference phage of known titre

14.4.3.1 Notes

Note 1: For each step of serial dilution, use a new pipet tip. Mix well by pipetting up and down several times. Do not use polystyrene vessels for dilution series.
Note 2: After addition of the washing solution, wait for 5 s. Shake well and take care to remove washing solution completely after each washing step.
Note 3: A color reaction should be visible after 5 min.

References

1. Hust M, Dübel S, Schirrmann T (2007) Selection of recombinant antibodies from antibody gene libraries. In: Ochs M (ed) Gene function analysis. Humana Press, Totowa, NY, pp 243–255 ISBN 1597455474
2. Rondot S, Koch J, Breitling F, Dübel S (2001) A helper phage to improve single-chain antibody presentation in phage display. Nat Biotechnol 19:75–78
3. Soltes G, Hust M, Ng KKY, Bansal A, Field J, Stewart DIH, Dübel S, Cha S, Wiersma E (2007) On the influence of vector design on antibody phage display. J Biotechnol 127: 626–637
4. Barbas CF III, Kang AK, Lerner RA, Benkovic SJ (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci USA 88:7978–7982
5. Breitling F, Dübel S, Seehaus T, Klewinghaus I, Little M (1991) A surface expression vector antibody screening. Gene 104:147–153
6. Hoogenboom HR, Griffith AD, Johnson KS, Chiswell DJ, Hudson P, Winter G (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res 19:4133–4137
7. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbour Laboratory Press, New York
8. Koch J, Breitling F, Dübel S (2000) Rapid titration of multiple samples of filamentous bacteriophage (M13) on nitrocellulose filters. Biotechniques 29:1196–1202

9. Micheel B, Heymann S, Scharte G, Böttger V, Vogel F, Dübel S, Breitling F, Little M, Behrsing O (1994) Production of monoclonal antibodies against epitopes of the main coat protein of filamentous fd phage. J Immunol Methods 171(1):103–109
10. Taussig MJ, Stoevesandt O, Borrebaeck C, Bradbury A, Dübel S, Frank R, Gibson T, Gold L, Herberg F, Hermjakob H, Hoheisel J, Joos T, Konthur Z, Landegren U, Plückthun A, Ueffing M, Uhlén M (2007) ProteomeBinders: planning a European resource of affinity reagents for analysis of the human proteome. Nat Methods 4:13–17

Chapter 15
Yeast Display and Selections

Kelly Davis Orcutt and K. Dane Wittrup

15.1 Introduction

Since its introduction, yeast surface display (Boder and Wittrup 1997) has become a widely used platform for protein engineering. Yeast surface display has been used to isolate single-chain variable fragments (scFvs) from immune and nonimmune libraries with binding specificity to a variety of protein, peptide, and small molecule targets (Feldhaus et al. 2003), including fluorogens (Szent-Gyorgyi et al. 2008), cancer antigens (Yeung et al. 2007), plasma membrane proteins (Wang et al. 2007), and viral envelope proteins (Bowley et al. 2007). Yeast surface display has also been used to affinity mature scFvs originally isolated by other methods (Boder et al. 2000; Kieke et al. 1997; Razai et al. 2005; Graff et al. 2004). In addition to scFvs, many other proteins, peptides, and enzymes have been expressed on the surface of yeast for a variety of applications and have been recently reviewed (Gai and Wittrup 2007; Pepper et al. 2008).

Yeast surface display provides a direct connection between genotype and phenotype; a plasmid containing the gene of interest is contained within the yeast cell, while the encoded protein is expressed on the surface. Although the protocols described in this chapter focus on the display of proteins as fusions to the Aga2p-mating protein, there are a number of alternative methods that can be used to display proteins on the surface of cells while maintaining a genotype–phenotype linkage. Like the Aga2p system, some of these methods rely on the fusion of a protein of interest to an endogenous or recombinant protein that is localized at or near the surface of the cell (Matsumoto et al. 2002; Van der Vaart et al. 1997). Other methods utilize a cell surface capture molecule that noncovalently interacts with the protein of interest, obviating the need for the construction of fusion proteins.

K.D. Orcutt and K.D. Wittrup (✉)
Chemical Engineering Department, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
e-mail: wittrup@mit.edu

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_15,

In one cell surface capture method, cell surfaces are chemically modified and coupled to a capture molecule that recognizes the protein of interest (Manz et al. 1995). The protein of interest is then captured by the capture molecule, where it can be quantified or further manipulated. A high viscosity medium (e.g., containing polyethylene glycol) can be used to prevent the diffusion of protein(s) of interest between cells to maintain the genotype–phenotype linkage. Similar secretion capture methods have been developed for yeast (Rakestraw et al. 2006; Frykman and Srienc 1998), and used to capture secreted full-length IgG on the yeast surface for Fc receptor engineering (Sazinsky et al. 2008). Cell surface capture methods are advantageous in that they do not require the construction of fusion proteins, which can perturb the intrinsic secretion characteristics of the protein of interest (Wentz and Shusta 2007). However, chemical modification of the cell surface can influence transport and secretion of the protein of interest as well as cell viability, and bioconjugation protocols with living cells are often difficult to reproduce quantitatively.

Fandl and colleagues have addressed this problem by expressing the capture molecule (e.g., FcγRI) in the same cell that produces the protein of interest, obviating the need for chemical modification of the cell surface (Fandl et al. 2008). However, by design, this method results in intracellular association between the capture molecule and the protein of interest. Therefore, the transport and secretion of the protein of interest may still be perturbed by the capture molecule in a manner similar to that observed with fusion proteins (Wentz and Shusta 2007). The influence of the capture molecule on the secretion of the protein of interest may be minimized or eliminated by controlling the expression of the respective molecules so that they occur in a temporally distinct manner, for example, by placing the capture molecule and the protein of interest under the control of different promoters. Expression of the capture molecule can then be induced, while suppressing expression of the protein of interest, followed by suppression of capture molecule and induction of the protein of interest. This will result in the extracellular formation of a complex between the capture molecule and the protein of interest, enabling the protein of interest to be secreted completely on its own merits. In yet another variant of the secretion capture approach, it has been found that most secreted proteins nonspecifically "stick" to the surface of CHO cells, thereby enabling flow cytometric isolation of production cell lines (Brezinsky et al. 2003); this phenomenon also occurs with yeast.

The yeast surface display system described here and the one that is most widely used is the Aga2p system. An Aga2p fusion protein associates with the Aga1p-mating protein through two disulfide bonds, resulting in a covalent attachment to the yeast cell wall (Fig. 15.1). The Aga1p gene is stably integrated into the chromosome of the yeast and the Aga2p fusion gene is contained in a circular plasmid, both under the control of galactose promoters. The plasmid DNA also contains a nutritional marker to allow for selective growth. Induced yeast express on average, 10,000–100,000 copies of the Aga2p fusion per cell.

Fluorescence activated cell sorting (FACS) can be used in combination with yeast surface display to allow for high throughput quantitative screening of antigen

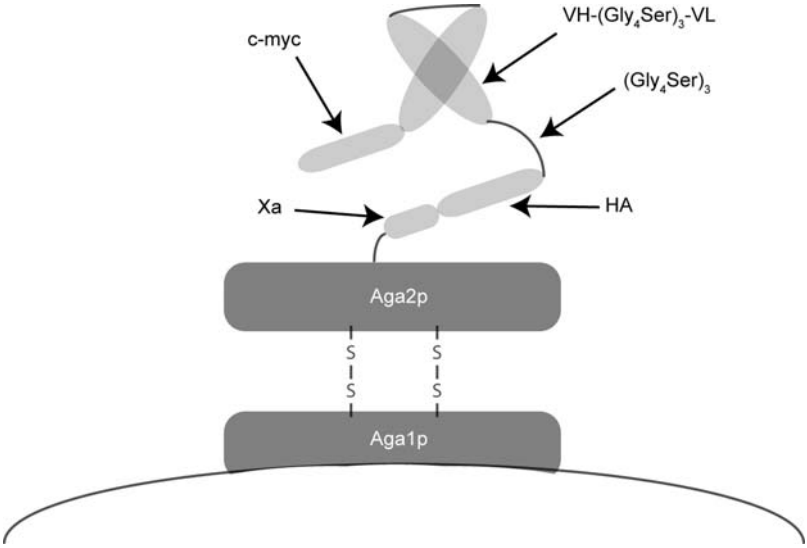


Fig. 15.1 Aga2p yeast display system. The scFv is directly fused to the Aga2p mating protein. The scFv is flanked by epitope tags, an *N*-terminal hemaglutinin (HA) epitope and a *C*-terminal c-myc epitope, both of which can be detected by antibodies to quantify scFv expression. Xa, Factor Xa cleavage site; $(Gly_4Ser)_3$, peptide linker, VH, variable heavy chain; VL, variable light chain

binding and protein expression in real time. Normalization for protein expression using antibodies specific for the *N*-terminal HA or *C*-terminal c-myc epitope tags (Fig. 15.1), used with two-color FACS, allows for fine affinity discrimination between clones (VanAntwerp and Wittrup 2000). In addition, expedient characterization of binding kinetics as well as thermal stability of proteins expressed on the surface of yeast can be performed without soluble expression and purification.

One of the main advantages of yeast surface display over other display technologies such as phage, mRNA, and bacterial display, is the eukaryotic expression bias of yeast, permitting the study and engineering of complex proteins that require posttranslational assembly and modification. A recent study directly compared yeast and phage display systems with respect to the isolation of clones that bind the HIV-1 gp120 antigen from an immune scFv cDNA library (Bowley et al. 2007). Yeast display not only identified all clones that were isolated using phage display, but also identified twice as many additional binders, many of which were nonfunctional when expressed by phage. This indicates that for this scFv DNA library, the functional library diversity in yeast display format is significantly greater than that of phage.

One disadvantage of yeast display is the relatively small library sizes on the DNA level that can be achieved compared to other display methods due to limitations in yeast transformation efficiencies. Yeast library sizes are typically in the order of 10^7–10^9 while phage and mRNA library sizes can be as large as 10^{11}–10^{13}. However, functional library sizes may be more similar because of yeast eukaryotic processing, as discussed above.

Detailed protocols for yeast surface display and selection methods using the Aga2p fusion method have been published previously (Chao et al. 2006; Feldhaus and Siegel 2004; Boder and Wittrup 2000; Colby et al. 2004). Presented here are updated protocols and new methods, including a highly avid magnetic bead selection process. The methods here describe (1) isolation of naïve scFv binders to a target antigen from a human nonimmune scFv library, (2) affinity maturation of naive binders, (3) characterization of individual clones, and (4) soluble expression of engineered scFvs from yeast. While these protocols are specific for scFvs, they can be easily modified to be used with other scaffolds, such as the 10th human fibronectin type III domain (Hackel et al. 2008; Lipovsek et al. 2007) or other antibody fragments such as Fabs (van den Beucken et al. 2003; Weaver-Feldhaus et al. 2004). A flow chart describing the process of isolating weak binders beginning with magnetic bead selections to a target antigen, followed by affinity maturation using FACS is provided in Fig. 15.2. Once antigen binding is visible by flow cytometry, further selections are performed using two-color FACS with either streptavidin-preloaded-antigen or two-step labeling and expression normalization to achieve better discrimination between clones. A pictorial representation of the three selection methods described is provided in Fig. 15.3.

15.2 Materials

15.2.1 Cells and Plasmids

- Nonimmune human scFv yeast library in pPNL6 plasmid vector, available by request from Pacific Northwest National Laboratories (http://www.sysbio.org/dataresources/singlechain.stm)
- Yeast strain EBY100 (trp-, leu-): *Saccharomyces cerevisiae* (*a GAL1-AGA1::URA3 ura3-52 trp1 leu2 leu2Δ1 his 3Δ200 pep4::HIS2 prb1Δ1.6R can 1 GAL*), available from the authors
- Yeast strain YVH10 (ura-, trp-): *S. cerevisiae* (*PDI::ADHII-PDI-Leu2 ura3-52 trp 1 leu2Δ1 his 3Δ200 pep4::H153 prb 1Δ1.6p can 1 GAL*), available from the authors
- Yeast display vector pCTCON2, available from the authors
- Yeast secretion vector prs314-GAL, available from the authors

15.2.2 Media and Buffers

- YPD: 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose; filter sterilize or autoclave, store at room temperature for up to 1 month or at 4°C for up to 2 months.
- YPG pH 6.0: 5.4 g/L Na_2HPO_4, 8.6 g/L $NaH_2PO_4{\cdot}H_2O$, 10 g/L yeast extract, 20 g/L peptone, 20 g/L galactose; filter sterilize or autoclave, store at room temperature for up to 1 month or at 4°C for up to 2 months.

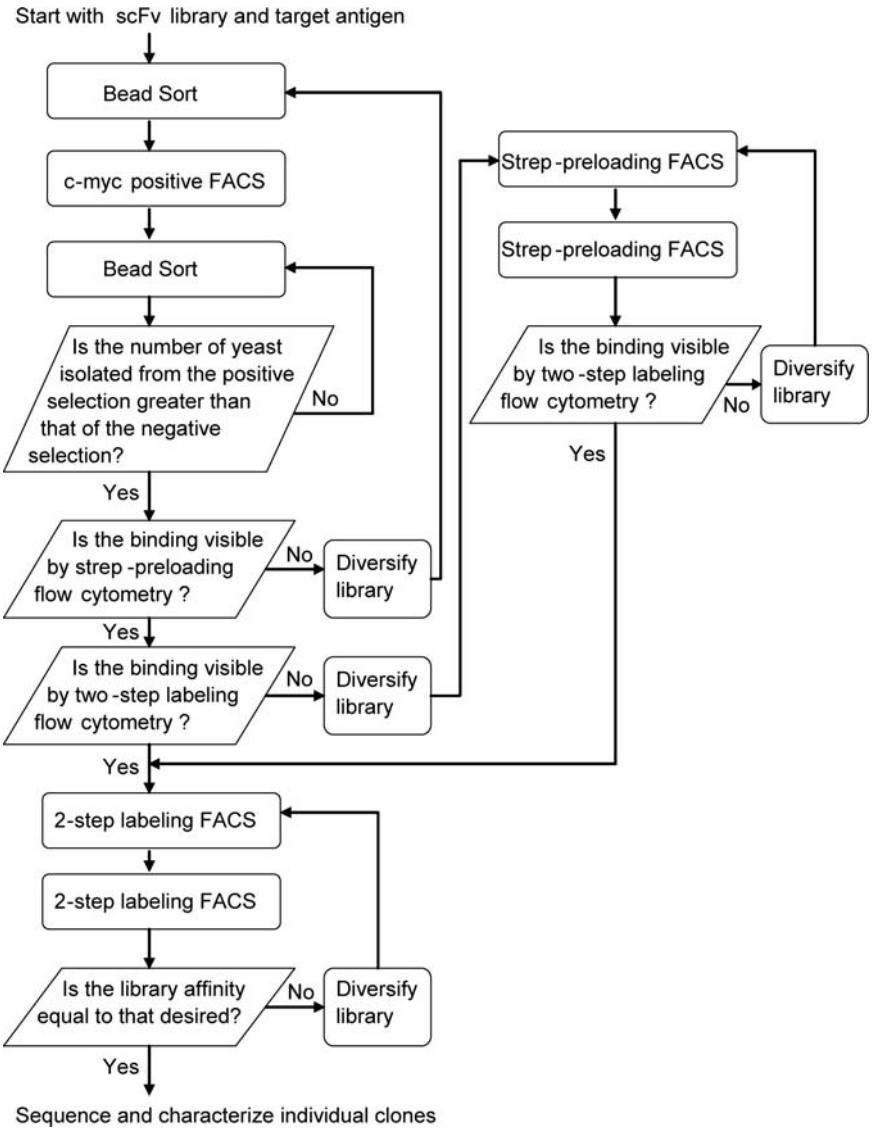


Fig. 15.2 Flow chart for scFv engineering to a target antigen. To engineer a high affinity scFv to a target antigen, the first step is to isolate weak antigen binders using magnetic bead selections. Once binding is visible by flow cytometry, subsequent selections can be performed with streptavidin preloaded antigen or two-step labeling

– SD-CAA pH 4.5: 10.4 g/L sodium citrate, 7.4 g/L citric acid monohydrate, 20 g/L dextrose, 6.7 g/L yeast nitrogen base, 5 g/L casamino acids (-ade, -ura, -trp), 100 kU/L penicillin, 0.1 g/L streptomycin; filter sterilize, store at room temperature for up to 3 months or at 4°C for up to 6 months.

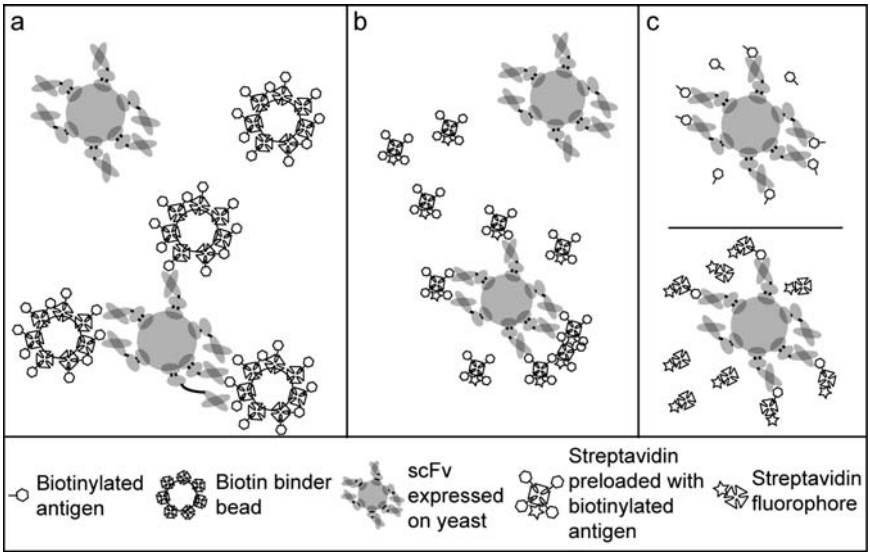


Fig. 15.3 Selection strategies. Yeast surface displayed libraries can be screened for binding to a target antigen using magnetic beads (**a**), streptavidin-fluorophore preloaded with biotinylated antigen for FACS (**b**), and/or two-step labeling for FACS with a primary incubation with biotinylated antigen (**c**, *top*) followed by a secondary incubation with a streptavidin-fluorophore conjugate (**c**, *bottom*). Note that magnetic beads and preloaded streptavidin result in an increase in antigen avidity. When using an antigen with multiple biotins, a "daisy chain" or polymer of antigen-streptavidin complexes can form

Note: Bacterial contamination is minimized by growth in low pH media supplemented with penicillin and streptomycin.

- SG-CAA pH 6.0: 5.4 g/L Na_2HPO_4, 8.6 g/L $NaH_2PO_4 \cdot H_2O$, 19 g/L galactose, 1 g/L dextrose, 6.7 g/L yeast nitrogen base, 5 g/L casamino acids, 100 kU/L penicillin, 0.1 g/L streptomycin; filter sterilize, store at room temperature for up to 3 months or at 4°C for up to 6 months.
  Note: A small amount of dextrose added to the induction media improves expression levels; however, high levels of dextrose suppress the GAL1 promoter.
- LB Amp media: 5 g/L yeast extract, 10 g/L NaCl, 10 g/L tryptone, 50 mg/L ampicillin; filter sterilize, store at 4°C for up to 4 months.
- SD-CAA plates: (1) dissolve 5.4 g Na_2HPO_4, 8.6 g $NaH_2PO_4 \cdot H_2O$, 182 g sorbitol, and 15 g agar in 900 mL H_2O and autoclave, (2) dissolve 20 g dextrose, 6.7 g yeast nitrogen base, and 5 g casamino acids in 100 mL H_2O and filter sterilize, (3) cool (1) to below 50°C, add (2), mix and pour plates; store at 4°C for up to 6 months.
- YPD plates: dissolve 20 g dextrose, 20 g peptone, 10 g yeast extract, and 15 g agar in 1 L H_2O, autoclave, mix, and pour plates; store at 4°C for up to 6 months.
- LB Amp plates: (1) dissolve 5 g yeast extract, 10 g NaCl, 10 g tryptone, and 15 g agar in 1 L H_2O and autoclave, (2) dissolve 50 mg ampicillin in 1 mL H_2O and

filter sterilize, (3) cool (1) to below 50°C, add (2), mix and pour plates; store at 4°C for up to 4 months.
- Tris-dithiothreitol (DTT): 0.39 g/mL 1,4-ditiothreitol in 1 M Tris, pH 8.0; filter sterilize, prepare fresh or store at −20°C for up to 6 months.
- Electroporation buffer (Buffer E): 10 mM Tris, pH 7.5, 270 mM sucrose, 1 mM $MgCl_2$; filter sterilize, store at 4°C.
- Phosphate buffered saline with bovine serum albumin (PBSA): 10 mM $NaHPO_4$, 137 mM NaCl, 2.7 mM KCL, 1 g/L BSA filter sterilize, pH 7.4; filter sterilize, store at 4°C for up to 6 months.
- Low-dextrose SD-CAA pH 6.0: 5 g/L dextrose, 6.7 g/L yeast nitrogen base, 5 g/L casamino acids, 5.4 g/L Na_2HPO_4, 8.6 g/L $NaH_2PO_4H_2O$; filter sterilize, store at room temperature for up to 3 months or at 4°C for up to 6 months.
- Freezing solution: 2% glycerol, 6.7 g/L yeast nitrogen base; filter sterilize, store at room temperature.

15.2.3 Bead Selection Reagents

- Biotinylated antigen (biotinylation kits available from Thermo Scientific and Invitrogen)
- DynaMag™-2 magnet (Invitrogen 123-21D)
- Biotin Binder Dynabeads (Invitrogen 110-47)

15.2.4 FACS Selection Reagents

- Biotinylated antigen, refer to Sect. 14.2.3
- Mouse anti-c-myc IgG clone 9E10 (Covance, MMS-150R) or chicken anti-c-myc IgY (Invitrogen, A21281)
- Mouse anti-HA clone 16B12 (Covance, MMS-101R)
- Alexa Fluor 647 goat anti-chicken IgG (H+L) (Invitrogen, A21449) or Alexa Fluor 647 goat anti-mouse IgG (H+L) (Invitrogen, A21235)
- Streptavidin R-phycoerythrin conjugate (Invitrogen, S-866)
  Note: The above primary and secondary antibodies are only suggestions; many other antibodies and fluorophores can be substituted

15.2.5 Diversification and Characterization Reagents

- Zymoprep I or Zymoprep II yeast plasmid miniprep kit (Zymo Research D2001 or D2004)
- QIAquick PCR Purification kit (Qiagen, 28104)
- XL1-blue Supercompetent *E. coli* (Stratagene, 200236)

- *Taq* DNA polymerase with 10× *Taq* buffer (Invitrogen, 10342-053)
- *Mutazyme*® II polymerase with 10× *Mutazyme*® buffer (Stratagene GeneMorph® II Random Mutagenesis Kit, 200550)
- 2′-deoxynucleoside 5′-triphosphates (dNTPs, Invitrogen, 18427-013)
- 8-oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP, TriLink, N-2034)
- 2′-deoxy-*p*-nucleoside-5′-triphosphate (dPTP, TriLink, N-2037)
- 10× gel-loading buffer (50% glycerol and 0.1% bromophenol blue)
- SYBR gold nucleic acid gel stain (Invitrogen, S-11494)
- QIAquick Gel extraction kit (Qiagen, 28704)
- PelletPaint co-precipitant (EMD Biosciences, 69049)
- Restriction enzymes (New England Biolabs, *Nhe*I R0131, *Sal*I R0138, *Bam*HI R0136)
- Frozen-EZ Yeast Transformation II (Zymo Research, T2001)
- 0.2-cm electroporation cuvettes (BioRad, 652086)
- Gene Pulser (BioRad, 1652076)

15.2.6 Yeast Soluble Expression Reagents

- Frozen-EZ Yeast Transformation II (Zymo Research, T2001)
- Restriction enzymes (New England Biolabs, *Nhe*I R0131, *Xho*1 R0146)
- Monoclonal anti-FLAG M2 Affinity Gel (Sigma, A2220)
- Monoclonal anti-FLAG M2-Peroxidase (HRP) (Sigma, A8592)
- Quick Ligation Kit (New England BioLabs, M2200)

15.3 Protocols

15.3.1 Yeast Growth and Induction

EBY100 grows optimally at 30°C with significant agitation and aeration. EBY100 containing the pCTCON2 plasmid with tryptophan marker requires growth in minimal media lacking tryptophan for selective pressure to maintain the plasmid. Induction in galactose containing media is typically carried out at 20°C to improve the surface display of scFv proteins with low thermal stability. However, induction at higher temperatures can be performed to select for more stable clones.

1. Incubate the human scFv yeast library in SD-CAA for 24–48 h at 30°C, 250 rpm. *Note*: Recommended vessels: Tunair shake flasks for 0.5–1 L culture, glass shake flasks for 50–100 mL culture, and glass culture tubes for 5 mL culture.
2. Pellet at least 10× the estimated library diversity at 2,500 *g* for 5 m or 12,000 *g* for 30 s and resuspend cells in fresh media. Subculture to a concentration of 0.2–0.8 × 10^7 cells/mL ($OD_{600} = 1$ corresponds to 1 × 10^7 cells/mL) in fresh SD-CAA.

Note: Always ensure that the number of cells subcultured is at least 10× the estimated library diversity to avoid losing unique clones.

3. Incubate at 30°C, 250 rpm until the cells are in mid-log phase ($OD_{600} = 2–5$ and yeast have doubled at least twice).
   Note: EBY100 doubles about once every 2 h in SD-CAA at 30°C.
4. Subculture the cells to a concentration of 1×10^7 cells/mL in SG-CAA, again ensuring at least ten-fold oversampling.
5. Incubate at 20°C, 250 rpm for 24–48 h.
6. Cells are ready for selection or characterization (store at 4°C for up to 1 month, or for long-term storage refer to Sect. 15.3.2).

15.3.2 Yeast Storage and Revival

15.3.2.1 Yeast Library Freezing

1. Inoculate at least a ten-fold oversampling of a freshly grown library into low-dextrose SD-CAA and grow at 30°C, 250 rpm for ~3 days to stationary phase ($OD_{600} = 8–12$).
   Note: It is highly recommended to check for bacterial contamination at this point to prevent storage of contaminated cultures.
2. Pellet cells and remove supernatant.
3. Resuspend cells in freezing solution to a final concentration of 6×10^{10} cells/mL.
4. Aliquot cells into cryogenic vials ensuring each vial has a ten-fold oversampling of the library diversity. Incubate at room temperature for 10 m.
5. Place the vials in an isopropanol bath at room temperature and slow-freeze the cells to −80°C.
6. Transfer frozen aliquots to liquid nitrogen.

15.3.2.2 Yeast Library Revival

1. Thaw cryogenic vial at room temperature.
2. Transfer yeast to at least 50 mL SD-CAA. Grow at 30°C 250 rpm 24–48 h.
3. Subculture cells to $0.2–0.8 \times 10^7$ in SD-CAA. Grow cells for induction or storage.

15.3.3 Isolation of Naïve Binders Using Magnetic Beads

Most libraries are orders of magnitude smaller than their theoretical diversity, and it is likely that nanomolar binders to some antigen targets simply do not exist in the

library population. However, it is possible that weaker binders exist; unfortunately, FACS is unable to select for binders with dissociation rates on the order of seconds or faster, such as those generally associated with micromolar binders, due to limitations in sorting speed (~1 h for 10^8 cells). A recently developed high throughput method using highly avid magnetic beads has demonstrated the ability to isolate binders undetectable by FACS, potentially as weak as millimolar affinity (Ackerman et al. 2009).

Streptavidin coated magnetic beads used in combination with biotinylated antigen provides a simple and straightforward screening method. Multiple copies of protein expressed on each yeast cell combined with multiple antigen targets coated on each bead results in a highly avid system (Fig. 15.3a) capable of isolating very weak binders. A library of 10^{10} yeast can be screened in a few hours by magnetic bead selection, while FACS screening of this number of yeast would take days.

The protocol provided below describes a negative selection to deplete the library of streptavidin binders followed immediately by a positive selection against the target antigen. The number of binders isolated to the antigen-coated beads should be larger than that for the negative selections after several rounds of selection. If this is indeed the case, the population is enriching for antigen binders and should be diversified.

15.3.3.1 Prepare Antigen-Coated Beads

1. Wash biotin binder beads twice with PBSA using DynaMag-2 magnet.
2. Incubate 10× molar excess of biotinylated antigen with 2.5×10^6 biotin binder beads (~6×10^6 biotin binding sites per bead) overnight at 4°C, rotating.
3. Wash antigen-coated beads twice with PBSA using magnet, store at 4°C.

15.3.3.2 Negative Selection (to Select Against Secondary Binders)

1. Pellet at least 10× the estimated diversity of the yeast library by centrifugation at 12,000 *g* for 30 s or 2,500 *g* for 2 m. Discard supernatant.
2. Wash the cells with PBSA: resuspend the cells in 1 mL PBSA and repellet the library.
3. Resuspend the yeast in 1 mL PBSA and uncoated biotin binder beads (about 1:20 bead to yeast ratio and no less than 10^7 beads per mL) for 1 hr rotating at 4°C or room temperature.
4. Collect unbound yeast: apply the microcentrifuge tube to the magnet for 5 m, collect the supernatant, and resuspend beads in 1 mL SD-CAA.
5. Plate serial dilutions of the bead solution on SD-CAA plates to estimate the number of streptavidin binders.

6. Repeat negative selection: incubate collected yeast with fresh uncoated beads for 1 hr. Collect the supernatant containing unbound yeast.
7. Immediately proceed to positive selection.

15.3.3.3 Positive Selection (to Select for Antigen Binding Clones)

1. Incubate the collected yeast with antigen coated beads (about 1:20 bead to yeast ratio and no less than 10^7 beads per mL) for 1 hr rotating at 4°C or room temperature.
2. Remove unbound yeast: apply the microcentrifuge tube to the magnet for 5 m, discard supernatant containing unbound yeast.
3. Optional: to increase stringency of selection, wash beads 1–3 times by resuspending the beads in 1 mL PBSA and incubating for 15 m, 4 or 20°C.
   Note: The selection stringency can be increased with (1) multiple washes, (2) decreasing the avidity by either reducing the amount of antigen on the beads or reducing the protein expression on yeast by shortening the induction time. Note that reducing the amount of antigen on the beads will increase the selection pressure toward streptavidin.
4. Resuspend beads in 1 mL SD-CAA. Plate serial dilutions on SD-CAA plates to estimate the number of antigen binders and library diversity.
5. Place beads with retrieved yeast in 50 mL SD-CAA. Incubate at 30°C, 250 rpm overnight.
6. Remove beads: centrifuge yeast culture, discard supernatant, resuspend in 1 mL SD-CAA, apply magnet for 5 m, collect supernatant and dilute in SD-CAA for continued growth.
   Note: A FACS selection for c-myc positive cells should be performed after the first bead selection of a new library to ensure selection of full-length clones. Refer to Sect. 15.3.5 for a detailed protocol of FACS labeling.
   Note: After two or three bead sorts, label the library with (1) 100–500 nM streptavidin-PE:antigen preloaded complex and/or (2) micomolar quantities of biotinylated antigen followed by secondary labeling with streptavidin-PE to determine if binding is visible by flow cytometry. If so, selections can now be performed using FACS; refer to Sect. 15.3.5.

15.3.4 Diversification

There are many methods used to diversify libraries in directed evolution applications. A protocol using error-prone PCR with both degenerate nucleotides and error prone DNA polymerases is described below.

The protocol describes yeast transformation with linearized pCTCON2 vector and overlapping error-prone PCR product resulting in *in vivo* homologous recombination.

Yeast transformation can also be accomplished with circular plasmid DNA; however, homologous recombination generates larger libraries, eliminates ligation and bacterial transformation, and results in additional recombination events yielding greater library diversity (Swers et al. 2004).

15.3.4.1 Prepare Cut Vector

1. Digest pCTCON2 plasmid to completion with *Sal*1, *Nhe*1, and *Bam*H1 following the manufacturer's protocol.
2. Concentrate using PelletPaint following the manufacturer's instructions. Resuspend in sterile water to a concentration of 1 μg/uL. Store vector at −20°C.

15.3.4.2 Mutagenesis

1. Zymoprep 10×10^7 cells following the manufacturer's protocol with two modifications. First, centrifuge the cell lysate supernatant in a fresh tube before applying to the spin column to remove residual precipitate. Second, clean up zymoprep DNA with a Qiagen PCR purification following the manufacturer's protocol. Store DNA at 4°C overnight or −20°C indefinitely.
2. Error prone PCR: prepare PCR mix for error prone PCR with degenerate nucleotides. Primers for pCTCON2 are forward, 5′-CGACGATTGAAGGTAGATACCCATACGACGTTCCAGACTACGCTCTGCAG-3′, and reverse, 5′-CAGATCTCGAGCTATTACAAGTCCTCTTCAGAAATAAGCTTTTGTTC-3′. The nonimmune human scFv library is in a slightly different yeast secretion vector, pPNL6; thus, for clones isolated directly from the library, use the reverse primer 5′-CGAGCTATTACAAGTCTTCTTCAGAAATAAGCTTTTCTTCTAGAATTCCGGA-3′. These primers amplify the scFv and provide a 50 base pair overlap to the pCTCON2 vector used in homologous recombination.

Volume	Component
5 μL	10× Thermopol buffer
2.5 μL	5′ primer (10 μM)
2.5 μL	3′ primer (10 μM)
1 μL	dNTPs (10 mM each)
10 μL	zymoprepped DNA
5 μL	8-oxo-dGTP 20 μM
5 μL	dPTP 20 μM
0.5 μL	*Taq* DNA polymerase (5 U/μL)
in 50 μL final volume	

3. Optional: Prepare PCR mix for error prone PCR with *Mutazyme* II.

Volume	Component
5 μL	10× Mutazyme buffer
2.5 μL	5′ primer (10 μM)
2.5 μL	3′ primer (10 μM)
1 μL	dNTPs (10 mM each)
10 μL	zymoprepped DNA (or 670 pg template DNA)
1 μL	*Mutazyme* II polymerase (2.5 U/μL)
in 50 μL final volume	

Note: *Mutazyme* II is a combination of two error prone DNA polymerases that results in a more unbiased mutational spectrum in contrast to error prone PCR with degenerate nucleotides with a bias to mutations at A's and T's and transition mutations (Wong et al. 2006).

4. Set up a thermal cycler with the following protocol.

Cycle	Condition
1	94°C for 3 m
2–11	94°C for 45 s, 60°C for 30 s, 72°C for 90 s
12	72°C for 10 m

5. Store mutated DNA at 4°C overnight or −20°C indefinitely.
6. Add 5.6 μL of 10× gel-loading buffer to 50 μL PCR product. Perform gel electrophoresis with a 1.5% agarose gel, 100 V for 45–60 m. Stain with SYBR gold nucleic acid gel stain. Excise the appropriate PCR product out of the gel (700–800 bp for an scFv). PCR product may not be visible on the gel; use a DNA ladder or a control amplified scFv as a standard and excise the appropriate site. Perform a QIAquick gel extraction following the manufacturer's protocol.
7. Optional: combine DNA from degenerate nucleotide PCR and *Mutazyme* II PCR.
8. Amplification PCR: prepare PCR sample in a 1.5 mL microcentrifuge tube.

Volume	Component
40 μL	10× Thermopol buffer
20 μL	5′ primer (10 μM)
20 μL	3′ primer (10 μM)
8 μL	dNTPs (10 mM each)
16 μL	gel extracted DNA
4 μL	*Taq* DNA polymerase (5 U/μL)
in 400 μL final volume	

9. Aliquot 100 μL into four 0.2 mL PCR tubes.
10. Set up a thermal cycler with the following protocol.

Cycle	Condition
1	94°C for 3 m
2–31	94°C for 45 s, 60°C for 30 s, 72°C for 90 s
32	72°C for 10 m

11. Combine amplification PCR products. Concentrate using PelletPaint according to the manufacturer's protocol. Resuspend in 2 μL sterile water.

15.3.4.3 Yeast Electroporation Transformation

1. Prepare EBY100 for electroporation transformation: streak frozen EBY100 onto a YPD plate and grow at 30°C for 2–4 days. Grow one colony in 5 mL liquid YPD overnight in 30°C 250 rpm. Inoculate 50 mL YPD to a concentration of 0.1–0.2 $\times$ 10^7 cells/mL. Incubate culture at 30°C, 250 rpm until cell density is 1.4–1.6 $\times$ 10^7 cells/mL (about 4–6 h).
2. Chill two electroporation cuvettes, buffer E, and all tubes for preparing the yeast on ice. Chill centrifuges to 4°C.
3. Prepare fresh DTT (refer to Sect. 15.2.2).
4. Add 0.5 mL DTT solution to 50 mL yeast culture prepared as described above.
5. Incubate at 30°C, 250 rpm for 15 m.
6. Pour culture into chilled 50 mL tube.
7. Centrifuge cells at 2,500 *g*, 4°C for 3 m. Discard supernatant.
8. Wash the pellet with 25 mL buffer E.
9. Centrifuge cells at 2,500 *g*, 4°C for 3 m. Discard supernatant.
10. Resuspend pellet in 1 mL buffer E. Transfer to a cold 1.5 mL microcentrifuge tube.
11. Centrifuge at 5,000 *g*, 4°C for 1 m. Discard supernatant.
12. Resuspend pellet to 100 μL final volume with buffer E.
13. Prepare DNA: add 2 μg cut vector to DNA insert.
14. Resuspend DNA with 100 μL competent yeast (pipette gently).
15. Transfer 50 μL of yeast mixture to each of two cold 0.2 cm electroporation cuvettes and keep on ice.
16. Electroporate at 25 μF, 0.54 kV.
17. Add 1 mL room temperature or 30°C prewarmed YPD to cuvette immediately.
18. Transfer yeast to a 15 mL round-bottomed Falcon tube. Wash each electroporation cuvette with an additional 1 mL YPD and transfer to the Falcon tube.
19. Incubate yeast at 30°C, 250 rpm for 1–2 h.
20. Centrifuge cells at 1,300 *g* for 1 m.
21. Resuspend in 1 mL SD-CAA. Plate serial dilutions on SD-CAA plates to determine electroporation efficiency and estimate library diversity. Transfer yeast to a flask with 100 mL SD-CAA.
    Note: Typical yeast transformation efficiencies are 10^6–10^7 transformed cells per 50 μL electroporation.
22. Incubate 30°C, 250 rpm overnight.
23. Subculture cells once and then induce as described above.

15.3.5 Sequence Characterization of Library Clones

1. Zymoprep library as described above.
2. Transform 1 μL of zymoprepped DNA into XL-1 Blue, DH5α, or other competent *E. coli* and select on LB Amp plates overnight at 37°C.

3. Grow individual colonies in 5 mL LB Amp media overnight at 37°C and purify plasmid DNA by miniprep.
4. Sequence the DNA; for pCTCON2, effective primers are forward, 5′-GTTCCA-GACTACGCTCTGCAGG-3′, and reverse, 5′-GATTTTGTTACATCTACAC-TGTTG-3′.

15.3.6 Screening by FACS

FACS allows quantitative real-time selection of improved binding to soluble antigen. Antigen-binding signal can be normalized by expression (Fig. 15.4), which allows for fine affinity discrimination between clones. Two-color labeling also allows for simultaneous stability and affinity engineering, as expression has been shown to correlate with protein stability (Shusta et al. 1999).

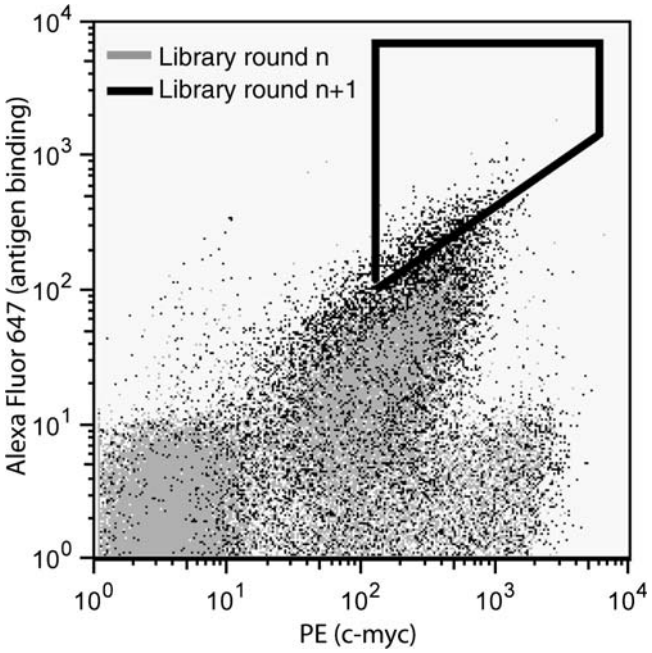


Fig. 15.4 FACS data. Typical flow cytometry data for an scFv affinity maturation are shown. Cells are labeled with biotinylated antigen and mouse anti-c-myc IgG followed by streptavidin Alexa Fluor 647 and R-phycoerythrin goat anti-mouse IgG secondary reagents. An example population is shown (library round n) alongside the same population that has been sorted once by FACS to enrich for higher affinity binders (library round n+1). The sort gate is drawn such that the highest displaying clones (PE signal) with the highest antigen binding signal relative to expression are selected. This sort gate will enrich for both high affinity and high stability clones

15.3.6.1 Labeling and FACS Selection

1. Pellet at least a five- to ten-fold oversampling of the induced library by centrifugation at 12,000 *g* for 30 s or 2,500 g for 2 m. Discard supernatant.
2. Wash the cells with PBSA: resuspend the cells in 1 mL PBSA, and repellet the library.
3. Resuspend the cells in PBSA with primary labels (1:100 dilution or ~10 mg/L mouse anti-cmyc, chicken anti-cmyc, or mouse anti-HA and an appropriate concentration of biotinylated antigen). Incubate at room temperature or 37°C to allow for a sufficient approach to equilibrium.
   Note: Alternate labeling between HA and c-myc expression has been found to eliminate the isolation of artifacts, such as c-myc mutants that bind with weaker affinity to the anti-c-myc antibody (Kieke et al. 1997), while still retaining full-length clones.
   Note: An appropriate antigen labeling concentration depends on the antigen binding affinity of the population. The goal is to choose a concentration that results in a visible diagonal, but with sub-saturating signal to achieve the greatest difference in binding signal between clones with different affinities (Boder and Wittrup 1998). Ensure a ten-fold molar excess of all primary and secondary labels to the number of surface expressed scFv molecules, assuming 10^5 scFv molecules per cell.
   Note: The half life to approach equilibrium can be calculated from the formula $t_{1/2} = \ln(2)/(k_{on}[Ag]+k_{off})$, where k_{on} is the association rate, k_{off} is the dissociation rate, and [Ag] is the antigen concentration. For most protein–protein interactions, the k_{on} is 10^5–10^6 $M^{-1}s^{-1}$. Four half lives is sufficient time for greater than 90% of the approach to equilibrium.
4. Pellet the library. Wash the cells with cold PBSA.
5. Resuspend the cells in cold PBSA with secondary labels (1:200 dilution or ~10 mg/L goat anti-chicken 647 or goat anti-mouse 647 and 1:100 dilution or ~10 mg/L streptavidin-PE). Incubate at 4°C for 30 m.
6. Pellet the library. Wash the cells with cold PBSA.
7. Resuspend the library in cold PBSA to a final concentration of 10^7–10^8 cells/mL for FACS. Select 0.02–0.5% of cells with the highest antigen binding with respect to scFv expression; refer to Fig. 15.5.
8. Grow the collected yeast cells in SD-CAA at 30°C 250 rpm.
   Note: Antigen labeling at 37°C can be performed to select for clones stable at physiological temperatures. Yeast can also be induced at 30°C for shorter time periods to increase selection pressure toward more stable clones.
   Note: For affinity maturation of very high affinity binders, large incubation volumes are required to ensure a molar excess of antigen to scFv. Generally, volumes larger than 50 mL tend to be impractical. Kinetic competition can thus be used for selections of high affinity populations to select for clones with slow dissociation rates. For kinetic competition, label the library population with a saturating concentration of biotinylated antigen. Wash the cells and resuspend them in PBSA with an excess of unbiotinylated antigen competitor. Incubate at

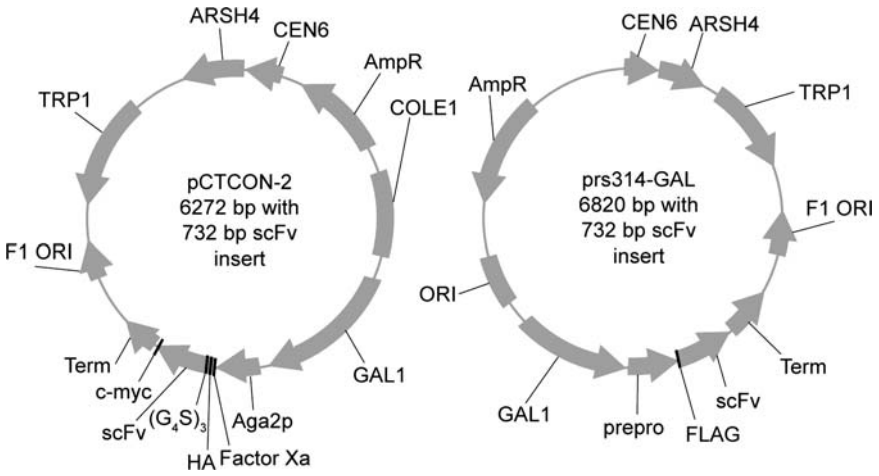


Fig. 15.5 Surface display and soluble expression plasmid maps. pCTCON-2 surface display plasmid (left) and pRS314-GAL soluble expression plasmid (right). Autonomously replicating sequence H4 (ARSH4), centomeric sequence 6 (CEN6), ampicillin resistance gene (AmpR), colicin E1 origin of replication (COLE1), origin of replication (ORI), F1 origin of replication (F1 ORI), GAL1 promoter (GAL1), agglutinin 2p protein subunit (Aga2p), factor Xa cleavage site (Factor Xa), hemaglutinin epitope tag (HA), $(Gly_4Ser)_3$ linker ($(G_4S)_3$)), single chain variable fragment VH-$(Gly_4Ser)_3$-VL (scFv), c-myc epitope tag (c-myc), terminator (Term), Trp1 gene (TRP1), alpha prepro leader sequence (prepro), FLAG epitope tag (FLAG)

room temperature or 37°C for an appropriate time to allow for dissociation of biotinylated antigen. Wash the cells in PBSA and incubate with primary antibody to detect HA or c-myc expression at 4°C for 30 m. Wash the cells in PBSA, incubate with secondary reagents as described above, and sort by FACS.

Note: For affinity maturation of very weak clones, ensure that all wash steps are performed with ice cold PBSA. In addition, secondary labeling can be performed for shorter times and the final wash step can be eliminated. Also refer to 14.3.6.2.

Note: A K_D curve for the library can be performed (refer to Sect. 15.3.7) to determine the average K_D of clones in the library. This analysis can help determine affinity maturation progress.

15.3.6.2 Optional Protocol for Streptavidin Preloading to Increase Antigen Avidity

Biotinylated antigen preloaded on tetravalent streptavidin can be used to increase avidity for FACS (Fig. 15.3b) to select for weak antigen binders.

1. Incubate streptavidin-PE with biotinylated antigen at 4°C for 30 m at a molar ratio of 1:4 streptavidin to biotinlyated antigen.

2. Label yeast library as described above for FACS. For step 3, incubate with anti-cmyc or anti-HA antibody only. For step 5, incubate with both goat anti-chicken 647 or goat anti-mouse 647 and preloaded streptavidin-PE:antigen complex. Ensure at least a three-fold molar excess of streptavidin-PE:antigen complex to scFv.

15.3.7 Characterization of Individual Clones

Engineered scFvs can be conveniently characterized while expressed on the surface of yeast, eliminating the need for soluble expression and purification of individual clones. The dissociation constant of proteins measured when expressed on yeast correlates well with measurements of soluble protein (Lipovsek et al. 2007, Gai and Wittrup 2007). The protocols here describe antigen binding kinetic characterization as well as stability analysis.

15.3.7.1 Prepare Clonal Yeast

1. Transform clonal miniprep DNA into EBY100 yeast using the Zymo Research Frozen-EZ Yeast Transformation II kit following the manufacturer's instructions. Select on SD-CAA plates.
2. Grow a single colony in SD-CAA pH 4.5 media and induce in SG-CAA as described in Sect. 15.3.1.

15.3.7.2 Equilibrium Dissociation Constant (K_D) Measurement

1. Pellet 1×10^7 induced clonal yeast by centrifugation at 12,000 *g* for 30 s or 2,500 g for 2 m. Discard supernatant.
2. Wash the cells with PBSA: resuspend the cells in 1 mL PBSA and repellet the library. Resuspend the cells in 1 mL PBSA.
3. Set up 8–12 tubes with 50 μL of cells and varying concentrations of biotinylated antigen in PBSA. Ensure that each tube contains a molar excess of antigen with respect to the number of surface expressed scFv molecules. Concentrations and volumes for an example titration are:

[Ag]	Tube	Cells	PBSA	Biotinylated Antigen		
				20 μM	2 μM	200 nM
0	1	50 μL	150 μL			
1 μM	2	50 μL	150 μL	10.53 μL		
316 nM	3	50 μL	150 μL		37.53 μL	
100 nM	4	50 μL	150 μL		10.53 μL	
31.6 nM	5	50 μL	500 μL		8.83 μL	
10 nM	6	50 μL	1 mL			55.26 μL

(*continued*)

[Ag]	Tube	Cells	PBSA	Biotinylated Antigen		
				20 μM	2 μM	200 nM
3.16 nM	7	50 μL	1 mL			16.86 μL
1 nM	8	50 μL	10 mL			50.50 μL
316 pM	9	50 μL	10 mL			15.90 μL
100 pM	10	50 μL	40 mL			20.04 μL
31.6 pM	11	50 μL	40 mL			6.33 μL
10 pM	12	50 μL	50 mL			2.5 μL

4. Incubate tubes for a sufficient time for all tubes to reach equilibrium. Refer to note on approaching equilibrium in Sect. 15.3.6. Most K_D curves are performed at 25°C or 37°C.
5. Centrifuge tubes, discard supernatant.
6. Wash the cells with cold PBSA.
7. Resuspend yeast in 100 μL cold PBSA with 1:100 dilution (10 mg/L) streptavidin-PE. Incubate at 4°C for 10–15 m.
8. Centrifuge tubes, discard supernatant, and wash the cells with cold PBSA.
9. Resuspend the library in cold PBSA and analyze on a flow cytometer.
10. Fit the total mean PE fluorescence, MFU_{tot}, versus antigen concentration, [Ag], by least squares regression to

$$MFU_{tot} = MFU_{\min} + \frac{MFU_{range} \times [Ag]}{K_D + [Ag]}$$

varying K_D, MFU_{min}, and MFU_{range}.
Note: If concentrations of <10 pM are necessary for titrations of high affinity binders, the entire titration can be performed with fewer cells (2.5×10^4 per tube). However, yeast cells at low concentrations do not pellet effectively. Thus, nonbinding cells (noninduced yeast) can be added (7.5×10^5 per tube) to aid in pelleting the cells while ensuring antigen excess over the scFv (Hackel et al. 2008).
Note: Nonspecific sticking at high concentrations can result in nonsaturating K_D titrations for weak binders. Yeast expressing an irrelevant scFv can be titrated identically and the mean total fluorescence due to nonspecific binding can be measured. This value can then be subtracted from the MFU_{tot} for the yeast of interest and the resulting data fit by least squares regression.

15.3.7.3 Competition Dissociation Constant (K_D) Measurement

Depending on the size of the antigen and the degree of biotinylation, the biotinylated antigen may have different binding properties than the unlabeled antigen. The K_D of the unlabeled antigen can be determined by competition. This is performed by labeling yeast with a constant concentration of biotinylated antigen and a varying concentration of unlabeled antigen competitors, both in molar excess over the number of scFv molecules.

1. Prepare yeast as described above for a K_D curve.
2. Set up 8–12 tubes with 50 μL of cells, a constant concentration of biotinylated antigen (a saturating concentration is recommended for best signal to background ratio) and varying concentrations of unlabeled antigen. Ensure that each tube contains a molar excess of both biotinylated and unlabeled antigen with respect to the number of surface-expressed scFv molecules. Concentrations and volumes for an example titration are:

[Ag]	Tube	Cells	PBSA	bAg 200 nM	Unlabeled Antigen		
					200 μM	2 μM	200 nM
0	1	50 μL	5 mL	2.53 μL			
1 μM	2	50 μL	5 mL	2.54 μL	25.39 μL		
316 nM	3	50 μL	5 mL	2.53 μL	8.00 μL		
100 nM	4	50 μL	5 mL	2.53 μL	2.53 μL		
31.6 nM	5	50 μL	5 mL	2.57 μL		81.11 μL	
100 nM	6	50 μL	5 mL	2.54 μL		25.39 μL	
3.16 nM	7	50 μL	5 mL	2.53 μL		8.00 μL	
1 nM	8	50 μL	5 mL	2.53 μL		2.53 μL	
316 pM	9	50 μL	10 mL	5.04 μL			15.91 μL
100 pM	10	50 μL	10 mL	5.03 μL			5.03 μL
31.6 pM	11	50 μL	40 mL	20.0 μL			6.33 μL
10 pM	12	50 μL	40 mL	20.0 μL			2.00 μL

3. Incubate tubes for a sufficient time to approach equilibrium for all tubes. Continue labeling and analysis as described above for a K_D curve. Fit the data by least squares regression to

$$MFU_{tot} = MFU_{\min} + \frac{MFU_{range} \times [Ag_{biot}]}{K_{D,biot} + [Ag_{biot}] + \left(\frac{K_{D,biot}}{K_D}\right)[Ag]} \tag{3.1}$$

varying K_D, MFU_{min}, and MFU_{range}. $[Ag_{biot}]$ is the biotinylated antigen concentration, [Ag] is the unlabeled antigen concentration, and $K_{D,biot}$ is the dissociation constant of the biotinylated antigen.

15.3.7.4 Association Rate (k_{on}) Measurement

1. Pellet 1×10^7 induced clonal yeast by centrifugation at 12,000 *g* for 30 s or 2,500 *g* for 2 m. Discard supernatant.
2. Wash the cells with PBSA: resuspend the cells in 1 mL PBSA and repellet the library. Resuspend the cells in 1 mL PBSA.
3. Set up 8–12 tubes each with 50 μL cells and a constant concentration of biotinylated antigen in PBSA. Ensure that each tube contains a molar excess of biotinylated antigen with respect to the number of surface expressed scFv molecules.
   Note: The concentration of biotinylated antigen should be chosen to be above the K_D value to provide sufficient saturation signal but low enough such that the

observed association rate, $k_{obs} = k_{on}[Ag] + k_{off}$, is not so fast that the signal saturates before several samples can be collected.
4. Incubate tubes at a constant temperature of interest.
5. At various times, remove an aliquot, wash with cold PBSA and incubate pellet at 4°C until all tubes have been pelleted.
6. Resuspend yeast in 100 μL cold PBSA with 1:100 dilution (10 mg/L) streptavidin-PE. Incubate at 4°C for 10–15 m.
7. Centrifuge tubes, discard supernatant, and wash the cells with cold PBSA.
8. Resuspend the library in cold PBSA and analyze on a flow cytometer.
9. Fit the total mean PE fluorescence (MFU_{tot}) versus time by least squares regression to

$$MFU_{tot} = MFU_{\min} + MFU_{range}(1 - e^{-k_{obs}t}) \tag{3.2}$$

varying k_{obs}, MFU_{min}, and MFU_{range}.

15.3.7.5 Dissociation Rate (k_{off}) Measurement

1. Pellet 1×10^7 induced clonal yeast by centrifugation at 12,000 *g* for 30 s or 2,500 *g* for 2 m. Discard supernatant.
2. Wash the cells with PBSA: resuspend the cells in 1 mL PBSA, and repellet the library.
3. Resuspend the cells in 1 mL PBSA with a saturating concentration of biotinylated antigen. Incubate for a sufficient time to approach equilibrium at the temperature of interest. Refer to note on approaching equilibrium in Sect. 15.3.6.
4. At various times, remove a 100 μL aliquot, wash cells with cold PBSA, and resuspend in PBSA with excess of unbiotinylated antigen or soluble scFv as competitor. Incubate at the temperature of interest.
5. Centrifuge all samples. Wash with 1 mL cold PBSA.
6. Resuspend yeast in 100 μL cold PBSA with 1:100 dilution (10 mg/L) streptavidin-PE. Incubate at 4°C for 10–15 m.
7. Centrifuge tubes, discard supernatant, and wash the cells with cold PBSA.
8. Resuspend the library in cold PBSA and analyze on a flow cytometer.
9. Fit the total mean PE fluorescence (MFU_{tot}) versus time by least squares regression to

$$MFU_{tot} = MFU_{\min} + MFU_{range}e^{-k_{off}t} \tag{3.3}$$

varying k_{obs}, MFU_{min}, and MFU_{range}.
Note: To determine the dissociation rate for nonbiotinylated antigen, the protocol can be followed as described above, except initially saturate with nonbiotinylated antigen and use biotinylated antigen as the competitor. The data will follow the expression

$$MFU_{tot} = MFU_{\min} + MFU_{range}\left(1 - e^{-k_{off}t}\right) \tag{3.4}$$

15.3.7.6 Protein Stability Analysis

The stability of a protein can be analyzed by thermal denaturation of the protein expressed on the surface of yeast. The midpoint of thermal denaturation measured using this assay correlates very well with that obtained by differential scanning calorimetry and circular dichroism of purified soluble protein (Hackel et al. 2008).

1. Pellet 1×10^7 clonal yeast by centrifugation at 12,000 *g* for 30 s or 2,500 *g* for 2 m. Discard supernatant.
2. Wash the cells with PBSA: resuspend the cells in 1 mL PBSA and repellet.
3. Resuspend the cells in 1 mL PBSA and set up ten 100 μL aliquots.
4. Incubate each aliquot at a different temperature for 30 m. The gradient function on a thermal cycler works well.
5. Incubate the aliquots for 15 m at 4°C.
6. Label the cells for quantification of antigen binding: incubate each aliquot with a saturating concentration of biotinylated antigen for 30 m at 4°C.
7. Pellet the library. Wash the cells with cold PBSA.
8. Resuspend the cells in cold PBSA and 1:100 dilution or ~10 mg/L streptavidin-PE. Incubate for 10–15 m at 4°C.
9. Pellet the library. Wash the cells with cold PBSA.
10. Resuspend the library in cold PBSA for flow cytometry analysis.

15.3.8 Soluble Protein Expression

Engineered scFv proteins can be easily secreted from yeast for further *in vitro* or *in vivo* characterization. The protocol below describes a method for digestion and ligation of an scFv sequence into the prs314-GAL yeast secretion vector (Fig. 5.5) with an *N*-terminal FLAG tag for purification and characterization. The scFv also contains a *C*-terminal c-myc tag. The resulting plasmid is transformed into YVH10, a yeast strain that over expresses the yeast protein disulfide isomerase (PDI). Basic molecular biology techniques can be used to remove either or both epitope tags.

1. Digest clonal miniprep DNA (pCTCON2 plasmid with scFv of interest) with *Nhe*1 and *Xho*1 following the manufacturer's protocol. Also digest prs314-GAL with *Nhe*1 and *Xho*1.
2. Gel purify the insert (~700–800 bp) and the vector (~6,000 bp) on a 1% agarose gel using the QIAquick gel purification kit following the manufacturer's protocol.
3. Ligate together (the New England BioLabs Quick Ligation kit is suggested, following the manufacturer's instructions) and transform into *E. Coli* with selection on LB Amp plates.
4. Grow colonies in LB Amp media and purify plasmid DNA by miniprep. Sequence to confirm presence of insert. An effective reverse sequencing primer for prs314-GAL is 5′-GATTTTGTTACATCTACACTGTTG-3′.
5. Transform plasmid DNA into YVH10 yeast using the Zymo Research Frozen-EZ Yeast Transformation II kit. Select on SD-CAA plates supplemented with 40 μg/L uracil.

6. Grow single colonies in SD-CAA media supplemented with 40 μg/L uracil at 30°C 250 rpm. When the culture has reached a concentration of about 5×10^7 cells/mL, centrifuge culture and resuspend in SG-CAA supplemented with 40 μg/L uracil to a final concentration of 5×10^7 cells/mL.
   Note: some scFvs may secrete better in YPG pH 6.0 media.
7. Incubate at 20–37°C 250 rpm for 2–3 days.
   Note: The optimal temperature for scFv expression varies. To determine the optimal temperature for a particular clone, set up several secretions at various temperatures and analyze the resulting yields.
8. Centrifuge culture and collect supernatant. Filter sterilize the supernatant. Confirm presence of scFv in supernatant by Western blot. After blocking step, incubate nitrocellulose membrane with a 1:500 dilution of anti-FLAG M2-Peroxidase 1 h at room temperature.
9. Purify with FLAG resin following the manufacturer's protocol. Ensure sample has proper pH and ionic strength before applying to the column. After purification, buffer exchange eluted protein into 1× PBS. Determine concentration by absorbance at 280 nm.
10. Confirm size and purity of purified scFv (MW ~ 25 kDa) by gel electrophoresis with Coumassie staining. Note that dimers may be present.
    Note: Typical scFv yields are 1–10 mg/L.

15.4 Results

Magnetic bead selections used in combination with c-myc positive FACS to select for full-length clones should result in the isolation of naïve binders to a target antigen after two to three magnetic bead selections with one intermediate c-myc positive selection. However, binding may not yet be visible by flow cytometry if the naïve binders possess affinities that are micromolar or weaker. Mutagenesis of the library followed by additional bead selections with increasing stringency can be performed until stronger binders have evolved. The total number of rounds of selection and mutagenesis will depend on the target and the desired affinity.

Antigen homogeneity and purity is essential for productive selections. Poor antigen quality can result in the enrichment of secondary binders or the isolation of binders to biotin or a misfolded epitope.

Engineered scFv clones can be characterized directly on the surface of yeast. In addition, scFv genes can be easily ligated into a yeast secretion vector for expression of soluble protein for further *in vitro* and *in vivo* characterization.

15.5 Troubleshooting

1. Problem: Very high optical density readings and/or cloudy supernatant after centrifugation

   Possible reason: bacterial contamination

Solution: Make sure to use sterile procedure and ensure all media and buffers are sterile. Yeast cultures can be treated with 50 mg/L tetracycline or 100 mg/L ampicillin to eliminate bacterial contamination.

2. Problem: Selections result in enrichment of secondary binders
   Possible reason(s): Heterogeneous antigen population, antigen not biotinylated and/or excess biotin in antigen stock
   Solution: Ensure antigen is pure and homogenous. Ensure complete saturation of magnetic beads with antigen. Ensure antigen is biotinylated with 1–2 biotins/protein – the Pierce Biotin Quantitation Kit, 28005 is recommended for biotin quantification.

3. Problem: Low yeast transformation efficiency
   Possible reason(s): Cell temperature too high and/or impurities
   Solution: Maintain cells at 4°C after DTT treatment. Avoid touching metal electrodes of electroporation cuvettes. Ensure PelletPaint protocol is followed properly; ethanol washes are critical to reduce salt content. Ensure buffer E washes of the yeast are performed thoroughly.

4. Problem: Data points at lower concentrations lie below the fit K_D curve
   Possible reason: Incubation time is not sufficient for antigen-binding to approach equilibrium
   Solution: Label cells for a longer period of time (refer to note on approaching equilibrium in Sect. 15.3.6).

5. Problem: Cultures grow to a very high OD_{600} (>12) and/or a loss of c-myc or HA expression is observed
   Possible reason: Yeast contamination (such as *C. Parapsilosis*)
   Solution(s): Yeast contamination can be confirmed with Biomerieux Vitek API 20 C AUX Test (Fisher, B20210RRP) (Colemann and Baird 2006). Make sure to use sterile procedure and ensure all media and buffers are sterile. Avoid prolonged growth. Freeze aliquots of each library for long-term storage. A previous uncontaminated library can be thawed and re-sorted. A c-myc positive selection can be performed followed immediately by Zymoprep to isolate DNA. Transfect DNA into a fresh culture of EBY100.

15.6 Conclusions

Yeast surface display used in combination with directed evolution is a robust platform for protein engineering, allowing the discrimination of subtle phenotype differences using FACS, and the characterization of protein kinetics and thermal stability measured directly on the cell surface. Yeast surface display also confers a eukaryotic expression bias, resulting in posttranslational assembly such as that mediated by foldases and chaperones, and posttranslational modification such as glycosylation. A large variety of proteins have been successfully engineered using

yeast surface display. In addition to protein engineering, more recently applications of this platform include enzyme engineering (Antipov et al. 2008), epitope mapping (Chao et al. 2006), and cell panning for the discovery of novel surface receptors (Wang et al. 2007).

Because yeast glycosylation is of the high mannose type and immunogenic in humans and prokaryotic hosts lack glycosylation machinery, current antibody and other glycoprotein therapeutics are produced from mammalian cells. There is a growing appreciation for the effect of protein glycosylation, influencing pharmacokinetics, solubility, immunogenicity, and therapeutic efficacy (Raju 2008; Sola et al. 2007; Sinclair and Elliott 2005). Many glycoproteins are expressed as heterogeneous mixtures from mammalian cells, resulting in protein therapeutics with heterogeneous properties.

Two recent promising developments underscore the potential for the future production of homogenous active full-length antibodies and other proteins requiring posttranslational processing in yeast. An engineered yeast strain with a humanized glycosylation pathway has been developed and is capable of producing homogenous glycoproteins with human N-linked glycosylation (Hamilton et al. 2003). In addition, it has recently been demonstrated that N-linked glycosylation of the IgG Fc domain is not necessary for Fcγ receptor activation, as aglycosylated antibodies have been engineered to retain Fcγ binding (Sazinsky et al. 2008). Production of antibodies in yeast would eliminate the current requisite mammalian reformatting step, result in shorter fermentation times, and likely effect higher yields. Already a widely used platform for protein engineering, yeast surface display may yet serve as a faster and more streamlined approach to the development of protein therapeutics from target identification and lead isolation followed by affinity and stability engineering for final production in yeast.

References

Ackerman ME, Levary D, Tobon G, Hackel BJ, Orcutt KD, Wittrup KD (2009) Highly avid magnetic bead capture; an efficient selection method for de novo protein engineering utilizing yeast surface display. Biotechnol Prog 25:774–783

Antipov E, Cho AE, Wittrup KD, Klibanov AM (2008) Highly L and D enantioselective variants of horseradish peroxidase discovered by an ultrahigh-throughput selection method. Proc Natl Acad Sci USA 105:17694–17699

Boder ET, Midelfort KS, Wittrup KD (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. Proc Natl Acad Sci USA 97:10701–10705

Boder ET, Wittrup KD (1997) Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol 15:553–557

Boder ET, Wittrup KD (1998) Optimal screening of surface-displayed polypeptide libraries. Biotechnol Prog 14:55–62

Boder ET, Wittrup KD (2000) Yeast surface display for directed evolution of protein expression, affinity, and stability. Methods Enzymol 328:430–444

Bowley DR, Labrijn AF, Zwick MB, Burton DR (2007) Antigen selection from an HIV-1 immune antibody library displayed on yeast yields many novel antibodies compared to selection from the same library displayed on phage. Protein Eng Des Sel 20:81–90

Brezinsky SC, Chiang GG, Szilvasi A, Mohan S, Shapiro RI, MacLean A, Sisk W, Thill G (2003) A simple method for enriching populations of transfected CHO cells for cells of higher specific productivity. J Immunol Methods 277:141–155

Chao G, Lau WL, Hackel BJ, Sazinsky SL, Lippow SM, Wittrup KD (2006) Isolating and engineering human antibodies using yeast surface display. Nat Protoc 1:755–768

Colby DW, Kellogg BA, Graff CP, Yeung YA, Swers JS, Wittrup KD (2004) Engineering antibody affinity by yeast surface display. Methods Enzymol 388:348–358

Colemann JR, Baird CL (2006) Indicators of candida contamination in the scFv yeast display library and method for its control. Pacific Northwest National Laboratory. http://www.sysbio.org/dataresources/candidascFvLibrary060207.pdf. Cited 14 Jan 2009

Fandl J, Stahl N, Chen G, Yancopoulos G (2008) Isolating Cells Expressing Secreted Proteins. US Patent 7(435):553

Feldhaus M, Siegel R (2004) Flow cytometric screening of yeast surface display libraries. Methods Mol Biol 263:311–332

Feldhaus MJ, Siegel RW, Opresko LK, Coleman JR, Feldhaus JM, Yeung YA, Cochran JR, Heinzelman P, Colby D, Swers J, Graff C, Wiley HS, Wittrup KD (2003) Flow-cytometric isolation of human antibodies from a nonimmune Saccharomyces cerevisiae surface display library. Nat Biotechnol 21:163–170

Frykman S, Srienc F (1998) Quantitating secretion rates of individual cells: design of secretion assays. Biotechnol Bioeng 59:214–226

Gai SA, Wittrup KD (2007) Yeast surface display for protein engineering and characterization. Curr Opin Struct Biol 17:467–473

Graff CP, Chester K, Begent R, Wittrup KD (2004) Directed evolution of an anti-carcinoembryonic antigen scFv with a 4-day monovalent dissociation half-time at 37 degrees C. Protein Eng Des Sel 17:293–304

Hackel BJ, Kapila A, Wittrup KD (2008) Picomolar affinity fibronectin domains engineered utilizing loop length diversity, recursive mutagenesis, and loop shuffling. J Mol Biol 381:1238–1252

Hamilton SR, Bobrowicz P, Bobrowicz B, Davidson RC, Li H, Mitchell T, Nett JH, Rausch S, Stadheim TA, Wischnewski H, Wildt S, Gerngross TU (2003) Production of complex human glycoproteins in yeast. Science 301:1244–1246

Kieke MC, Cho BK, Boder ET, Kranz DM, Wittrup KD (1997) Isolation of anti-T cell receptor scFv mutants by yeast surface display. Protein Eng 10:1303–1310

Lipovsek D, Lippow SM, Hackel BJ, Gregson MW, Cheng P, Kapila A, Wittrup KD (2007) Evolution of an interloop disulfide bond in high-affinity antibody mimics based on fibronectin type III domain and selected by yeast surface display: molecular convergence with single-domain camelid and shark antibodies. J Mol Biol 368:1024–1041

Manz R, Assenmacher M, Pfluger E, Miltenyi S, Radbruch A (1995) Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix. Proc Natl Acad Sci USA 92:1921–1925

Matsumoto T, Fukuda H, Ueda M, Tanaka A, Kondo A (2002) Construction of yeast strains with high cell surface lipase activity by using novel display systems based on the Flo1p flocculation functional domain. Appl Environ Microbiol 68:4517–4522

Pepper LR, Cho YK, Boder ET, Shusta EV (2008) A decade of yeast surface display technology: where are we now? Comb Chem High Throughput Screen 11:127–134

Raju TS (2008) Terminal sugars of Fc glycans influence antibody effector functions of IgGs. Curr Opin Immunol 20:471–478

Rakestraw JA, Baskaran AR, Wittrup KD (2006) A flow cytometric assay for screening improved heterologous protein secretion in yeast. Biotechnol Prog 22:1200–1208

Razai A, Garcia-Rodriguez C, Lou J, Geren IN, Forsyth CM, Robles Y, Tsai R, Smith TJ, Smith LA, Siegel RW, Feldhaus M, Marks JD (2005) Molecular evolution of antibody affinity for sensitive detection of botulinum neurotoxin type A. J Mol Biol 351:158–169

Sazinsky SL, Ott RG, Silver NW, Tidor B, Ravetch JV, Wittrup KD (2008) Aglycosylated immunoglobulin G1 variants productively engage activating Fc receptors. Proc Natl Acad Sci USA 105:20167–20172

Shusta EV, Kieke MC, Parke E, Kranz DM, Wittrup KD (1999) Yeast polypeptide fusion surface display levels predict thermal stability and soluble secretion efficiency. J Mol Biol 292:949–956

Sinclair AM, Elliott S (2005) Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. J Pharm Sci 94:1626–1635

Sola RJ, Rodriguez-Martinez JA, Griebenow K (2007) Modulation of protein biophysical properties by chemical glycosylation: biochemical insights and biomedical implications. Cell Mol Life Sci 64:2133–2152

Swers JS, Kellogg BA, Wittrup KD (2004) Shuffled antibody libraries created by in vivo homologous recombination and yeast surface display. Nucleic Acids Res 32:e36

Szent-Gyorgyi C, Schmidt BF, Creeger Y, Fisher GW, Zakel KL, Adler S, Fitzpatrick JA, Woolford CA, Yan Q, Vasilev KV, Berget PB, Bruchez MP, Jarvik JW, Waggoner A (2008) Fluorogen-activating single-chain antibodies for imaging cell surface proteins. Nat Biotechnol 26:235–240

van den Beucken T, Pieters H, Steukers M, van der Vaart M, Ladner RC, Hoogenboom HR, Hufton SE (2003) Affinity maturation of Fab antibody fragments by fluorescent-activated cell sorting of yeast-displayed libraries. FEBS Lett 546:288–294

Van der Vaart JM, te biesebeke R, Chapman JW, Toschka HY, Klis FM, Verrips CT (1997) Comparison of cell wall proteins of Saccharomyces cerevisiae as anchors for cell surface expression of heterologous proteins. Appl Environ Microbiol 63:615–620

VanAntwerp JJ, Wittrup KD (2000) Fine affinity discrimination by yeast surface display and flow cytometry. Biotechnol Prog 16:31–37

Wang XX, Cho YK, Shusta EV (2007) Mining a yeast library for brain endothelial cell-binding antibodies. Nat Methods 4:143–145

Weaver-Feldhaus JM, Lou J, Coleman JR, Siegel RW, Marks JD, Feldhaus MJ (2004) Yeast mating for combinatorial Fab library generation and surface display. FEBS Lett 564:24–34

Wentz AE, Shusta EV (2007) A novel high-throughput screen reveals yeast genes that increase secretion of heterologous proteins. Appl Environ Microbiol 73:1189–1198

Wong TS, Roccatano D, Zacharias M, Schwaneberg U (2006) A statistical analysis of random mutagenesis methods used for directed protein evolution. J Mol Biol 355:858–871

Yeung YA, Finney AH, Koyrakh IA, Lebowitz MS, Ghanbari HA, Wands JR, Wittrup KD (2007) Isolation and characterization of human antibodies targeting human aspartyl (asparaginyl) beta-hydroxylase. Hum Antibodies 16:163–176

Chapter 16
The Generation of Transgenic Mice Expressing Human Antibody Repertoires

Xiangang Zou, Guocheng Lan, Michael J. Osborn, and Marianne Brüggemann

16.1 Introduction

Antibody production in transgenic mice is a success story with the first human antibody repertoires created some 20 years ago (Brüggemann et al. 1989) and the derivation of many highly effective, fully human monoclonal Igs in clinical use (summarized in Nature Biotech Sept 2005 and Dec 2007).

Initially, the transgenic methodologies used for insertion of antibody genes in the germline of animals involved microinjection of purified DNA into zygotes (Isola and Gordon 1991). Establishing embryonic stem (ES) cell lines from mouse blastocysts, followed by their manipulation in tissue culture, ensured rapid progress of transgene expression in an endogenous KO or silenced mouse background. Parallel approaches to insert large DNA fragments, or Ig loci over 1,000 kb into ES cells, resulted in germline transmission animals (Davies et al. 1993; Zou et al. 1996; Mendez et al. 1997). Many of these approaches are now routinely carried out by companies or transgenic core facilities associated with many research laboratories, which will microinject the DNA of your choice or produce transgenic mice from manipulated ES cells.

Here our aim is to provide detailed methods for the experienced researcher as well as the novice with limited knowledge in molecular biology and tissue culture. Protocols rarely provided in detail include DNA preparation for microinjection and protoplast transformation, which seamlessly insert very large regions (e.g., human Ig YACs) or whole gene loci into ES cells.

X. Zou and G. Lan
Cancer Research UK/Cambridge Research Institute, Robinson Way, Cambridge, CB2 0RE, UK
e-mail: xiangang.zou@cancer.org.uk

M.J. Osborn (✉) and M. Brüggemann
The Babraham Institute, Babraham, Cambridge, CB22 3AT, UK
e-mail: marianne.bruggemann@bbsrc.ac.uk

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_16,

16.2 Microinjection into Zygotes

Speed, efficiency and costs are important factors for producing transgenic mice, and in-house facilities managed by an experienced team are probably the most successful route. Setting up new transgenic technologies is inadvisable as several companies have a proven track record in producing transgenic animals. For example: "http://www.binet.bbsrc.ac.uk," "www.genoway.com," "www.caliperls.com," "www.genetargeting.com" or "www.ozgene.com," offer everything from microinjection to ES cells manipulation and mouse breeding, including testing.

The derivation of transgenic mice involves the microinjection of purified DNA into the pronucleus of fertilized one-cell embryos. The limitations of this method are: random DNA integration within the genome and that the DNA concentration injected determines the copy number, which frequently results in multiple integration.

16.2.1 DNA Preparation

Although DNA preparation, including construct making and purification, can be included in the transgenic service, many laboratories prefer to prepare the DNA constructs themselves. This may be linked to a novel project, in vitro expression studies of available genes or envisaged patent protection at a later stage. For DNA transfection in ES cells, gene targeting constructs should contain a 5′ and 3′ homology region of ideally several kb in size. For embryo transfer and integration in the mouse genome, small transgenic constructs are much easier to inject and have a high success rate, while large constructs may fragment and a region of less then 100 kb is advisable. It is also possible to co-inject 2 or more regions, which can integrate in tandem (Brüggemann et al. 1991) or even recombine as shown for the reconstitution of a human heavy chain locus of 180 kb from three overlapping P1 clones (Wagner et al. 1996).

For DNA preparation and fragment purification, extensive information can be found on various webpages. For example: "http://www1.qiagen.com/literature/Default.aspx?Term=gel+extraction &Language=EN&LiteratureType=4%3b8%3b9%3b10&ProductCategory=0" and "http://www.promega.com/search/default.aspx?query=plasmid%20 purification."

Protocol 1: Purification of DNA for pronuclear injection
Equipment and reagents (kits)

- For DNA preparation and fragment purification from agarose gels, a variety of kits are available. Such as, GENECLEAN Kit from MP Biomedicals (cat. no. 1001-200, up to 20 kb); or Qiaex II Gel Extraction Kit from Qiagen (cat. no. 20021, up to 50 kb) and Elutip-D kit from Whatman (cat. no. 10462615) or others
- Restriction enzymes, for example, from Fermentas or NewEngland Biolabs, which include 10× buffer

- Double distilled and autoclaved H_2O
- Ultra pure agarose (or low melting)
- DNA size marker and loading buffer (Invitrogen; Sambrook and Russell 2001)
- Ethidium bromide or SYBR Green or GelRed (Cambridge Bioscience, UK)
- TAE buffer: 40 mM Tris-acetate, 1 mM EDTA
- TE buffer: 10 mM (high grade) Tris–HCl pH 7.4, 1 mM EDTA
- 10 mM high grade Tris-EDTA pH 7.5
- NaAc: Sodium acetate (3 M)
- Ethanol (100 and 70%)
- Large and small gel boxes; power pack; transilluminator (UV light); centrifuge; Gilson pipette; autoclaved tips, tubes and forceps; new razor blades
- DNA injection buffer: 10 mM Tris–HCl pH7.4, 10 mM NaCl, 0.25 mM EDTA
- 0.22 μm sterile filter

Method

1. Prepare plasmid DNA with Qiagen plasmid midi kit
2. Digest with restriction enzyme(s) to release DNA fragment:

DNA (~50 μg)	50 μl
Enzyme 1	10 μl
Enzyme 2	10 μl
Buffer	20 μl
BSA (1 mg/ml, optional)	20 μl
Add H_2O to a total volume of	200 μl

   For difficult digests and/or to avoid incomplete fragmentation, incubate overnight and add another 0.5 μl of enzyme(s) for a further 1–2 h digestion in the morning. Check that the digest is complete and results in the expected size fragment by running a small aliquot on a minigel.
3. Run a 0.8% agarose gel in TAE and cut out the DNA fragment. (Identify the required band by avoiding ethidium bromide as much as possible, or other DNA staining dyes and UV illumination. The removal of vector sequences is advisable)
4. Release the DNA fragment with GENECLEAN (MP Biomedicals, cat. no. 1001-200, up to 20 kb) or Qiaex II Gel Extraction Kit (Qiagen, cat. no. 20021, up to 50 kb) and elute with 200 μl TE
5. Precipitate the DNA by adding one drop of 3 M NaAc, and then 420 μl 100% ice-cold ethanol and store at −20°C for at least 30 min
6. Spin at 13,000 rpm, 4°C for 10 min, wash the DNA with 70% ethanol, remove the liquid and briefly air dry (avoid over-drying)
7. Clean the DNA with the Elutip-d^R purification columns (Whatman, cat. no. 10462615)
8. Precipitate the eluted DNA solution again and dissolve in DNA injection buffer, which has been passed through a 0.22 μm filter
9. Run 0.8% agarose gel with molecular weight standards to quantify the DNA concentration
10. Freshly dilute DNA into a final concentration of 1–3 ng/μl in an injection buffer ready for microinjection

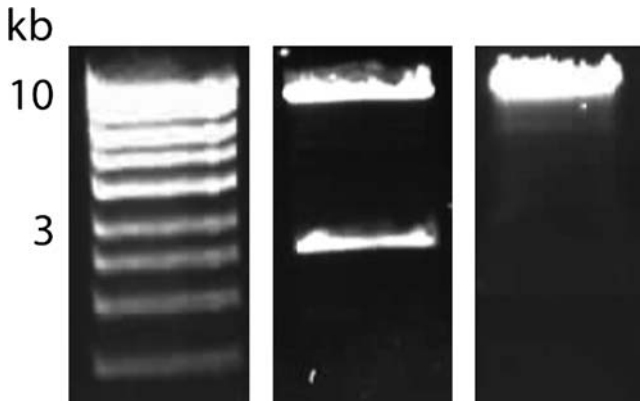


Fig. 16.1 Digest and purification of DNA

Tips for preparation of large DNA:

Dialysis of the purified DNA against microinjection buffer, which makes precipitation unnecessary, uses "V" series membranes VMWP02500 (0.05 μm pore size) from Millipore ("http://www.millipore.com/catalogue/item/vmwp02500"). For this, the filter is placed on the desired buffer (up to 50 ml) in a petri dish and 100–150 μl of purified DNA are added (Marusyk and Sergeant 1980). Dialysis time is ~30 min, after which the DNA sample can be stored or used for microinjection (Fig. 16.1).

16.2.2 Pronuclear injection

Immediately after fertilisation, zygotes are used for DNA microinjection at the one-cell stage. This allows a precise positioning of the DNA into one of the two pronuclei. After injection, the eggs are incubated at 37°C for up to 24 h and implanted into the oviducts of pseudo-pregnant females.

Protocol 2: DNA pronuclear injection
Equipment and reagents (kits)

- Mice: DNA is most commonly injected into C57/Bl6xCBA F2 embryos as egg recovery is easy. However, if required, DNA can also be injected into other genetic backgrounds, such as FVB/N, CD1 or C57/Bl6 mice
- Nikon inverted microscope with DIC (differential interference contrast) or Hoffman modulation setup
- Microinjection system such as Leica, Narishige or Eppendorf manipulators
- FemtoJet (http://www.eppendorf.ca/int/img/na/lit/pdf/8301-C121B-04.pdf) with the following setting:

Compensation pressure (Pc):	5–15 hPa
Injection Pressure (Pi):	80–120 hPa
Injection Time (Ti):	0.5–1 s.

- Injection pipette and embryo-holding pipette
- DNA injection buffer (as above)
- Pregnant mare's serum gonadotropin (PMSG) and Human chorionic gonadotropin (HCG)
- M2 and M16 embryo culture medium (Sigma-Aldrich, cat. no. MFCD00283761 and M7292)
- Hyaluronidase (Sigma-Aldrich, cat. no. 37326-33-3)
- Incubator

Method

1. Several 3–4 weeks old C57/Bl6xCBA F1 females are superovulated by intraperitoneal injection with 5–7.5 IU of PMSG, followed 48 h later by a similar dose of HCG. The females are then mated with C57/Bl6XCBA F1 stud males. (The use of animals in experimental procedures is strictly regulated and government permission is required. In the UK, this necessitates approval by the Home Office, documented in project and personal licences.)
2. One-cell embryos are collected at 0.5 day after plugging (indicated by vaginal discharge) and the surrounding cumulus cells are removed by culturing in M2 medium containing ~0.3 mg/ml hyaluronidase and pipetting.
3. Embryos are washed with M16 medium and cultured in an incubator at 37°C with 5% CO_2 until microinjection.
4. The FemtoJet microinjector is programmed.
5. A Nikon microscope with 40× objective in DIC module is set.
6. 20–30 embryos are transferred into a drop of M2 medium on the dipping of a glass slide and covered with mineral oil.
7. Use holding pipette to position an embryo such as to obtain a clear view of one of the two pronuclei (usually the larger male pronucleus).
8. Move injection pipette into the pronucleus and push the foot pad of the FemtoJet once to inject. You should see an expansion of the pronucleus indicating a successful injection. If no expansion is seen, repeat once.
9. Release the injected embryo at a separate collection point and continue with the other embryos.
10. After all the embryos (groups of 20–30) are injected, they should be washed three times with M16 medium and cultured in M16 overnight.
11. Next day, the oviduct transfer should be carried out. For this, about 20 embryos are transferred into both sides of the oviduct of one mouse (this is usually done by a skilled research assistant).
12. The new pups will be born after 18–19 days and can be weaned and tagged when they are ~21 days old. Tissue samples (a minute sample of the tip of the tail or ear) are sent to the scientist for analysis.
13. Normally 200–250 embryos are injected and transferred into recipients. This should result in 30-40 mice being born with the expectation of 3–5 transgenic founders (Fig. 16.2).

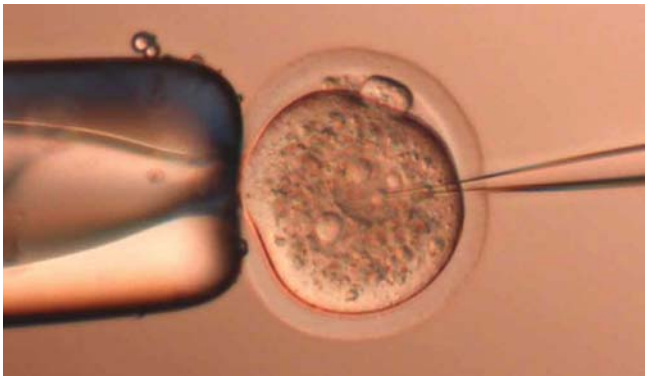


Fig. 16.2 Pronuclear injection

16.3 Embryonic Stem Cells

A sizable number of embryonic stem cells have been described and germline transmission, even after multiple gene targeting approaches, have been obtained regularly (Ren et al. 2004). However, preference should be given to the more recently derived lines that produce a high level of chimerism and successful transmission.

ES cell line[a]	Germline transmission	Reference
AB1	Yes	McMahon and Bradley (1990)
BRUCE4	Yes	Köntgen et al. (1993)
CCE	Difficult	Robertson et al. (1986)
D3	Difficult	Gossler et al. (1986)
E14	Yes	Handyside et al. (1989)
HM-1	Yes	Selfridge et al. (1992)
HPRT	Difficult	Hooper et al. (1987)
PC3	Yes	O'Gorman et al. (1997)
TMDB10	Yes	(Babraham Institute)
ZX3	Yes	Ren et al. (2004)

[a]This is an incomplete list and contains only the lines that have been used and tested in our laboratories and institutes

16.3.1 Culture Conditions

Maintaining ES cell lines, and also their derivation, needs a dedicated researcher with some experience in tissues culture and familiarity with aseptic techniques, easily obtained by culturing myeloma cells. An (ideally) inverted microscope with 4×, 10× and 20× objectives should be routinely available (Fig. 16.3).

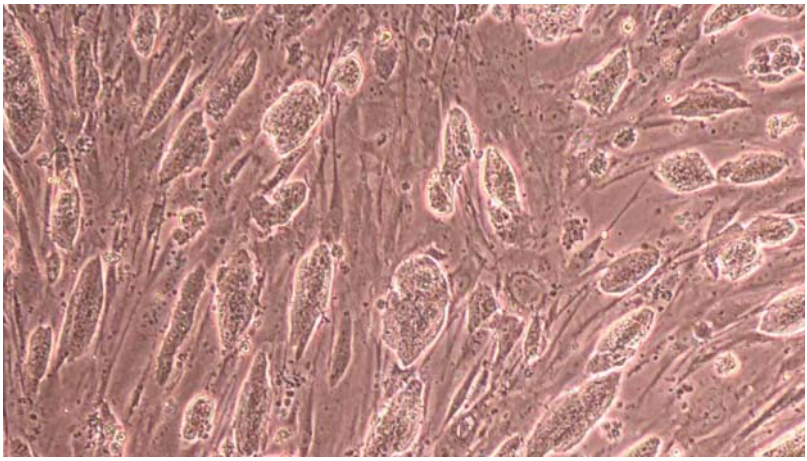


Fig. 16.3 Embryonic stem cells at 100× magnification

Protocol 3: Feeder cell preparation
Equipment and reagents

- Inverted microscope
- Class II safety cabinet
- 37°C CO_2 cell culture incubator
- Water bath
- Freezer (−80°C) and liquid nitrogen (N_2) storage
- Sterile bottle filter
- Plastic pipettes and pipette-aid, 10 or 14 cm cell culture dishes, 6, 24 and 96-well plates, 2.0 ml vials, 15 and 50 ml (Falcon) tubes
- Fetal calf serum (FCS)
- PBS, without CaCl and MgCl (Sigma-Aldrich cat. no. D1408)
- Sodium pyruvate (200 mM, =100×)
- L-Glutamine (200 mM, =100×)
- Penicillin/streptomycin (pen/strep) solution (Sigma-Aldrich, cat. no. P 4458, =100×)
- DMEM medium (Sigma-Aldrich, cat. no. D 6655)
- DMSO (Dimethyl sulfoxide)
- Mitomycin-C or γ-ray irradiator
- Trypsin-EDTA solution (Sigma T 3924)

Feeder tissue culture (T/C) medium

- T/C medium: DMEM containing 10% FCS, sodium pyruvate, L-Glutamine and pen/strep
- 0.1% Gelatin solution. (Weigh 500 mg gelatin into a glass bottle with 500 ml ddH_2O and autoclave)

- Mitomycin-C solution: 2 mg Mitomycin-C dissolved in 200 ml T/C medium and sterile filtered
- 2× freezing medium: DMEM with 20% FCS and 20% DMSO

Method

SNL cells (an immortalized cell line) or DR4 mouse fetal fibroblast cells are commonly used as feeder cells in ES cell culture (Soriano et al. 1991; Tucker et al. 1997).

1. Thaw one vial of frozen (N_2) SNL or DR4 cells by briefly placing it into a 37°C waterbath
2. Transfer cells with 5 ml of T/C medium into 15 ml tube and spin down
3. Pour or pipette cells into one 10 cm cell culture dish with ~10 ml T/C medium
4. When ~90% confluent, passage the cells onto three 14 cm cell culture dishes with ~20 ml T/C medium each
5. After 3 days in culture (or when ~90% confluent), passage the cells into thirty 14 cm cell culture dishes

6a. Mitomycin-c treatment:

When the cells are ~90% confluent, add 15 ml of Mitomycin-C medium to each dish
Culture for 2–3 h at 37°C
Wash three times with PBS, add 3 ml of 0.05% Trypsin-EDTA to the collected cells for 5 min, then add T/C medium

6b. Or γ-irradiation:

When the cells are ~90% confluent, wash twice with PBS, trypsin-treat as above and place the cells into 50 ml tubes. Expose the cells to γ-irradiation of 3,000–4,000 rad

7. Count the cells and aliquot (2.5×10^6 ml^{-1}) and store in freezing medium (1 ml/vial) at −80°C (up to 3 months) and in liquid N_2 for longer
8. To coat feeder cell plates, add 0.1% gelatin, 2 ml for a 6-well plate and 8 ml for a 10 cm dish, leave for at least 30 min, remove the solution and dry in the class II cabinet
9. To prepare feeder cell plates, thaw the mitomycin-C treated or irradiated cells in a 37°C water bath, transfer into a 15 ml conical tube with 5 ml T/C medium. Spin down, resuspend in 12 ml T/C medium and transfer equally into one 6-well plate or into one 10 cm dish. Culture for more than 2 days to allow the feeder cells to form a layer of support on the bottom of the culture plate

Protocol 4: ES cell maintenance

Equipment and reagents (in addition to above)

- Centrifuge (such as Heraeus, Megafuge 1.0R)
- Fetal Calf serum (ES cell tested)
- Knockout DMEM or Knockout DMEM-F12

- 2(β)-mercaptoethanol (2-ME)
- mLIF: ESGRO (Lif) (Millipore, ESG1107)

Embryonic stem cell culture (ES) Medium

- Knockout DMEM plus 15% FCS (ES cell tested) with the following reagents to be added (see Protocol 3, sterile filtered if needed): 2-ME (to 0.1 mM), sodium pyruvate (to 2 mM), L-Glutamine (to 2 mM), pen/strep (1×) and ESGRO(Lif) at 1,000 units/ml
- 2× freezing medium: ES medium containing 20% FCS and 20% DMSO

Method

There are many ES cell lines available commercially or from research institutions. Suitable FCS is one of most important factors for ES cell maintenance, and each ES cell line requires testing to establish that its totipotency or germ cell potential is retained.

1. Thaw one vial of ES cells, from liquid N_2, in a 37°C water bath
2. Transfer into 15 ml conical tube with 5 ml warm ES medium
3. Centrifuge at 1,200 rpm (~300 g) for 5 min and remove the supernatant
4. Add 3 ml of ES medium, resuspend the ES cells by gentle pipetting and transfer all cells into one well of a 6-well plate with feeder cells
5. Replace the medium with 3 ml of fresh ES cell medium every day
6. At ~80% confluency, wash twice with 2 ml PBS (without $CaCl_2$ and $MgCl_2$), add 0.5 ml of 0.05% Trypsin/EDTA into each well and incubate for 5 min
7. Add 2 ml of ES medium to stop Trypsin action
8. Transfer cells into 6 wells of a 6-well plate (at 1:6 dilution)
9. Change ES medium every day as above
10. At ~80% confluency, passage ES cells equally into 6 wells of a 6-well plate (at 1:6 dilution)
11. Collect all ES cells, count, aliquot ($2.5 \times 10^6 ml^{-1}$) and store (1 ml/vial) at −80°C or subsequently in liquid N_2

16.3.2 Transfection and Selection

For establishing ES cell transfectants, a selectable marker gene is added to the integration or targeting construct. Marker genes most commonly used confer resistance to neomycin or G418, hygromycin or puromycin (Tucker et al. 1997). The concentration of the selective drug has to be established precisely as publications only provide a rough guidance and also because of quality variations from batch to batch. For G418 selection, the concentration that results in apoptosis of ES cells without the neomycin resistance gene after 3 days in culture is ~200 μg/ml. However, sometimes this may be lower, 180 μg/ml, or up to 220 μg/ml, and should be titrated out precisely, ideally, with positive and negative control cells.

The use of ES cells for DNA integration has the advantage that single-copy constructs can be transferred and semi-randomly integrated into a mouse chromosome. This allows gene targeting in up to a few percent of insertions. In some experiments, the removal of the selection gene is advisable and this can be done by, for example, flanking with *lox*P sequences and Cre transfection or breeding with Cre expressors (Zou et al. 2003). For the integration of large gene loci, several 100 kb in size, DNA transfer via spheroplasts has been established using yeast artificial chromosomes (YACs). The construction and alteration of YACs to add a selectable marker has been described and various modification vectors are available, which make use of the high degree of homologous integration in yeast (Davies et al. 1996).

16.3.2.1 DNA Electroporation

DNA transfer into cells by electroporation is highly efficient, with the critical parameter being the purity and concentration of the DNA, and the exponential growth rate and number of cells.

Protocol 5: ES cell electroporation and selection
Equipment and reagents (in addition to above)

- Bio-Rad Gene Pulser Xcell
- Cuvettes (0.4 cm, Bio-rad)
- Selection drugs in ES cell medium:
  - Neomycin or G418 selection at 180–220 μg/ml
  - Puromycin selection at 1–2 μg/ml
  - Hygromycin B selection at 150–200 μg/ml

Method

1. ES cells cultured on one 6-well plate at 80% confluency
2. Treat the cells with trypsin/EDTA (as above) and count
3. Pellet cells at 1,200 rpm (~300 g) for 5 min and resuspend at a concentration of 1×10^7 cells/ml in ES medium (15% FCS)
4. Transfer 0.8 ml cell suspension into one 0.4 cm cuvette
5. Add 20–30 μl DNA solution (15–20 μg) into ES cell suspension and mix gently
6. Leave at room temperature (RT) for 10 min
7. Electroporation on Gene Pulser Xcell (eukaryotic system) at the following conditions:

   Type: Exponential Protocol
   Voltage: 230 V
   Capacitor: 500 μF
   Resistor (Pulse Controller) Maximum (Ω none)
   Number of Pulses: 1

8. Incubate the cells in the cuvette at 37°C for 30 min
9. Transfer cells into a 50 ml tube and dilute with 30 ml ES medium
10. Aliquot equally into five 6-well plates with feeder cells. Add another 1 ml of ES medium into each well
11. Next day; remove all medium and add 3 ml selection medium per well
12. Replenish selection medium every day for 8–10 days, when clones should appear

16.3.2.2 YAC Transfer by Protoplast Fusion

The relatively thick wall of a yeast cell surrounding the membrane must be removed to allow cell fusion to take place. This is done by lyticase or zymolyase treatment. There do not seem to be limitations concerning the ES cell line that can be used for the fusion process, and YAC integration has been achieved with the following lines: D3 (Gossler et al. 1986), E14 (Handyside et al. 1989), CCE (Robertson et al. 1986), AB1 (McMahon and Bradley 1990), HM-1 (Selfridge et al. 1992) and ZX3 (Ren et al. 2004). Our preferred line, HM-1, consistently produced germline transmission (Zou et al. 1996; Nicholson et al. 1999; Popov et al. 1999) and this appeared to be linked to the quality or early passage number of the ES cell line rather then the fusion process (Davies et al. 1993; Jakobovits et al. 1993).

Protocol 6: Spheroplast fusion of YAC-containing yeast with ES cells
Equipment and reagents

- 37°C CO_2 cell culture incubator
- 30°C incubator for yeast cells
- 30°C shaking incubator for yeast cells
- Phase contrast microscope
- Spectrometer
- ST (1 M Sorbitol, 10 mM Tris–HCl pH7.0, sterile filtered)
- Drop-out agar plates and liquid media (Davies et al. 1996; Invitrogen)
- Falcon tubes, 15 and 50 ml
- Double distilled autoclaved water (ddH_2O)
- 1 M Sorbitol, sterile filtered
- SCE (1 M Sorbitol, 0.1 M Sodium Citrate pH 5.8, 10 mM EDTA, sterile filtered)
- β-Mercaptoethanol (βME)
- Yeast lytic enzyme (Lyticase or Zymolyase from Sigma or AMS biotechnology)
- ES-cell culture medium
- 6-well tissue culture plates
- G418 or appropriate selective drug
- DMEM
- Trypsin-EDTA (Sigma)
- PEG 1500 provided as a 50% solution containing 75 mM HEPES
- PEG solution (PEG 1500, 50% w/v with the final concentration of 50 mm β-ME, 5 mM $CaCl_2$, 5% DMSO)

Preparation of yeast spheroplasts

1. Streak the YAC containing yeast on the appropriate drop-out plate and grow in a 30°C incubator until individual colonies are 1–2 mm
2. Inoculate a single colony into 10 ml drop out medium and grow at 30°C in a shaker at about 350 rpm
3. When OD_{600} is 1–2, expand into 100 ml and continue growing until OD_{600} is 1–2
4. Cells are collected by centrifugation for 5 min at 600 g at RT in 50 ml Falcon tubes. Subsequent manipulations are done at RT in the same tubes, unless otherwise stated
5. Resuspend the cells in 20 ml ddH_2O and centrifuge as before
6. Resuspend the cells in 20 ml 1 M Sorbitol and centrifuge again
7. Finally resuspend the cells in 20 ml SCE, count the cells and dilute in SCE to obtain 20 ml cell suspension with 7.5×10^7 cells/ml
8. Add 46 µl βME
9. Add yeast lytic enzyme: 60–80 µl Zymolyase 20T (10 mg/ml) and place tube at 30°C
10. Monitor the extent of spheroplast formation[a]
11. When OD_{600} is reduced to 20% as compared with the initial suspension, centrifuge cells for 5 min at 200 g at RT
12. Decant the supernatant and resuspend the cells in 20 ml ST. Centrifuge again as before, resuspend in ST, count the cells and transfer 2×10^7 cells into a 50 ml tube
13. Centrifuge as before. Carefully aspirate the supernatant so as not to disturb the pellet

[a]Other enzymes such as lyticase can be used. The amount of enzyme used should take spheroplasting to 90% in 20–30 min. The extent of spheroplast formation should be tested after 5, 10, 15 and 20 min. This is done by adding equal amounts of 5% SDS, which bursts spheroplasts and makes them appear like "ghosts" under the phase contrast microscope. A more precise way to monitor spheroplasting is to check for the drop in OD at 800 nm as follows: during spheroplasting, add 100 µl cells to 900 µl of water every 5 min and monitor the drop in OD_{800}. When this value is about 20% of the start OD, the spheroplasts are ready for transformation.

The time of treatment with Zymolyase is not very critical as successful fusions have been obtained even after 40 min incubation, provided spheroplasting reached 90%.

Protoplast fusion with ES cells

1. By the time the protoplasts are ready for the fusion[b], ES cells should have been prepared as for electroporation (see above), then washed twice with serum-free DMEM and resuspended at 2×10^6 cells/ml
2. 1 ml of cells are carefully layered onto the spheroplast pellet and centrifuged at 150 g for 5 min
3. Aspirate the supernatant. Gently stir the pellet and add slowly and drop-wise 1 ml PEG solution

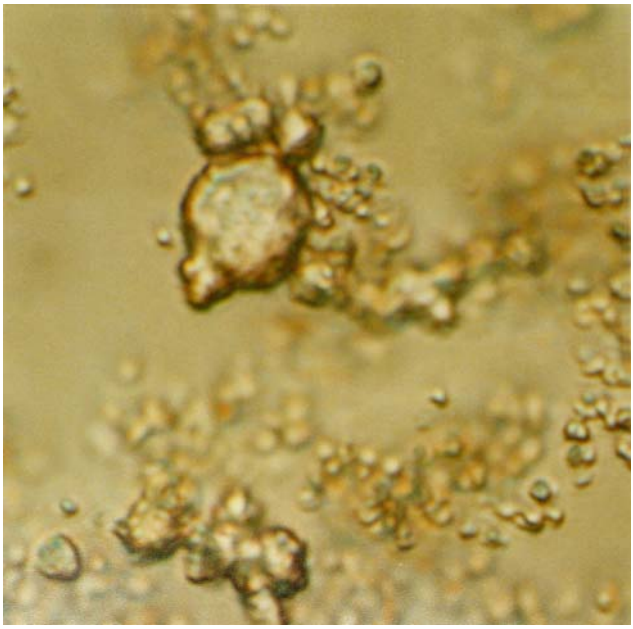


Fig. 16.4 Fusion of yeast spheroplasts with ES cells

4. Incubate at RT while gently stirring for no longer than 2 min
5. Dilute the suspension with prewarmed (37°C) serum-free DMEM. Dilution should be slow and drop-wise down the side of the tube. Approximately 1 ml in 1 min for the first 3 min and then quicker to a final volume of 20 ml
6. Carefully invert once and place the tube in a 37°C incubator for 20–30 min
7. Centrifuge the cells at 130 g for 5 min
8. Resuspend the cells in ES cell culture medium and plate them out in one 6-well tissue culture plate with feeder cells (Fig 16.4)
9. After 24 h, aspirate the supernatant and add selection medium containing ~200 μg/ml G418. Medium is changed every day and 5–50 colonies will be visible in 8–14 days

[b]A negative control such as yeast cells without YAC should be performed in parallel.

16.3.3 Picking Colonies

After electroporation or spheroplast fusion and growth in selective medium, clones are visible after 8–14 days and can be picked and expanded. Selection is continued

for a further few days after picking. It is important to choose clones with growth rates and morphology similar to unmanipulated ES cells, which ensures that the selection of clones with integrated yeast DNA is a rare event (Davies et al. 1993).

Protocol 7: Picking ES cell colonies and cell expansion
Equipment (reagents and medium for ES cells as above)

- Inverted microscope
- Stereomicroscope
- 100 µl (Gilson) pipette
- Sterile (autoclaved) tips

Method

1. ES clones are big enough for picking when they can be seen as small dots with the naked eye, i.e., 12–14 days after transformation. (An inverted microscope is useful for checking the growth and size of the ES cell clones)
2. Change the culture medium for PBS without $CaCl_2$ and $MgCl_2$
3. Set the pipette to 20 µl and use a 100 or 200 µl tip
4. Push the yellow tip to gently detach the ES cell clone, and suck it into the tip with ~20 µl PBS
5. Transfer into one well of a 96-well plate
6. Repeat with new tip until the desired number of colonies is transferred. (For each gene target construct 200–300 ES cell colonies should be picked)
7. Add 30 µl 0.05% trypsin/EDTA to each well and culture at 37°C for 7 min
8. Add 50 µl ES cell medium and disrupt ES cell clumps by pipetting
9. Transfer all ES cell suspension into a new well of another 96-well plate with feeder cells
10. Within 1–2 h, remove trypsin and PBS without disturbing the cells and replace with selective medium
11. Change selection medium daily for the next 3 days, after which half of the cells of each clone are frozen in the 96-well plate and the other half is used for DNA preparation to identify transgene or homologous integration
12. Thaw the desired (e.g., homologous integration) ES cell colonies into one well of a 24-well plate with feeder cells, expand and re-store by freezing
13. Prepare DNA and re-analyse the integration event (e.g., discard clone with yeast strain sequence)

16.3.3.1 Blastocyst Injection

After transformation, by DNA electroporation or protoplast fusion, ES cell colonies with the desired integration are identified. This may be done by PCR or Southern blotting. Colony expansion and re-testing should verify the result. Analysis of YAC integration should also include restriction digests by rare cutters such as NotI and pulsed field gel electrophoresis (Davies et al. 1996). This verifies that a large integrated translocus is in an uninterrupted or continuous configuration.

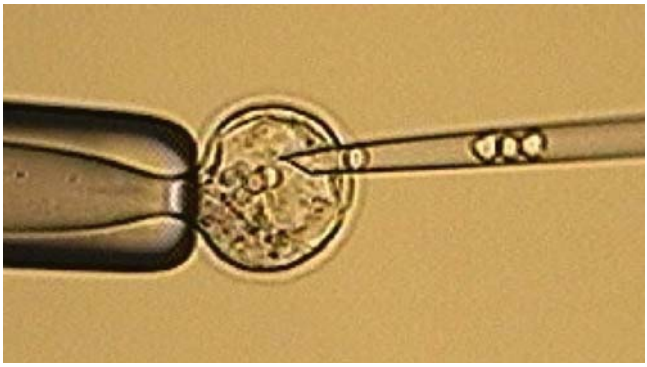


Fig. 16.5 Blastocyst injection. The position of the bastocyst is fixed with a holding pipette (*left*) and ES cells are transferred via a drawn out and cut capillary (*right*)

Most ES cell lines are derived from embryos of the 129 mouse strain of light-bellied agouti colour. However, recently, hybrid mouse ES cell lines, such as C57/Bl6x129 F1 have become available and are increasingly popular because of a good germ line transmission ability. Most researchers or transgenic core facilities choose C57/Bl6 blastocyst donors, which transmit the black coat. This mouse line is also available in white (albino) coat colour (C57Bl/6^{c-}, Tyr$^{c\text{-}Brd}$, ID: MGI:3640303, Liu et al. 1998). In most cases ES cell lines are male, and male chimeras are usually bred with females of a different coat colour to identify germ-line transmission of pups by their coat colour (Fig. 16.5).

Protocol 8: Blastocyst injection

Equipment and reagents used for pronuclear injection, Protocol 2, can also be used for blastocyst injection. These are an inverted microscope, the microinjection apparatus and M2 and M16 medium.

Method

1. Adult naive (or superovlated) C57/Bl6 females are set up with C57/Bl6 stud males (as in Protocol 2)
2. Blastocysts are collected on day 3.5 after plugging. Mice are killed and both uterines are cut out into a dish. Blastocysts are flushed out from the uterines with M2 medium. Blastocysts are collected and cultured in M16 medium until injection
3. Blastocsyts are positioned on a 10 cm dish in a large drop of M2 medium
4. ES cells are collected and resuspended in M2 medium
5. ES cells are transferred into the drop of M2 medium on the dish
6. With a holding pipette, one blastocyst is positioned such that the inner cell mass (ICM) with its cavity is facing the injection pipette
7. Move the injection pipette to pickup 10–15 ES cells (ES cells are round, smaller and brighter in comparison to the feeder cells)
8. Adjust holder and injection pipettes to level the blastocyst injection point and the tip of the injection pipette

Fig. 16.6 Chimeric mice. Transfer of 129 (agouti) ES cells into Balb/c (*white*) blastocysts (*top*) and C57Bl/6 (*black*) blastocysts (*bottom*)

9. Move injection pipette gently forward inside the cavity of a blastocyst and inject 10–15 ES cells
10. The injected blastocyst is released
11. The procedure is repeated until all blastocysts are injected
12. The injected blastocysts are washed three times with and kept in M16 medium in a culture dish in a 37°C 5% CO_2 incubator. This is followed by uterine transfer on the same day into pseudopregnant recipients at 2.5 days after plugging (see Protocol 2). Usually 10–15 injected blastocysts are transferred into each recipient

Recovery and pregnancy are carefully monitored and chimeric animals should be born after ~18 days. Weaning is usually at 3 weeks of age and each chimeric male, identified by its extensive coat colour chimerism, should be set up at ~8 weeks of age for breeding with 2 (C57Bl/6) females to obtain germline transmission. The resulting agouti pups are weaned, tagged and sampled when ~ 21 days old (Fig. 16.6).

16.4 Molecular Analysis of Integrated Exogenous DNA

For the DNA analysis by PCR and Southern blot, cells are harvested from ES cell clones or small ear or tail tip clips (1–2 mm) from mice. Tissue culture cells are washed in PBS or saline solution and 1–10^6 cells are pelleted.

Protocol 9: DNA preparation from tissue
Equipment and reagents

- Heat block
- Centrifuge
- 1.5 ml Eppendorf tubes
- Tris-base
- HCl
- EDTA
- SDS
- NaCl
- Boric acid
- Proteinase K
- Double distilled and autoclaved H_2O
- Ultra pure agarose
- DNA size marker and loading buffer
- Ethidium bromide
- Phenol
- Chlorophorm
- Isoamyl alcohol
- Isopropanol
- Sodium acetate
- Ethanol
- DNA lysis buffer:

  100 mM Tris–HCl, pH 8.5
  5 mM EDTA
  0.2% SDS
  200 mM NaCl
  120 μg/ml Proteinase K (add freshly).

- Electrophoresis buffer (0.5× TBE):

  45 mM Tris-borate
  0.5 mM EDTA

- TE buffer:

  10 mM Tris–HCl, pH 7.4
  1 mM EDTA

Method

1. Add tail tip or cell pellet into a 1.5 ml Eppendorf tube with 500 μl DNA lysis buffer
2. Place the tube in a 55°C heat block overnight
3. Add 500 μl Phenol:chloroform:isoamyl alcohol (25:24:1) and mix gently
4. Spin down at 13,000 rpm for 10 min

5. Transfer the supernatant into a new tube
6. Add 500 µl Isopropanol, mix gently
7. Spin down and wash pellet with 70% Ethanol once
8. Dry on a 60°C heat block for 10 min or at RT on the bench (avoid overdrying).
9. Dissolve DNA in 50–100 µl TE

- Extensive protocols for DNA analysis are described in "Molecular Cloning" (Sambrook and Russell 2001), and can also be found on the Internet.
- Southern blotting: Invaluable details are described by Southern (2006) and reagents and protocols, including labelling with nonradioactive digoxigenin (DIG), by several companies (e.g. "https://www.roche-applied-science.com/prodinfo_fst.jsp?page=/DIG/dig_compare01.htm")
- Restriction nuclease digest: "http://www.molecularstation.com/protocol-links/Molecular-Biology-Protocols/Restriction-Enzyme-Digestion/"
- PCR: "http://info.med.yale.edu/genetics/ward/tavi/PCR.html"

16.5 Conclusions

The practical tools provided in the protocols focus on germ cell manipulation by purified DNA for gene transfer, to establish knock-outs/ -ins, and the use of vesicular mini-chromosomes to facilitate translocus expression. These methodologies provided the foundation to express human antibody repertoires in transgenic animals without interference by the endogenous Ig loci. The alteration of ES cells and cross-breeding of the derived mutant mouse lines resulted in animals expressing antigen-specific fully human antibodies after immunisation (Brüggemann et al. 2007).

Acknowledgements The work in our laboratories is supported by the Cancer Research UK and the Babraham Institute. We are particularly grateful to the MRC and BBSRC for grant funding over many years to carry out the transgenic experiments.

References

Brüggemann M, Caskey HM, Teale C, Waldmann H, Williams GT, Surani MA, Neuberger MS (1989) A repertoire of monoclonal antibodies with human heavy-chains from transgenic mice. Proc Natl Acad Sci USA 86:6709–6713

Brüggemann M, Spicer C, Buluwela L, Rosewell I, Barton S, Surani MA, Rabbitts TH (1991) Human antibody production in transgenic mice: expression from 100 kb of the human IgH locus. Eur J Immunol 21:1323–1326

Brüggemann M, Smith JA, Osborn MJ, Zou X (2007) Selection strategies III: transgenic mice. In: Dübel S (ed) Handbook of therapeutic antibodies. Part I Selecting and shaping the antibody molecule. Wiley-VHC, Braunschweig, pp 69–93

Davies NP, Rosewell IR, Richardson JC, Cook GP, Neuberger MS, Brownstein BH, Norris ML, Brüggemann M (1993) Creation of mice expressing human antibody light chains by

introduction of a yeast artificial chromosome containing the core region of the human immunoglobulin κ locus. Biotechnology 11:911–914
Davies NP, Popov AV, Zou X, Brüggemann M (1996) Human antibody repertoires in transgenic mice: Manipulation and transfer of YACs. In: McCafferty J, Hoogenboom HR, Chiswell DJ (eds) Antibody engineering, a practical approach. IRL, Oxford, pp 59–76
Gossler A, Doetschman T, Korn R, Serfling E, Kemler R (1986) Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc Natl Acad Sci USA 83:9065–9069
Handyside AH, O'Neill GT, Jones M, Hooper ML (1989) Use of BRL-conditioned medium in combination with feeder layers to isolate a diploid embryonal stem cell line. Roux Arch Dev Biol 198:48–56
Hooper M, Hardy K, Handyside A, Hunter S, Monk M (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326:292–295
Isola LM, Gordon JW (1991) Transgenic animals: a new era in developmental biology and medicine. Biotechnology 16:3–20
Jakobovits A, Moore AL, Green LL, Vergara GJ, Maynard-Currie CE, Austin HA, Klapholz S (1993) Germ-line transmission and expression of a human-derived yeast artificial chromosome. Nature 362:255–258
Köntgen F, Süss G, Stewart C, Steinmetz M, Bluethmann H (1993) Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. Int Immunol 5:957–964
Liu P, Zhang H, McLellan A, Vogel H, Bradley A (1998) Embryonic lethality and tumorigenesis caused by segmental aneuploidy on mouse chromosome 11. Genetics 150:1155–1168
Marusyk R, Sergeant A (1980) A simple method for dialysis of small-volume samples. Anal Biochem 105:403–404
McMahon AP, Bradley A (1990) The *Wnt*-1 (*int*-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 62:1073–1085
Mendez MJ, Green LL, Corvalan JR, Jia X-C, Maynard-Currie CE, Yang X-D, Gallo ML, Louie DM, Lee DV, Erickson KL, Luna J, Roy CM, Abderrahim H, Kirschenbaum F, Noguchi M, Smith DH, Fukushima A, Hales JF, Klapholz S, Finer MH, Davis CG, Zsebo KM, Jakobovits A (1997) Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. Nat Genet 15:146–156
Nicholson IC, Zou X, Popov AV, Cook GP, Corps EM, Humphries S, Ayling C, Goyenechea B, Xian J, Taussig MJ, Neuberger MS, Brüggemann M (1999) Antibody repertoires of four and five feature translocus mice carrying human immunoglobulin heavy chain and kappa and lambda light chain YACs. J Immunol 163:6898–6906
O'Gorman S, Dagenais NA, Qian M, Marchuk Y (1997) Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. Proc Natl Acad Sci USA 94:14602–14607
Popov AV, Zou X, Xian J, Nicholson IC, Brüggemann M (1999) A human immunoglobulin λ locus is similarly well expressed in mice and humans. J Exp Med 189:1611–1619
Ren L, Zou X, Smith JA, Brüggemann M (2004) Silencing of the immunoglobulin heavy chain locus by removal of all 8 constant region genes on a 200 kb region. Genomics 84:686–695
Robertson EJ, Bradley A, Kuehn M, Evans MJ (1986) Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323:445–448
Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cols Spring Harbor Laboratoy Press, New York
Selfridge J, Pow AM, McWhir J, Magin TM, Melton DW (1992) Gene targeting using a mouse HPRT minigene/HPRT-deficient embryonic stem cell system: inactivation of the mouse ERCC-1 gene. Somat Cell Mol Genet 18:325–336
Soriano P, Montgomery C, Geske R, Bradley A (1991) Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell 64:693–702
Southern E (2006) Southern blotting. Nat methods 1:518–525
Tucker KL, Wang Y, Dausman J, Jaenisch R (1997) A transgenic mouse strain expressing four drug-selectable marker genes. Nucl Acids Res 25:3745–3746

Wagner SD, Gross G, Cook GP, Davies SL, Neuberger MS (1996) Antibody expression from the core region of the human IgH locus reconstructed in transgenic mice using bacteriophage P1 clones. Genomics 35:405–414

Zou X, Xian J, Davies NP, Popov AV, Brüggemann M (1996) Dominant expression of a 1.3 Mb human Igκ locus replacing mouse light chain production. FASEB J 10:1227–1232

Zou X, Piper TA, Smith JA, Allen ND, Xian J, Brüggemann M (2003) Block in development at the pre B-II to immature B-cell stage in mice without Igκ and Igλ light chain. J Immunol 170:1354–1361

Chapter 17
Selection of Antibody Fragments by Means of the Filter-Sandwich Colony Screening Assay

Sabine Rauth, Martin Schlapschy, and Arne Skerra

17.1 Introduction

The selection and screening of antigen-specific antibody fragments from cloned natural or synthetic variable (V) gene libraries is a widely used methodology in the field of antibody engineering (Hoogenboom 2005). Despite the power of phage display panning against antigens for the selection of cognate antibody fragments, this technique intrinsically provides only an enrichment of clones from the original repertoire, while specific antigen-binding activity is usually not the only driving force during this process. In fact, it is a common problem that several functional and biological aspects – most of them with undesired effect – play a role in the selection of cloned antibody fragments or their corresponding phage particles, for example: hydrophobic/multispecific binding properties, high expression/folding efficiency (despite moderate or even poor antigen-binding activity) and/or better compliance with the bacterial host cell (i.e. low toxicity of the foreign gene product). Thus, phage display enrichment is usually followed by an extensive screening effort in order to identify single clones with defined and specific antigen-binding activity.

Optimally, such a screening is performed under 'orthogonal' conditions, by avoiding fusion of the recombinant antibody fragment to a phage particle and by presenting the antigen in a different format, in order to exclude systemic sources of nonspecific interaction. This screening is often performed using ELISA, which is, however, difficult to operate at high throughput in a research laboratory if large numbers of clones have to be investigated.

Here, we describe a protocol for filter-sandwich colony screening as a simple alternative assay to identify antibody fragments from preselected libraries or from

S. Rauth, M. Schlapschy, and A. Skerra (✉)
Lehrstuhl für Biologische Chemie, Technische Universität München, 85350 Freising-Weihenstephan, Germany
e-mail: skerra@wzw.tum.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_17,

small repertoires, e.g. during *in vitro* affinity maturation (Skerra et al. 1991; Schlehuber et al. 2000; Fiedler et al. 2002).

In this assay, individual *Escherichia coli* colonies produce antibody fragments – after subcloning from the phage display vector on a suitable plasmid for soluble periplasmic expression – whereby separation from the bacterial colonies is achieved via a two filter membrane system. While the bacterial colonies that secrete the antibody fragments are supported on a hydrophilic upper membrane placed on an agar plate, the released antibody fragments are specifically captured onto a lower hydrophobic membrane (Fig. 17.1). After separation of the two membranes, the upper membrane, carrying the still viable colonies, is stored for later recovery, and the lower membrane with the captured antibody fragments is then probed for antigen-binding activity – in a manner resembling the Western blot – by applying the soluble antigen in an appropriately labelled format.

Two technical aspects are crucial for this assay. First, the periplasmically secreted antibody fragments have to be efficiently released from the bacterial colonies. Initially, this was an unexpected phenomenon (Skerra et al. 1991), which is probably related to the observation that prolonged induction of periplasmic expression leads to leakiness of the bacterial outer membrane, ultimately followed by cell lysis (Plückthun and Skerra 1989). However, under the induction conditions described here, a sufficient number of viable cells survive in each colony such that

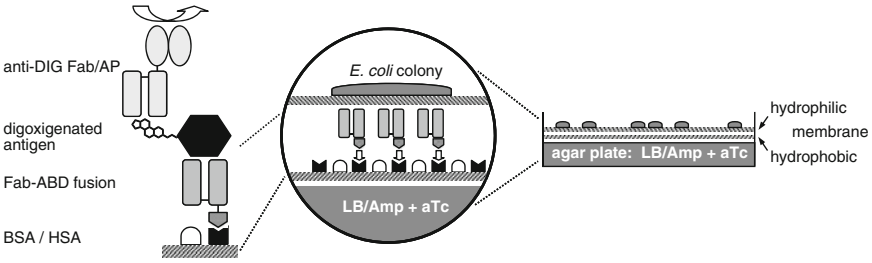


Fig. 17.1 Schematic overview of the filter-sandwich colony screening assay for the identification of Fab variants with affinity for a DIG-labelled antigen. After transformation of an appropriate *E. coli* strain with a pool of pASK106 plasmids harbouring different variable gene inserts, the cell suspension is spread onto a hydrophilic membrane on an LB/Amp plate and incubated at 37°C. Meanwhile, a hydrophobic membrane is coated with HSA, blocked with BSA and then placed onto another LB/Amp plate supplemented with aTc for induction. The hydrophilic membrane carrying the colonies is transferred onto the hydrophobic membrane with the colonies facing upwards. During the incubation period, the Fab-ABD fusion proteins are secreted into the periplasm of *E. coli* and become partially released from the bacterial colonies. As illustrated here, the proteins diffuse through the pores of the hydrophilic membrane and are captured on the hydrophobic membrane underneath via specific interaction between the ABD and HSA. After the induction period, the hydrophobic membrane, with the Fab-ABD fusion proteins functionally immobilised at positions corresponding to the bacterial colonies, can be probed for binding activity towards the labelled antigen. To this end, the membrane is consecutively incubated with the digoxigenated antigen and with an anti-DIG Fab/alkaline phosphatase conjugate, followed by a chromogenic reaction. Colonies corresponding to intensely stained spots are identified on the first membrane and applied to further analysis

later recovery for propagation and analysis of selected clones is not hampered. Thus, there is no need for specific lysis of the cells – for example, by adding detergents or chemical denaturants – which preserves the biochemical activity of the recombinant antibody fragments and contributes to the specificity of the binding signal, because the release of host cell proteins from the bacterial cytoplasm is minimised.

Second, the specificity and sensitivity of this method is further enhanced by the selective capturing strategy – in contrast to nonspecific adsorption to the hydrophobic membrane, which usually is accompanied by partial protein denaturation. Several capturing reagents have been successfully applied, for example, an antiserum directed against the C_κ domain of the recombinant Fab fragment (Skerra et al. 1991) or the bacterial receptor protein L, which specifically recognises human V_κ domains within their framework region (de Wildt et al. 2000). Furthermore, the specific interaction between the small (46 residue) albumin-binding domain (ABD) of *streptococcal* protein G and human serum albumin (HSA) has been established as another inexpensive and efficient capturing strategy (König and Skerra 1998). To this end, the recombinant Fab fragment – or any other immunoglobulin (Ig) fragment, for example, scFv or even a single domain antibody fragment (Holliger and Hudson 2005) – can be secreted as a fusion protein with the ABD and captured on the second membrane that has been excessively coated (and also blocked) with HSA.

The vector pASK106 (Fiedler et al. 2002) was specifically developed for the filter-sandwich colony screening assay of Fab fragments carrying the ABD and serves as an example in the presently described protocol (Fig. 17.2). The heavy and light chains of the Fab fragment are fused at their *N*-termini with the bacterial OmpA or PhoA signal sequences, respectively, to direct co-secretion into the bacterial periplasm, where folding and disulphide formation can take place. The artificial dicistronic operon is under tight transcriptional control by the *tet* promoter/operator (Skerra 1994b) and its expression can be conveniently induced with anhydrotetracycline (aTc). While the heavy chain of the Fab fragment carries a His_6-tag, enabling facile protein purification under conditions of preparative expression in liquid culture (cf. Chapter 22 on "Purification and characterisation of His-tagged antibody fragments"), the light chain is fused to the ABD.

In the course of the filter-sandwich colony screening assay, the Fab-ABD fusion proteins become secreted across the inner bacterial membrane and then partially released from the bacterial colonies via leakage from the periplasm. The upper hydrophilic membrane, which possesses defined pore size and low protein binding capacity, serves as a filter between the bacterial colonies and the soluble antibody fragments. It permits their diffusion and localised immobilisation on the hydrophobic second membrane via specific interaction between the ABD and the HSA that has been used for coating. Following incubation with the labelled antigen and subsequent colour development, stained spots on the hydrophobic membrane can be assigned to corresponding colonies on the hydrophilic first membrane. Thus, 1,000–10,000 colonies can be easily analysed on a single 82 mm diameter petri dish, provided that the bacteria were evenly plated.

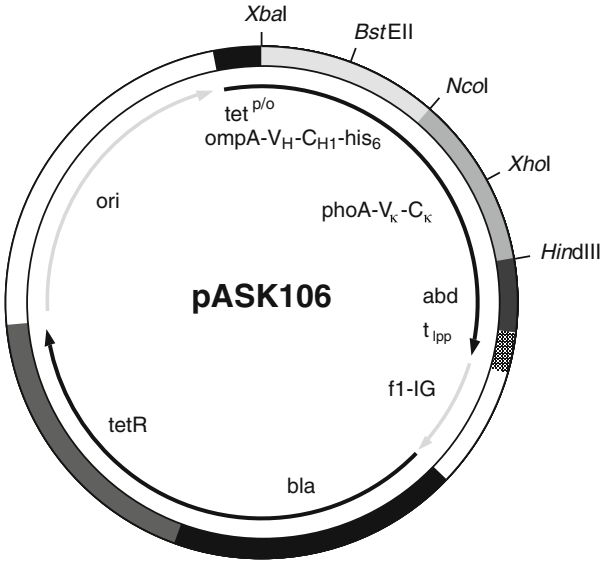


Fig. 17.2 Expression vector pASK106. The heavy and light chains of the Fab fragment are arranged in a dicistronic operon (ompA-V_H-C_{H1}-his_6 and phoA-V_κ-C_κ), which is under tight transcriptional control of the *tet* promoter/operator (*tet*$^{p/o}$) and ends with the lipoprotein transcription terminator (t_{lpp}). The light chain of the Fab fragment is fused to the ABD at its *C*-terminus via a Pro-Ala-Ser linker. The plasmid carries an ampicillin resistance gene (bla). *Xba*I, *Bst*EII, *Nco*I, *Xho*I and *Hin*dIII indicate conserved restriction sites for cloning or exchange of domains (Skerra, 1994a). *tet*R: *tet* repressor gene, ori: origin of replication, f1-IG: intergenic region of filamentous phage, useful for the preparation of single-stranded plasmid DNA to facilitate site-directed mutagenesis

For probing of the second membrane, which initially carries an invisible replica of the colonies on the first membrane in the form of the functionally immobilised Fab fragments, several strategies can be applied. For example, the antigen can be labelled with biotin or digoxigenin (DIG) using commercially available labelling reagents followed by detection of the bound antigen using a suitable enzyme conjugate, e.g. streptavidin/alkaline phosphatase (AP) or anti-DIG-Fab/AP. In the case of biotin, however, one should be aware that the endogenous biotin carboxyl carrier protein (BCCP) of *E. coli* can lead to more or less strong background signals, particularly when using a long induction phase that can result in partial cell lysis. In contrast, the DIG group does not occur in *E. coli* such that this system generally yields a more specific staining. Alternatively, reagents that specifically recognise the antigen itself – without interfering with the epitope to be bound by the recombinant antibody fragment – for example, individual antisera or reagents which interact with an affinity tag, such as the *Strep*-tag II (Schmidt and Skerra 2007), may be utilised as well. Finally, the antigen can be directly labelled, for example, with a radionuclide or with colloidal gold (Skerra et al. 1991).

After signal development with a chromogenic reagent, typically the strongest signals are chosen, even though the morphology and appearance of the signals may also serve as criteria. Corresponding colonies on the hydrophilic first membrane are then identified and propagated for further analysis such as DNA sequencing, subcloning and/or soluble production of the recombinant antibody fragment.

17.2 Outline

As an example of the filter-sandwich colony screening assay, humanised Fab-ABD fusion proteins recognising the extracellular fragment of the CD30 antigen (Schlapschy et al. 2004) will be used here. A general overview of the assay for the identification of Fab variants with affinity for a DIG-labelled antigen is given in Fig. 17.1.

17.3 Materials

17.3.1 Filter-Sandwich Colony Screening Assay

Media and buffers should be sterilised by autoclaving or filtration.

- Incubator with flexible temperature setting (e.g. Friocell, MMM-Group)
- Bench-top shaker (e.g. GFL, Gesellschaft für Labortechnik)
- Hydrophilic PVDF membrane with low protein binding capacity (type GVWP, 0.22 μm; Millipore); cut to size as necessary
- Hydrophobic Immobilon-P membrane (0.45 μm; Millipore); cut to size as necessary
- Human serum albumin (HSA; Sigma-Aldrich)
- Bovine serum albumin (BSA; Carl Roth)
- Ampicillin (Amp; Carl Roth) stock solution at 100 mg/ml in water, sterile filtered
- Anhydrotetracycline (aTc; Acros Organics) stock solution at 2 mg/ml in dimethylformamide (DMF, Carl Roth)
- 5-Bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (BCIP; Carl Roth) stock solution at 50 mg/ml in DMF
- Nitroblue tetrazolium (NBT; Sigma-Aldrich) stock solution at 75 mg/ml in 70% (v/v) DMF
- *E. coli* strain suitable for this assay, e.g. TG1 (Gibson 1984), JM83 (Yanisch-Perron et al. 1985), KS303i (Strauch and Beckwith 1988; Skerra et al. 1991)
- Fab expression vector pASK106 (Fiedler et al. 2002); available from the authors upon request
- Labelled antigens, e.g. biotinylated or digoxigenated

- Suitable enzyme conjugates, e.g. ExtrAvidin-AP (Sigma-Aldrich) or anti-DIG-Fab/AP (Roche Diagnostics)
- Luria-Bertani (LB) medium and LB plates: 10 g/l Bacto Tryptone (Difco), 5 g/l Bacto Yeast Extract (Difco), 5 g/l NaCl; adjust to pH 7.5 with NaOH; for plates add 15 g/l Bacto Agar (Difco) before autoclaving, supplement with the required antibiotic (e.g. 100 mg/l Amp) and inducer of gene expression if needed (e.g. 200 µg/l aTc)
- Alkaline phosphatase (AP) buffer: 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$; adjust to pH 8.8 with HCl
- PBS: 4 mM KH_2PO_4, 16 mM Na_2HPO_4,115 mM NaCl, pH 7.4
- PBS/T: 0.1% (v/v) Tween 20 (Sigma-Aldrich) in PBS

17.3.2 Chemical Labelling of Antigens with Biotin or Digoxigenin

- Labelling reagents, e.g. D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Roche Diagnostics) or digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Roche Diagnostics) dissolved in water-free DMF or dimethylsulfoxide (DMSO; Carl Roth)
- Molecular sieve (Merck)
- Size exclusion column, e.g. Superdex (GE Healthcare) or PD-10 (GE Healthcare)
- Suitable enzyme conjugates, e.g. ExtrAvidin-AP (Sigma-Aldrich) or anti-DIG-Fab/AP (Roche Diagnostics)
- 1 M Tris/HCl pH 8.0
- BCIP, NBT and AP buffer (see **17.3.1**)
- PBS (see **17.3.1**)

17.4 Protocols

17.4.1 Filter-Sandwich Colony Screening Assay

All incubation steps of the hydrophobic membrane are performed under gentle shaking and at ambient temperature. Local drying of the membrane should be avoided.

Day 1

1. In the morning, transform a suitable *E. coli* strain with the Fab fragment expression library in the vector pASK106 – either from a pooled plasmid preparation or directly from a ligation reaction – using standard methodology, e.g. $CaCl_2$ competent cells or electrotransformation (Sambrook et al. 2001).

2. Place the hydrophilic PVDF membrane onto an LB/Amp agar plate and carefully plate 100–200 µl of the *E. coli* suspension according to 500–1,000 colonies onto the membrane (see **Note 1**). Make sure that the bacterial suspension is spread evenly, to get a uniform distribution of the colonies covering the whole membrane (see **Note 2**). Avoid mechanical stress on the cells – e.g. by tightly pressing a Drigalski spatula onto the membrane – as this may reduce the number of colonies.
   Incubate the plate at 37°C for a period of 7.5–8 h until the colonies are just visible, i.e. with a diameter of approximately 0.5 mm. Incubation times for bacterial growth can vary depending on the kind of expression plasmid (choice of promoter, antibiotic resistance) and on the *E. coli* strain used. Incubation times indicated in this protocol correspond to the use of pASK106 and *E. coli* TG1 (see **Note 3** and **Note 4**).
3. Meanwhile, activate the hydrophobic Immobilon-P membrane with methanol for 5 min and immerse the membrane first in sterile water and then in sterile PBS for 5 min each.
4. Coat the membrane with 10 mg/ml HSA in PBS (sterile-filtered) for 4 h.
5. Block remaining binding sites on the membrane with 3% (w/v) BSA, 0.5% (v/v) Tween 20 in PBS (sterile-filtered) for 2 h. If sufficient amounts are available, HSA may be used instead of BSA.
6. Immerse the membrane first in sterile PBS twice for 10 min and then in LB/Amp containing 200 µg/l aTc for another 10 min.
7. Drain off excess liquid and place the hydrophobic membrane onto an LB/Amp inducer plate (containing 200 µg/l aTc). If necessary, the membrane may be stored at 4°C at this step until the first membrane with the colonies is ready.
8. Carefully transfer (using a pair of forceps) the hydrophilic membrane onto the hydrophobic membrane with the colonies facing upwards. Avoid drying of the first membrane during this transfer and also the formation of air bubbles; there should be a uniform liquid film between the two membranes as well as the agar plate. For subsequent identification of the colonies, mark the two membranes at two or three points.
9. Incubate the agar plate covered with the membrane sandwich for approximately 11–15 h at 22°C (see **Note 5**).

Day 2

1. Transfer the upper membrane, which carries the colonies, onto a fresh LB/Amp plate and store it at 4°C for later use.
2. Remove the hydrophobic membrane from the agar plate and wash it immediately three times in 10–20 ml PBS/T for 5 min.
3. Incubate the hydrophobic membrane with the labelled (e.g. biotinylated or digoxigenated) antigen in PBS/T for 1 h (for labelling see **17.4.3**). The antigen concentration should be chosen according to the expected dissociation constants of the antibody fragments.
4. Wash the hydrophobic membrane three times for 5 min with PBS/T.

5. Incubate the hydrophobic membrane with a suitable enzyme conjugate diluted in PBS/T (e.g. ExtrAvidin-AP at a dilution of 1:5000 or anti-DIG-Fab/AP at 1:1500) for 1 h (see **Note 6**).
6. Wash the hydrophobic membrane twice for 5 min with PBS/T and twice with PBS.
7. Immerse the membrane in 10 ml AP buffer supplemented with 30 μl BCIP and 5 μl NBT (see **Note 6**). Incubate the membrane without shaking until clear colour signals appear (see **Note 7**).
8. To stop the chromogenic reaction, wash the membrane with water and dry it on air.
9. Identify those colonies which yield the strongest signals on the hydrophobic membrane. Then, recover corresponding colonies from the first membrane by means of a toothpick or a microliter pipetting tip and use them for inoculation of a fresh LB/Amp master plate (see **Note 8**).
10. Further, test these selected variants both for their antigen-binding activity and specificity via secondary filter-sandwich colony screening assay as described below in **17.4.2**.

17.4.2 Secondary Filter-Sandwich Colony Screening Assay

Day 1

1. For the secondary filter assay, place a fresh hydrophilic membrane onto an LB/Amp agar plate and stipple the selected variants – as duplicates or even higher multiples (see **Note 9**) – from a liquid overnight culture onto this membrane using a toothpick or a microliter pipetting tip. Incubate this membrane at 37°C for just 4 h until the colonies are clearly visible.
2. Prepare the hydrophobic membrane according to the primary filter screening assay (see **17.4.1**, day 1).
3. Perform the overnight incubation of the membrane sandwich at 22°C for only 10–11 h.

Day 2

1. Transfer the upper membrane, which carries the colonies onto a fresh LB/Amp plate and store it at 4°C.
2. Remove the hydrophobic membrane from the agar plate and wash it immediately three times in 10–20 ml PBS/T for 5 min.
3. To confirm choice of the proper colonies, test the selected variants for their specific binding to the labelled target antigen again and also, using additional membranes, test for lack of binding to control proteins, e.g. labelled ovalbumin (see **Note 10**).
4. Visualise protein binding according to the primary filter screening assay (see **17.4.1**, day 2).

17.4.3 Chemical Labelling of Antigens with Biotin or Digoxigenin

There are many commercially available reagents for the labelling of proteins using various chemistries. We recommend the use of N-hydroxysuccinimide esters of biotin or digoxigenin (Schlehuber et al. 2000), which react with the free amino groups of exposed Lys side chains under mild conditions:

1. Dissolve the NHS ester in water-free DMF or DMSO to a concentration ranging from 15–30 mg/ml. If necessary, store an aliquot of the solvent over molecular sieve in a sealed vessel for a couple of days prior to use.
2. Determine the molar concentration of the protein dissolved in PBS using its measured absorption at 280 nm (corrected against a buffer blank) and its calculated specific extinction coefficient at this wavelength (Gill and von Hippel 1989). Avoid any buffer components that may contain free amino groups, e.g. Tris, as these would react with the NHS ester first. If such components are present, the protein solution should either be dialysed three times against PBS or a corresponding buffer exchange may be accomplished by passing the sample through a size exclusion column (e.g. PD-10) equilibrated with PBS and using the same buffer for elution.
3. Quickly (but gently) mix the protein solution with a twofold molar amount from the stock solution of the labelling reagent and rotate at room temperature for 1 h. In order to avoid a denaturing effect of the DMF on the antigen, make sure that the volume of the protein in PBS exceeds the volume of the labelling reagent at least by a factor of 20. While the optimal molar ratio of labelling reagent to protein may be empirically determined, a ratio of 2:1 is usually successful. A larger excess of the NHS ester is not advisable because there is an increasing risk that the epitope may become obscured due to chemical modification.
4. After the labelling, add 1 M Tris/HCl pH 8.0 to a final concentration of 10 mM and incubate for several hours to block all remaining NHS ester groups.
5. To remove the reagents, dialyse the labelled protein against PBS at 4°C overnight or subject it to size exclusion chromatography.
6. Labelling of the antigen may be checked on a Western blot using the corresponding enzyme conjugate (e.g. ExtrAvidin-AP at a dilution of 1:5000 or anti-DIG-Fab/AP at 1:1500) followed by the chromogenic reaction with BCIP and NBT (see **17.4.1**, day 2).

17.5 Result

An example of a filter with Fab-ABD fusion proteins recognising the CD30 antigen is shown in Fig. 17.3. This filter illustrates a step during the *in vitro* affinity maturation following humanisation of the mouse monoclonal antibody HRS3 via CDR grafting (Schlapschy et al. 2004). The filter-sandwich colony screening assay

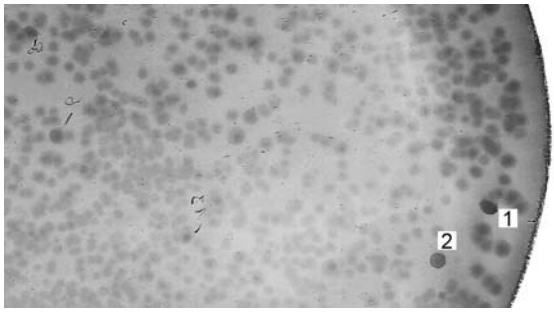


Fig. 17.3 Section of a filter-sandwich colony screening assay for humanised HRS3 Fab variants with improved affinity for the extracellular fragment of the CD30 antigen. The mutagenised V_H gene obtained by error-prone PCR from the initially humanised version was cloned on the vector pASK106. *E. coli* JM83 cells were transformed with the ligation mixture and subjected to the filter-sandwich colony screening assay by incubating the lower hydrophobic membrane with a 500 nM solution of the digoxigenated recombinant CD30 fragment (amino acids 185–335 of the mature CD30 antigen). Bound antigen was detected with an anti-DIG-Fab/AP conjugate followed by chromogenic reaction with BCIP and NBT. The variants EP1/1 and EP1/2 that were identified in this assay are marked with numbers. Variant EP1/2 was subjected to further cycles of affinity maturation, leading to the finally optimised Fab fragment (Schlapschy et al. 2004)

– employing the pASK106 expression vector as described in this protocol – in conjunction with error-prone polymerase chain reaction (PCR) of the variable genes proved to be a useful strategy to significantly increase the antigen affinity of the original humanised version. The optimised Fab fragment had an affinity similar to the original murine Fab and was fully functional with respect to antigen-binding in ELISA experiments and in FACS analysis using CD30-positive cells (Schlapschy et al. 2004).

In the depicted example, approximately 90% of the colonies show a colour signal, which is in agreement with the fact that these clones did not represent a naive antibody library but mutant versions of the humanised Fab fragment that already exhibits antigen-binding affinity with a K_D of approximately 280 nM. Eight promising colonies, corresponding to the most intense signals, were identified on this hydrophilic membrane and one of it served as starting point for further affinity maturation cycles, finally leading to the successful candidate.

17.6 Notes

1. To obtain a suitable number of colonies on the hydrophilic membrane, a test transformation should be performed a day before the actual assay. For this purpose, different volumes of the transformation reaction should be plated onto

LB/Amp agar plates (without filter membrane), allowing estimation of the required volume for approximately 500–1,000 colonies. If there are more or fewer colonies than considered appropriate, the bacterial suspension to be used for the filter-sandwich colony screening assay should either be diluted with LB medium or concentrated (by spinning down the cells in a microfuge and resuspending them in a smaller volume of the supernatant) in order to meet the recommended volume for spreading on the membrane.

2. Uniform distribution of the colonies on the hydrophilic membrane can be easily achieved using a Drigalski spatula in combination with a turntable for petri dishes. However, avoid pressing the Drigalski spatula too tightly onto the membrane since this exerts mechanical stress onto the cells and may reduce the number of colonies.
3. Instead of the $tet^{p/o}$ the $lac^{p/o}$ may be used (Skerra et al. 1991; Skerra 1994a). In this case the inducer aTc has to be replaced by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG).
4. The signal intensity is not strikingly increased when using a leaky *E. coli* strain such as KS303i instead of TG1 (Skerra et al. 1991), indicating sufficient release of the secreted antibody fragment from the periplasm under these assay conditions. Since TG1 has an amber suppressor genotype and also exhibits a relatively short generation time, this strain – or a version cured from the F′ episome (Kim et al. 2009) – can be recommended for the filter-sandwich colony screening assay.
5. It is advisable to perform protein expression at ambient temperature since a low temperature strongly favours the proportion of functional antibody fragments and the viability of *E. coli* cells.
6. Alternative to the chromogenic reaction using alkaline phosphatase in combination with BCIP and NBT, peroxidase-coupled reagents and their corresponding substrates can be used.
7. If no signal can be detected after the development of the hydrophobic membrane, the successful labelling of the target proteins should be analysed by Western blotting.
8. To facilitate the isolation of positive colonies, colour signals appearing on the second membrane as well as the label positions may be copied onto an overhead transparency. The hydrophilic membrane carrying the viable colonies is then placed onto this sheet and colonies overlapping with the marked signals are picked and plated onto an LB/Amp master plate. However, one should take care that the colonies do not dry out during this step, for example, by returning the membrane back onto its agar plate.
9. It is advisable to apply each sample in several copies – to account for signal variation due to varying cell numbers – and to investigate previously characterised clones side by side for control.
10. Additionally, the assay may be performed under competitive conditions, for example, by also applying an unlabelled target or a competing antibody (fragment) with known affinity or specificity along with the solution of the labelled antigen (Kim et al. 2009).

References

de Wildt RMT, Mundy CR, Gorick BD, Tomlinson IM (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. Nat Biotechnol 18:989–994

Fiedler M, Horn C, Bandtlow C, Schwab ME, Skerra A (2002) An engineered IN-1 Fab fragment with improved affinity for the Nogo-A axonal growth inhibitor permits immunochemical detection and shows enhanced neutralizing activity. Protein Eng 15:931–941

Gibson TJ (1984) Studies on the Eppstein-Barr virus genome. PhD thesis, Cambridge University, England

Gill SC, von Hippel PH (1989) Calculation of protein extinction coefficients from amino acid sequence data. Anal Biochem 182:319–326

Holliger P, Hudson PJ (2005) Engineered antibody fragments and the rise of single domains. Nat Biotechnol 23:1126–1136

Hoogenboom HR (2005) Selecting and screening recombinant antibody libraries. Nat Biotechnol 23:1105–1116

Kim HJ, Eichinger A, Skerra A (2009) High affinity recognition of lanthanide (III) chelate complexes by a reprogrammed human lipocalin 2. J Am Chem Soc 131:3565–3576

König T, Skerra A (1998) Use of an albumin-binding domain for the selective immobilisation of recombinant capture antibody fragments on ELISA plates. J Immunol Methods 218:73–83

Plückthun A, Skerra A (1989) Expression of functional antibody Fv and Fab fragments in *Escherichia coli*. Methods Enzymol 178:497–515

Sambrook J, Fritsch EF, Maniatis T (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schlapschy M, Gruber H, Gresch O, Schäfer C, Renner C, Pfreundschuh M, Skerra A (2004) Functional humanization of an anti-CD30 Fab fragment for the immunotherapy of Hodgkin's lymphoma using an *in vitro* evolution approach. Protein Eng Des Sel 17:847–860

Schlehuber S, Beste G, Skerra A (2000) A novel type of receptor protein, based on the lipocalin scaffold, with specificity for digoxigenin. J Mol Biol 297:1105–1120

Schmidt TGM, Skerra A (2007) The *Strep*-tag system for one-step purification and high affinity detection or capturing of proteins. Nat Protoc 2:1528–1535

Skerra A (1994a) A general vector, pASK84, for cloning, bacterial production, and single-step purification of antibody Fab fragments. Gene 141:79–84

Skerra A (1994b) Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in *Escherichia coli*. Gene 151:131–135

Skerra A, Dreher ML, Winter G (1991) Filter screening of antibody Fab fragments secreted from individual bacterial colonies: Specific detection of antigen binding with a two-membrane system. Anal Biochem 196:151–155

Strauch KL, Beckwith J (1988) An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. Proc Natl Acad Sci USA 85:1576–1580

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Chapter 18
Semi-automated Magnetic Bead-Based Antibody Selection from Phage Display Libraries

Zoltán Konthur, Jeannine Wilde, and Theam Soon Lim

18.1 Introduction

Antibodies are the fastest growing sector of biological therapeutics, and currently more than 400 monoclonal antibodies are in the pipeline (Dübel 2007). The quest to obtain human antibodies for therapy has led many researchers to design diverse display technologies as well as to generate larger and more diverse antibody display libraries (Mondon et al. 2008). Although many different display technologies have been introduced for human antibody generation, such as yeast display (Boder and Wittrup 1997), ribosome display (Hanes and Plückthun 1997) and mRNA display (Fukuda et al. 2006), phage display still remains the gold standard in human antibody generation (McCafferty et al. 1990; Barbas et al. 1991; Breitling et al. 1991). The accomplishment of phage display-derived human antibodies is highlighted by the increase of human antibody-based therapies being introduced into the market, and many more in clinical trials (Thie et al. 2008). Today, multiple companies exploit phage display technology worldwide for the development of therapeutic antibodies (Konthur 2007). Additionally, phage display of antibody libraries is being increasingly appreciated for the development of research reagents and a number of national and international initiatives already apply this method for antibody generation (Konthur et al. 2005; Taussig et al. 2007).

Z. Konthur (✉) and J. Wilde
Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Ihnestrasse 73, Berlin 14195, Germany
e-mail: konthur@molgen.mpg.de; wilde@molgen.mpg.de

T.S. Lim
Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Ihnestrasse 73, Berlin 14195, Germany
Free University Berlin, Faculty of Biology, Chemistry and Pharmacy, Takustrasse 3, Berlin 14195, Germany
Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800 Penang, Malaysia
e-mail: lim@molgen.mpg.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_18,

In phage display, panning is an iterative process, where specific binder molecules are continuously enriched and multiplied from a pool of predominantly nonbinders until the specific binders finally become the majority population (Konthur and Crameri 2003). This "panning" method has been referred to the tool used by gold washers to isolate gold (Parmley and Smith 1988). For panning, antigens can be anchored to various types of solid supports, such as magnetic beads (Walter et al. 2001), column matrix (Noppe et al. 2009), nitrocellulose (Hawlisch et al. 2001) or to a larger extent, plastic surfaces in the form of polystyrene tubes (Hust et al. 2002), or 96 well polystyrene microtiter plates (Krebs et al. 2001). The selection process is an affinity-based enrichment process and involves multiple rounds of selection. The antibody presenting phage particles are incubated with the immobilized antigens to allow interactions to occur. Next, nonbinders are removed from the selection matrix by washing off unbound phage particles. The bound phages are then used to infect *Escherichia coli* and are subsequently re-amplified to be used in the following round. This selection cycle is normally repeated until a satisfactory enrichment is achieved. Normal panning protocols usually constitute between two and four rounds. The infection of *E. coli* with phage particles along with the propagation of antibody presenting phages is a fairly robust but nevertheless laborious biological process involving multiple steps, which need to be performed with care, and is genuinely regarded as difficult to fully automate.

By definition, an automated system refers to pipelines in which all steps of the process or assay is carried out without any human intervention (Cohen and Trinka 2002). In contrast, unit automation requires human involvement in certain stages, and only individual stages in the process pipeline are partially automated independent of each other (Menke 2002). As the entire technology is a compilation of various stages of work, to structure the workflow in a fully automated fashion is indeed challenging in terms of compatibility and cost. Key stages in the selection procedure are panning, infection, propagation, colony picking, and ELISA evaluations. Principally, almost all stages can be automated, but the extent of the automation should be within the financial resources available. In practice, the extent of laboratory automation is dependent upon the scope and timeline of the project pursued (Hamilton 2002).

Despite the technology's potential in high-throughput platforms being regarded as low (Li 2000), the growing interest for human antibodies has kick-started various initiatives to streamline the processes involved in phage display. Automation and high-throughput approaches are required to circumvent the need for faster and more efficient screening protocols and to allow simultaneous selection and evaluation of enriched antibody phage libraries. For most of these methods, individual attempts to automate panning, colony picking, and ELISA have been successful (Buckler et al. 2008).

Possible panning procedures compatible with automation involve immobilization of antigens to either 96 well microtiter plates (Krebs et al. 2001) or magnetic beads (Walter et al. 2001). In microtiter plates, immobilization of antigens is carried out in two ways, by adsorption of the antigens to the plate surface, or in a directed

fashion using, for example, streptavidin-coated plates to capture biotinylated antigens. Alternatively, antigens can be attached to magnetic beads. The main advantage of magnetic beads against microtiter plates is the increase in surface area, leading to a more efficient panning process. Magnetic particles have been shown to be more efficient than polystyrene plates in the panning process (McConnell et al. 1999) and to assist in ELISA experiments detecting antigens otherwise not detected by conventional ELISA (Kala et al. 1997).

We use a pin-based magnetic particle processor (Kingfisher, Thermo) for unit-automation of the panning procedure, which enables the handling of 96 magnetic pins, corresponding to the positions of a 96-well microtitre plate (Walter et al. 2001; Rhyner et al. 2003). The processor can accommodate several microtitre plates filled with individual buffers for washing and incubation as depicted in Fig. 18.1a. The individual steps of the panning procedure are performed by transferring the magnetic particles between wells with rod-shaped magnets covered with plastic caps by a sequence of capture and release motions (Fig. 18.1b). The movements are software-driven and parameters such as time, position, frequency, and strength of shaking movements can be adjusted, allowing reproducible control of each step of the phage display selection protocol for as many as 96 parallel selections. In our eyes, using a magnetic bead-based, instead of a microtiter plate-based selection scheme has an additional advantage. Moving the magnetic particles from vessel to vessel reduces the background of unspecific binders to the surfaces and transfers minimal volumes. Changing solutions in microtiter plates with a liquid handling robot always leaves a dead volume of liquid behind and a background of non-specific binders can occur (Konthur and Walter 2002).

Our panning procedure is generally carried out using a magnetic particle processor over four rounds of selection (Fig. 18.1, Tables 18.1 and 18.2). Applying the processor allows standardization of panning parameters, such as washing conditions, incubation times, or to perform parallel selections on same targets under different buffer conditions. To keep things simple, we have not attempted to fully automate the selection process. Instead, we followed a unit-automation approach to the extent where it is easy and straightforward to do, as all steps require human intervention. However, setting up standard operating procedures and adapting all biological processes to 96-well microtiter plate format, manipulation by hand is kept as simple as possible and can be easily performed with multichannel pipettes reducing handling errors. The whole protocol has been streamlined to carry out bead loading, phage selection, phage amplification between selection rounds, and ELISAs for confirmation of binding activity in microtiter plate format. Once the phage display panning procedure is completed, the evaluation process to identify positive monoclonal antibodies is performed over two stages of ELISA. The first stage of evaluation is carried out on polyclonal level, in which the selection rounds are tested for target-specific enrichment of binders using a magnetic bead-based ELISA protocol (Fig. 18.2, Table 18.3). At the second stage, a set of monoclonal binders from individual selection rounds (chosen according to the results of the polyclonal ELISA) are analyzed for special binding by ELISA (Fig. 18.3).

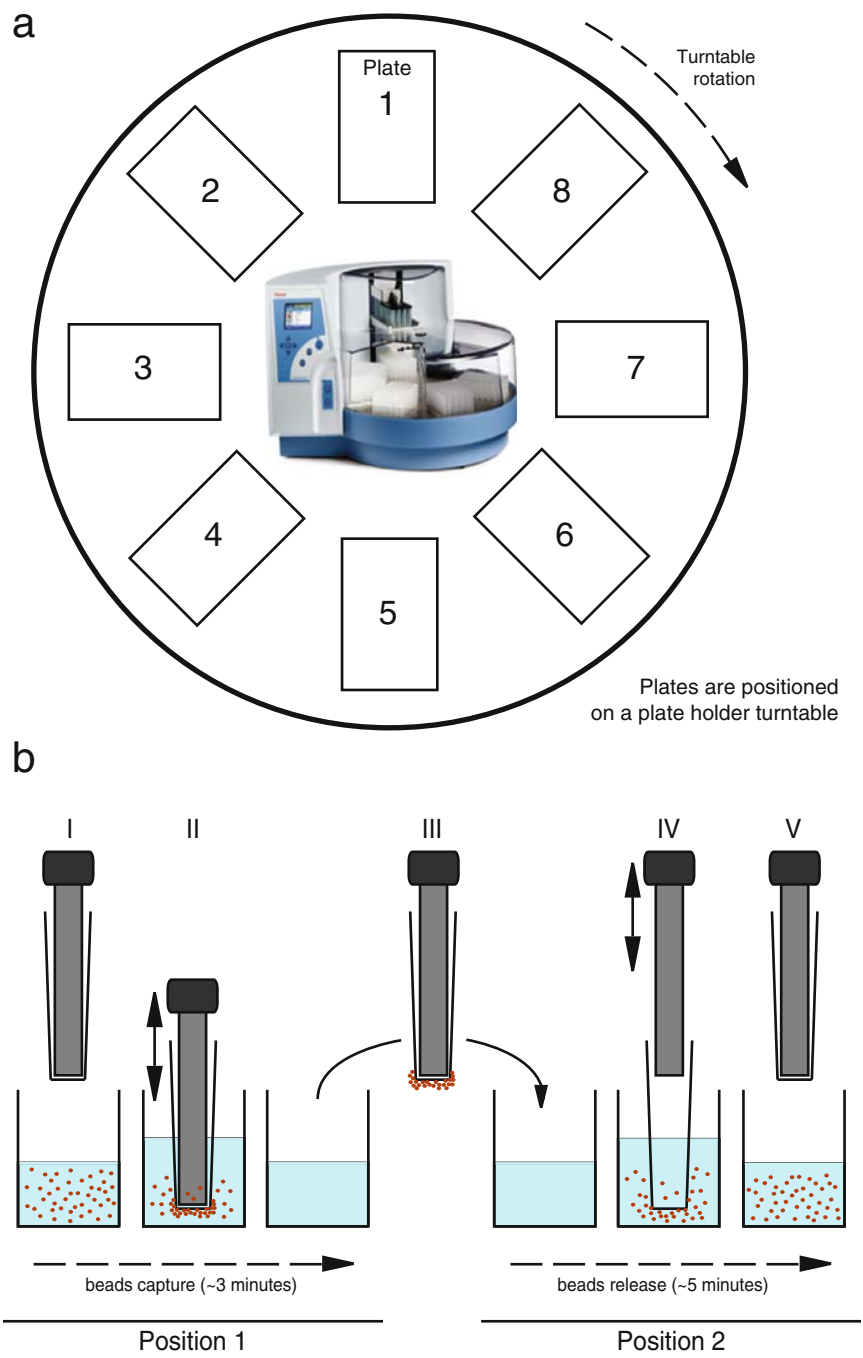


Fig. 18.1 Principle of the magnetic particle processor. (**a**) Rotating table of a Kingfisher 96 magnetic particle processor. The magnetic head is at a fixed loading position. For moving beads

Table 18.1 Overview of automated magnetic bead-based panning procedure on a Kingfisher 96

Plate no.	Panning round 1	Panning round 2	Panning round 3	Panning round 4
1	Bead plate	Bead plate	Bead plate	Bead plate
2	Phage plate	Phage plate	Phage plate	Phage plate
3	Wash plate 1	Wash plate 1	Wash plate 1	Wash plate 1
4	Release plate	Wash plate 2	Wash plate 2	Wash plate 2
5	*E. coli* culture plate	Release plate	Wash plate 3	Wash plate 3
6	–	*E. coli* culture plate	Release plate	Wash plate 4
7	–	–	*E. coli* culture plate	Release plate
8	–	–	–	*E. coli* culture plate
Total time:	~135 min	~145 min	~155 min	~165 min

Table 18.2 Automated magnetic bead-based panning protocol (round 4) for Kingfisher 96

Plate no.	Plate name	Work step	Volume (μL)	Time (min)
1	Bead plate	Blocking of antigen-loaded and control magnetic beads with PTM	200	60
2	Phage plate	Incubation of magnetic beads in antibody phage stocks of the selection rounds	200	60
3	Wash plate 1	Wash 1 of magnetic beads in PBST	200	10
4	Wash plate 2	Wash 2 of magnetic beads in PBST	200	10
5	Wash plate 3	Wash 3 of magnetic beads in PBST	200	10
6	Wash plate 4	Wash 4 of magnetic beads in PBST	200	10
7	Release plate	Waiting position for magnetic beads until *E. coli* culture plate is ready for infection[a]	200	5–10
8	*E. coli* culture plate	Infection of *E. coli* TG1 culture with bead-bound phage particles	200	–[b]
Total time:				170

[a]At this stage, 10 μl bead-bound phage solution can be collected for titration. (see Sect. 18.3.4)
[b]Incubation takes place outside Kingfisher 96 Instrument at 37°C

In summary, automating the panning process on its own can largely increase the number of targets against which antibodies are selected in parallel, but it also shifts the bottleneck of the overall selection pipeline further toward the isolation and evaluation of monospecific binders. Isolation and screening of monoclonal binders can be readily automated, and multiple strategies have been reported (de Wildt et al.

Fig. 18.1 (continued) from plate to plate, the beads are recovered from one plate and then the new plate is moved to the loading position by rotating the plate holder table clockwise. Photograph shows the latest model: Kingfisher Flex instrument, Thermo Scientific. (**b**) Operating mode of magnetic particle processors. (I) The rod-shaped magnet is covered by a plastic cap and moves into a solution containing suspended magnetic beads. (II) Moving slowly up and down, the beads are attracted to the cover, and (III) by moving the covered magnet to the next position, the beads are transferred to a new solution. (IV) Once the magnet is removed from the cap, the beads are slowly suspended again. (V) The magnet head and plastic covers are raised to the starting position to proceed to the next stage of the process

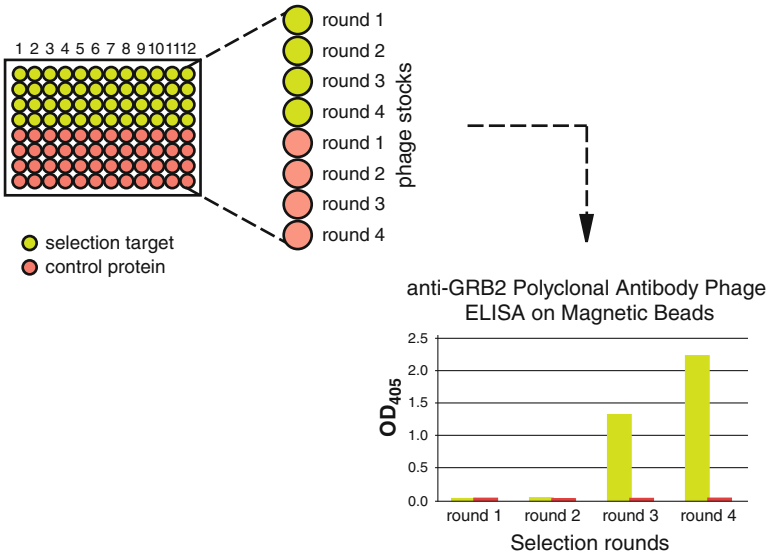


Fig. 18.2 Magnetic particle ELISA for monitoring polyclonal antibody phage enrichment. On the left, the plate layout for 12 individual selections is shown. For each selection, the phage stock solutions are split and pipetted into the positions A–D and E–H of the same column, respectively. Next, selection target-coated or control protein-coated magnetic beads are added to the phage solutions as indicated. All steps of the ELISA are performed in a 96-well magnetic particle processor. On the right, the enrichment of specifically binding antibody phage during four rounds of selection on GRB2 is shown. Specific enrichment is seen in rounds 3 and 4

Table 18.3 Automated magnetic bead-based polyclonal antibody ELISA protocol for Kingfisher 96

Plate no.	Plate name	Work step	Volume (μL)	Incubation time (min)
1	Bead plate	Blocking of antigen-loaded and control magnetic beads with PTM	200	60
2	Phage plate	Incubation of magnetic beads in antibody phage stocks of the selection rounds	200	60
3	Wash plate 1	Wash 1 of magnetic beads in PBST	200	10
4	Wash plate 2	Wash 2 of magnetic beads in PBST	200	10
5	Antibody plate	Incubation of antigen-loaded and control beads with mouse anti-M13 monoclonal Antibody, HRP-conjugated, 1:5000 in PTM	200	60
6	Wash plate 3	Wash 3 of magnetic beads in PBST	200	10
7	Wash plate 4	Wash 4 of magnetic beads in PBST	200	10
8	Substrate plate	Incubation of magnetic beads in ABTS-containing substrate buffer for horseradish peroxidase[a]	200	20
Total time:				240

[a]Prior to measurement of extinction, beads are transferred back to Wash plate 4

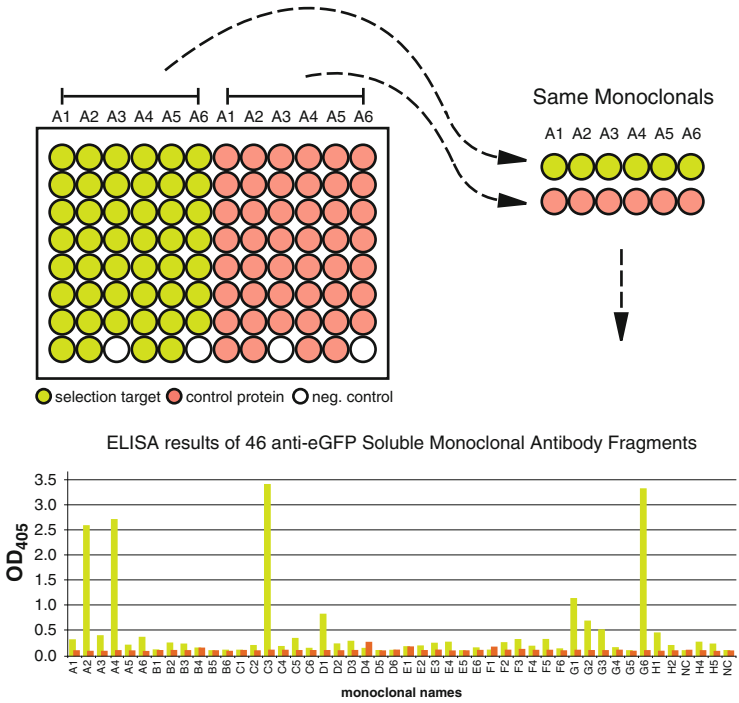


Fig. 18.3 ELISA design for evaluation of 46 soluble monoclonal antibody fragments. On the top, the layout for antigen coating is shown. The soluble monoclonal antibody fragments of the plate positions A1–H6 (or A7–H12, respectively) are added to each half of the Assay plate. On the bottom, the results for 46 anti-eGFP soluble antibody fragments are shown. All clones with a >tenfold signal to background ratio are considered as positive. In this case, all clones with an $OD_{405} > 1.0$

2000; Krebs et al. 2001; Hallborn and Carlsson 2002; Buckler et al. 2008; Turunen et al. 2009).

18.2 Materials

18.2.1 Loading of Magnetic Beads

- Dynabeads® M-280 Streptavidin (Invitrogen Dynal AS, Oslo, Norway)
- Phosphate-buffered saline (PBS): 8 g/L NaCl, 0.2 g/L KCL, 1.44 g/L $Na_2HPO_4 \cdot 2\ H_2O$, and 0.24 g/L KH_2PO4, pH 7.4
- Phosphate-buffered saline Tween (PBST): PBS + 0.1% Tween-20

18.2.2 Semi-automated Panning using a Magnetic Particle Processor

- *E. coli* TG1, genotype: K12 Δ*(lac-proAB) supE thi hsdD5*/F' *traD36 proA+B lacIq lacZΔM15*
- 96-well V-bottom polypropylene (PP) microtiter plates (Nunc, Wiesbaden, Germany)
- 96-well U-bottom polypropylene (PP) microtiter plates (Nunc, Wiesbaden, Germany)
- AeraSeal breathable sealing film (Sigma-Aldrich, Taufkirchen, Germany)
- Phosphate-buffered saline Tween Milkpowder (PTM): PBS + 1% Tween-20 + 2% nonfat dry milkpowder, prepare fresh
- 2YT medium: 1.6% (w/v) tryptone, 1% (w/v) yeast extract, and 0.5% NaCl, pH 7.0
- 10× Amp/Glu solution: 1 mg/mL ampicilin, and 20% (w/v) glucose in 2YT medium

18.2.3 Packaging of Phagemids

- M13K07 Helperphage (New England BioLabs, Frankfurt, Germany)
- 96-well filtration plate: MultiScreen$_{HTS}$ Plates with hydrophilic Durapore PVDF membrane with 0.65 μm pore size (Millipore, Schwalbach/Ts, Germany)
- 2YT-AG-2: 2YT medium containing 100 μg/mL ampicilin, 2% (w/v) glucose
- 2YT-AKG: 2YT medium containing 100 μg/mL ampicilin, 60 μg/mL kanamycin, 0.1% (w/v) glucose
- Glycerol solution: 80% (w/v)

18.2.4 Titration of Phage Particles

- 2YT-AG agar plates: 2YT medium containing 100 μg/mL ampicilin, 2% (w/v) glucose, and 1.5% (w/v) agar-agar
- 2YT-K agar plates: 2YT medium containing 60 μg/mL kanamycin, and 1.5% (w/v) agar-agar

18.2.5 Magnetic Particle ELISA of Polyclonal Antibody Phage

- Matrix 96-well polystyrene microtiter plates (Thermo Scientific, Dreieich, Germany)

- Anti-M13, horseradish peroxidise (HRP)-conjugated monoclonal antibody (GE Healthcare, München, Germany)
- Substrate buffer: 50 mM citrate buffer, pH 4.3. Mix 1:2 50 mM trisodium citrate and 50 mM citric acid shortly before use
- ABTS (2,2′-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium tablets (Sigma, Deisenhofen, Germany)
- Hydrogen peroxide (Perhydrol, 30% H_2O_2; Merck, Darmstadt, Germany)

18.2.6 Production of Soluble Monoclonal Antibody Fragments in Microtiter Plates

- *E. coli* HB2151, genotype: K12 *ara* Δ*(lac-proAB) thi*/F′ *proA+B lacIq lacZΔM15*
- 2YT-AG-0.1: 2YT medium containing 100 μg/mL ampicilin, 0.1 % (w/v) glucose
- 20 mM isopropyl-β-D-thiogalactopyranoside (IPTG)

18.2.7 ELISA of Soluble Monoclonal Antibody Fragments in Microtiter Plates

- Bovine Serum Albumin (BSA): 10 mg/mL stock solution in PBS
- Recombinant Protein L, horseradish peroxidise (HRP)-conjugated (Pierce, Thermo Scientific, Bonn, Germany)

18.3 Methods

All the protocols in this section are designed to allow handling of multiple selection targets in parallel. The protocols were set up with the human single-fold scFv libraries, I and J (MRC Cambridge, UK; see Sect. 18.5, Note 1). The complete phage display selection procedure could be streamlined to require only minimal user intervention. Conventional panning procedures require repetitive rounds of tedious phage infection and propagation, which makes large sample screening cumbersome. With the standard operating procedures and standardized templates outlined here, the migration from automated selection to manual biological processes throughout the selection procedure becomes straightforward. The 96-well microtiter plate layout allows the protocol to be adaptable for manual use by employing multichannel pipettes. When applied, the semi-automated method will provide high-throughput results with maximum convenience and minimal handling.

18.3.1 Loading of Magnetic Beads

This protocol provides sufficient antigen-coated beads per target to perform up to five rounds of semi-automated panning for antibody phage enrichment and the following polyclonal antibody phage ELISA (see Sect. 18.3.5). All steps are carried out in 1.5 mL Eppendorf cups and for ease of use, a magnetic stand and rotator is required (see Sect. 18.5, Note 2).

1. Take 1 mg (100 µL) Dynabeads M-280 Streptavidin magnetic beads and wash 3 × 5 min (min) with 1.5 mL PBST and 1 × 5 min with 1.5 mL PBS at room temperature (RT). Meanwhile, dissolve (a) 100–200 µg biotinylated protein antigen (see Sect. 18.5, Note 3) in 1 mL PBS, or alternatively, (b) 1–2 µg biotinylated peptide antigen in 1 mL PBS. Discard wash solution, resuspend magnetic beads gently in the 1 mL antigen solution, and incubate for 1 h at RT on a rotator. Alternatively, the incubation can be performed overnight at 4°C on a rotator
2. Take off antigen solution and wash magnetic beads 3 × 5 min with 1.5 mL PBST
3. Finally, discard last wash solution, resuspend magnetic beads in 200 µL PBS, and store *antigen-loaded bead stock* until further use at 4°C

18.3.2 Semi-automated Panning on Magnetic Particle Processor

For simplicity, the protocol refers to parallel selection on 12 antigens, which will be arranged throughout the whole protocol in the positions A1–A12 of a 96 well microtiter plate used in a Kingfisher 96 instrument. The number of plates and steps for each selection round is summarized in Table 18.1. As an example, the automated magnetic bead-based panning protocol on the Kingfisher 96 for the fourth round of selection is summarized in Table 18.2. If more or fewer selections are performed in parallel, add or remove microtiter plate positions accordingly.

Since the selection of specific binders occurs on magnetic beads, the microtiter plates are only reaction vessels. Therefore, the magnetic bead-based selection protocol can, in principle, be applied in all available magnetic particle processors employing the basic concept of moving beads from vessel to vessel (Fig. 18.1b).

1. Inoculate 5 mL of 2YT in a 15 mL polypropylene tube with a single clone of TG1 from an agar plate and grow shaking overnight at 37°C and 250 rpm (see Sect. 18.5, Note 4)
2. Inoculate 50 mL 2YT in a 250 mL Erlenmeyer flask with 0.5 mL of a fresh overnight TG1 culture and incubate shaking at 37°C and 250 rpm until OD_{600} = 0.4–0.5 (see Sect. 18.5, Note 5)

3. Arrange *bead-plate*. Fill positions A1–A12 of a 96-well V-bottom PP (PP) microtiter plate with 180 µL PTM, and for each antigen, add 20 µL from corresponding *antigen-loaded bead stocks* (see Sect. 18.3.1) to specified position. Add magnetic beads of antigen 1 to positions A1, beads of antigen 2 to positions A2, and so on
   For the first round of selection, continue with step 4. For later selection rounds, continue with step 7
4. Preincubate unselected antibody phage library with empty magnetic beads in PTM to deplete selection matrix binders. In a 15 mL PP tube, add 2 mg (200 µL) Dynabeads M-280 Streptavidin to 1×10^{13}–3×10^{13} phage particles in 10 mL PTM. Incubate for 1–2 h at RT on a rotator
5. Collect beads at the bottom of the tube by 2 min centrifugation, 2,000 rpm, and carefully transfer antibody phage library solution to a new 15 mL PP tube. Discard magnetic beads
6. Arrange *phage-plate for first round*. Fill positions A1–A12 of a 96-well V-bottom PP microtiter plate with 200 µL of the antibody phage library solution. Continue with step 8
7. Arrange *phage-plate for remaining rounds*. Fill positions A1–A12 of a 96-well V-bottom PP microtiter plate with 100 µL PTM. Add 100 µL of the amplified phage solutions of the previous round (see Sect. 18.3.3) according the same antigen order in positions A1–A12
8. Prepare *wash plate(s)*. Fill positions A1–A12 of a 96-well V-bottom PP microtiter plates with 200 µL PBST. Adjust number of plates according to Table 18.1 (see Sect. 18.5, Note 6)
9. Prepare *release plate*. Fill positions A1–A12 of a 96-well V-bottom PP microtiter plates with 200 µL PBS
10. Place plates in the Kingfisher 96 instrument according to the plate positions in Table 18.1 and start magnetic bead-based panning program. The program should be set to move magnetic beads from plate to plate and incubate the beads in each plate as indicated in Table 18.2. During all incubations, the beads should be kept in suspension by moving plastic tips up and down in the wells at medium speed (30–50 mm/s). The program ends by releasing the beads in the release plate (see Sect. 18.5, Notes 7 and 8)
11. Once the panning program has finished, prepare *E. coli culture plate*. Fill positions A1–A12 of a 96-well U-bottom PP microtiter plates with 200 µL of *E. coli* TG1 ($OD_{600} = 0.4$–0.5), place *E. coli culture plate* in Kingfisher 96 instrument and start Transfer Program. This program simply transfers the beads from the release plate to the *E. coli* culture plate
12. Take out *selection stock plate* from the Kingfisher 96 instrument, cover with plastic lid, and incubate for 30 min at 37°C (see Sect. 18.5, Note 9)
13. Add 20 µL 10× Amp/Glu solution, seal with breathable sealing film, and incubate in a microplate shaker for 2 h at 37°C and 1,400 rpm (see Sect. 18.5, Note 10)
14. Directly proceed with Packaging of Phagemids protocol, Sect. 18.3.3

18.3.3 Packaging of Phagemids

The steps described in this section are directly connected to the semi-automated selection protocol in Sect. 18.3.2. If packaging of phagemids from glycerol stock plates is required, see Sect. 18.5, Note 11.

1. Take *selection stock plate* from Sect. 18.3.2, and add 200 μL of prewarmed 2YT-AG-2 medium (37°C) to culture, mix thoroughly, and transfer 200 μL into 96-well filtration plate. Seal *selection stock plate* again with breathable sealing film and continue incubation in a microplate shaker overnight at 37°C and 1,200 rpm
2. To the filtration plate, add 20 μL M13K07 helperphage (~ 10^9 phage particles), cover with plastic lid, and incubate stationary for 30 min at 37°C (see Sect. 18.5, Note 12)
3. Place filtration plate on top of a 96-well U-bottom PP microtiter plate and fix with sticky tape. Prepare a counter balance plate in similar fashion
4. Filter bacterial culture by centrifugation in microtiter plate holders (swing out rotor) for 2–5 min at 2,000 rpm (see Sect. 18.5, Note 13)
5. Discard filtrate with remaining M13K07 helperphage
6. Resuspend bacteria in 220 μL prewarmed 2YT-AKG (30°C) and transfer to a fresh 96-well U-bottom PP microtiter plate. Seal *phage production plate* with breathable sealing film and incubate in a microplate shaker overnight at 30°C shaking at 1,400 rpm
7. The next day, add 160 μL glycerol solution to *selection stock plate*, mix, and store as glycerol stock at −80°C
8. Pellet bacteria in *phage production plate* by centrifugation for 10 min at 2,000 rpm. Transfer supernatant carefully without disturbing the pellet to a 96-well filtration plate
9. Place filtration plate on top of a new 96-well U-bottom PP microtiter plate and fix with sticky tape
10. Filter antibody presenting phage particles to remove possible contaminating *E. coli* cells by centrifugation for 2–5 min at 2,000 rpm
11. Store filtrate (*phage stock plate*) and discard bacteria pellets and used filtration plate
12. Add 50 μL PBS to each well of the *phage stock plate* and mix thoroughly. Use 100 μL for the next round of selection (see Sect. 18.3.2), use 10 μL for phage titration (see Sect. 18.3.4). Seal *phage stock plate* carefully with sticky tape and store until further use at 4°C

18.3.4 Titration of Phage Particles

During the selection process, the success of phage particle amplification before each following selection round is monitored by titration. Additionally, the titer of

bead-bound antibody phages during panning can also be monitored (see Sect. 18.5, Note 8).

1. Inoculate 5 mL of 2YT in a 15 mL PP tube with a single clone of TG1 from an agar plate and grow, shaking overnight at 37°C and 250 rpm (see Sect. 18.5, Note 4)
2. Inoculate 50 mL 2YT in a 250 mL Erlenmeyer flask with 0.5 mL of overnight TG1 culture and incubate, shaking at 37°C and 250 rpm until $OD_{600} = 0.4–0.5$ (see Sect. 18.5, Note 5)
3. Prepare a 1:10 serial dilution (until 10^{-9}) of enriched phage libraries from selection rounds (*phage stock plate*, see Sect. 18.3.3) by adding 10 µL phage to 90 µL PBST in a 96-well U-bottom PP microtiter plate (see Sect. 18.5, Note 12)
4. Add 100 µL of *E. coli* TG1 ($OD_{600} = 0.4–0.5$) to phage dilutions 10^{-5}–10^{-9}, cover with plastic lid, and incubate stationary for 30 min at 37°C
5. Mix infected *E. coli* cultures and plate 10 µL droplets of each dilution series on a single 2YT-AG and 2YT-K agar plates per enriched library. Once droplets are dried, incubate plates top-down overnight at 37°C (see Sect. 18.5, Note 14)
6. Next day, count the number of colonies in the droplets on all plates, and calculate from these the colony forming units (infectious phage particles/mL) using the formula:

$$\text{c.f.u.} = \text{numberofcolonies} \times \text{dilutionfactor} \times 100$$

   On an average, phage preparations in microtiter plates (200 µL culture volume) produce 10^{10}–10^{11} c.f.u.
7. Compare the c.f.u. values obtained on 2YT-AG and 2YT-K agar plates for each phage library. The helperphage genome containing population should be a minimum of 4–5 orders of magnitude smaller than the antibody fragment containing phagemid population

18.3.5 Magnetic Particle ELISA of Polyclonal Antibody Phage

The polyclonal antibody phage ELISA for evaluation of enrichment success is performed using a magnetic particle processor to maintain similar conditions as the initial selection process. At a maximum, polyclonal ELISA for 12 independent selections over four rounds can be performed simultaneously with the appropriate negative control. Proposed plate layout for 12 ELISAs in parallel and an example of a polyclonal ELISA result are shown in Fig. 18.2. The ELISA protocol on the Kingfisher 96 instrument takes 4 h and is summarized in Table 18.3.

1. Arrange *bead-plate*. Fill each position of a 96-well V-bottom PP microtiter plate with 180 µL PTM and add 20 µL of *antigen-loaded bead stock* (see

Sect. 18.3.1) according to plate layout in Fig. 18.2. Add magnetic beads of antigen 1 to positions A1–D1, beads of antigen 2 to positions A2–D2, and so on
2. As negative control, empty beads are used. Take 5 mg (500 µL) Dynabeads M-280 Streptavidin magnetic beads and wash 3 × 5 min with 1.5 mL PBST and 1 × 5 min with 1.5 mL PBS at RT. Discard last wash solution and resuspend in 1 mL. Add 20 µL to positions E1–H12
3. Arrange *phage-plate*. Fill each position of a 96-well V-bottom PP microtiter plate with 150 µL PTM. Add 50 µL of phage solution from the *phage stock plates* of the individual rounds to plate according layout in Fig. 18.2. Add phage stocks of selection rounds 1–4 on antigen 1 to position A1–D1 and E1–H1, respectively. Add phage stocks of selection rounds 1–4 on antigen 2 to position A2–D2 and E2–H2, respectively, and so on
4. Prepare *wash plates 1–3*. Fill 96-well V-bottom PP microtiter plates with 200 µL PBST
5. Prepare *wash plate 4*. Fill 96-well V-bottom PP microtiter plates with 200 µL PBS
6. Prepare *antibody plate*. Add 4 µL mouse monoclonal anti-M13, HRP-conjugated, to 20 mL PTM (1:5,000). Fill 96-well V-bottom PP microtiter plates with 200 µL antibody solution
7. Place plates in the Kingfisher 96 instrument and start magnetic bead-based ELISA program. The program should be set to move magnetic beads from plate to plate and incubate the beads in each plate as indicated in Table 18.3. During all incubations, the beads should be kept in suspension by moving plastic tips up and down in the wells at medium speed (30–50 mm/s)
8. While ELISA program is running, prepare *substrate plate*. Dissolve one ABTS tablet (10 mg) in 20 mL substrate buffer. Shortly after the antibody plate incubation step in the ELISA process is finished, add 10 µL hydrogen peroxide to substrate solution and pipette 200 µL to each well of a Matrix 96-well polystyrene microtiter plates (see. Sect. 18.5, Note 15) and place plate in Kingfisher 96
9. Once beads are incubated in the substrate and color developed for 20 min, beads are removed from the substrate by transferring them back to *wash plate 4*
10. Take out Substrate plate from the Kingfisher 96 instrument and measure substrate specific extinction at 405 nm in an ELISA reader (see Sect. 18.5, Note 16)
11. For each individual selection target, evaluate enrichment by plotting the obtained values for antigen-loaded and control beads of each phage selection rounds next to each other as depicted in Fig. 18.2

18.3.6 Production of Soluble Monoclonal Antibody Fragments in Microtiter Plates

Prior to the production of soluble monoclonal antibody fragments, individual clones are picked and the *E. coli* host strain is switched (see Sect. 18.5, Note 17).

1. Inoculate 5 mL of 2YT in a 15 mL PP tube with a single clone of HB2151 from an agar plate and grow shaking overnight at 37°C and 250 rpm. (see Sect. 18.5, Note 4)
2. Inoculate 50 mL 2YT in a 250 mL Erlenmeyer flask with 0.5 mL of overnight HB2151 culture and incubate shaking at 37°C and 250 rpm until OD_{600} = 0.4–0.5. (see Sect. 18.5, Note 5)
3. Meanwhile, prepare a 1:10 dilution series of the desired panning round from the corresponding *phage stock plate* (see Sect. 18.3.3) by adding 10 µL phage to 90 µL PBST (see Sect. 18.5, Note 12)
4. Add 100 µL of *E. coli* HB2151 (OD_{600} = 0.4–0.5) to phage dilutions 10^{-5}–10^{-8} and incubate for 30 min at 37°C
5. Mix infected *E. coli* cultures and plate 100 µL of each dilution series on a 2YT-AG agar plate. Once dried, incubate plates top-down overnight at 37°C
6. Pick 92 clones into 96-well U-bottom PP microtiter plate filled with 200 µL 2YT-AG-2. Leave positions H3, H6, H9, and H12 empty for controls. Seal *mother plate* with breathable sealing film and incubate in a microplate shaker overnight at 37°C and 1,400 rpm (see Sect. 18.5, Note 10)
7. Next day, inoculate fresh 96-well U-bottom PP microtiter plate containing 180 µL 2YT-AG-0.1 with 20 µL of the overnight culture and incubate *daughter plate* for 2 h at 37°C and 1,400 rpm
8. Add 150 µL glycerol solution to each well of the *mother plate* and store as glycerol stock at −80°C
9. Induce soluble antibody fragment production in *daughter plate* by adding 11 µL 20 mM IPTG (final conc. 1 mM) to each well and continue incubating overnight at 30°C and 1,400 rpm
10. Pellet bacteria by centrifugation of microtiter plates for 10 min at 3,000 rpm (see Sect. 18.5, Note 13)
11. Transfer soluble monoclonal antibody fragment containing culture supernatant into fresh 96-well U-bottom PP microtiter plate and store until further use at 4°C. Discard pellet-containing plate

18.3.7 ELISA of Soluble Monoclonal Antibody Fragments in Microtiter Plates

For each microtiter plate of soluble monoclonal antibody fragments (92 clones), two ELISA plates must be prepared. 46 individual clones (culture plate positions A1–H6 and A6–H12, respectively) will be evaluated per ELISA plate to allow for monitoring unspecific binding on a respective negative control antigen. Proposed plate layout and an example for a monoclonal ELISA result are shown in Fig. 18.3.

1. Coat half of a Matrix 96-well microtiter plate (positions A1–H6) by transferring (a) 1–2 µg protein antigen in 100 µL PBS or (b) 10–20 ng peptide antigen

in 100 μL PBS to each well. At the same time, coat the other half of the plate (positions A7–H12) with 100 μL/well of an appropriate negative control, such as Bovine Serum Albumin (10 μg/mL in PBS) or PTM and incubate microtiter plate overnight at 4°C
2. Discard coating solution and wash wells 2 × 5 min by completely filling them with PBST (see Sect. 18.5, Note 18)
3. Block wells by completely filling them with PTM and incubate for 1 h at RT
4. Discard blocking solution and wash wells 3 × 5 min by completely filling them with PBST
5. Fill each well with 50 μL PTM and add 50 μL soluble antibody fragment solution of the respective 46 clones to each half of the plate (containing target antigen and a negative control, respectively) and incubate for 1 h at RT. For ease of use and to avoid pipetting errors, use an eight-channel micropipette
6. Discard soluble antibody fragment solution and wash wells 3 × 5 min by completely filling them with PBST
7. Add 100 μL of recombinant Protein L-HRP (1:5,000 in PTM) to each well and incubate for 1 h at RT (see Sect. 18.5, Note 19)
8. Discard recombinant Protein L-HRP solution and wash wells 3 × 5 min with PBST and 2 × 5 min with PBS by completely filling them
9. Meanwhile, prepare substrate by dissolving one ABTS tablet (10 mg) in 20 mL substrate buffer. Immediately prior use, add 10 μL hydrogen peroxide to the substrate solution
10. Finally, add 100 μL of substrate to each well and leave to develop (change to dark green color) for 5–30 min at RT in the dark
11. Measure substrate-specific extinction at 405 nm in an ELISA reader (see Sect. 18.5, Note 16)
12. Plot the obtained values for antigen and negative control protein for each soluble monoclonal antibody fragment next to each other and identify positive candidates with an acceptable (usually >tenfold) signal to background ratio (Fig. 18.3)

18.4 Results and Conclusion

This semi-automated magnetic bead-based selection protocol allows the isolation of recombinant antibodies from phage display libraries against multiple targets simultaneously. The throughput is dependent on which magnetic particle processor is available in the laboratory. Currently, the highest number of parallel selections can be performed on Thermo Scientific's Kingfisher 96 and derivatives (Kingfisher Flex or Qiagen Biosprint 96). Essentially, any of these processors can be used to perform phage display selections. The selection protocol can be amended to any of the available systems since the key parameters, such as incubation time, speed of motion, and number and volume of washing steps, can be programmed accordingly.

Next to the automated panning protocol, a number of key methods are designed to accommodate the throughput of multiple simultaneous selections. Phage infection, propagation, isolation, and titration protocols are simplified and adapted for use on microtiter plate format. An additional benefit is the reduction of time required per selection round with streamlined processes. A single selection round can be performed within a day, thus allowing the entire panning process to be completed in a week. For the evaluation steps consisting of polyclonal antibody phage ELISA and soluble monoclonal antibody fragment ELISA, standard operating procedures have been set up. Use of standardized plate layouts and Microsoft Excel Worksheet templates allows fast and easy data handling of a multitude of assay results. Figure 18.2 shows a typical ELISA result highlighting the enrichment patterns of antibody phage selection over four rounds. Enrichment is frequently observed starting in round 3 and/or round 4. A typical result for soluble monoclonal antibody fragment ELISA is shown in Fig. 18.3. Signal intensities as well as signal-to-background ratios can vary because of the amount and quality of the selection target.

As an alternative to performing selections against up to 96 target proteins in parallel, the number of selection targets can be reduced and the complexity of selection parameters increased. For instance, biological parameters can be changed, i.e., different antibody libraries can be applied, or different helper phage systems can be used for initial packaging of the same library (e.g., Hyperphage versus M13K07; see Soltes et al. 2007), or even different *E. coli* strains can be used for phage propagation. Furthermore, different buffer conditions or counter selection strategies can be introduced. This could be of great value, for example, when antibodies against certain splice variants or post translational modifications of a protein need to be selected. In such a case, increased amounts of a nonbiotinylated unwanted protein variant can be added at various steps for counter selection, such as washing steps or even the selection step of rounds 2–4 itself.

In summary, the semi-automated selection protocol allows performing up to 96 phage display selections in parallel. While its strength is the use of standard operating procedures in respect to technical parameters of magnetic bead handling, the system allows a high degree of flexibility in selection design. Until now, we have successfully applied the protocols described here to select antibody fragments against many different types of targets, such as peptides, recombinant or homologous proteins, or chemical compounds.

18.5 Notes and Troubleshooting

1. The human single-fold scFv libraries I and J (Tomlinson I and J) were created in Greg Winter's lab at the MRC Laboratory of Molecular Biology and the MRC Center for Protein Engineering (Cambridge, UK). Further information on the libraries can be found at the distributor's website: http://www.geneservice.co.uk/products/proteomic/scFv_tomlinsonIJ.jsp (Cited 7 May 2009). Using

other than these combinatorial antibody phage display libraries might need some library-specific adaptation to the individual protocols.

2. The beads are retained in the cups while exchanging solutions by placing cups in a magnetic stand, such as a DynaMag™-2 magnet (Invitrogen Dynal AS). Incubations are carried out using a rotator, such as Rotators SB2 or SB3 (Carl Roth, Karlsruhe, Germany).
3. In case the target antigen is not already biotinylated, it can be in vitro biotinylated with commercially available biotinylation reagent kits, such as the NHS-SS-Biotin (sulfosuccinimidyl-2-(biotinamido)ethly-1,3-dithiopropionate) from Pierce.
4. Maintenance of F' episome in TG1 and HB2151 cells requires regular passaging on Minimal (M9) agar plates. For details see Sambrook et al. 1989.
5. For infection, filamentous bateriophages require the *E. coli* cells to possess pili. *E. coli* cells carrying the F' episome form pili mainly during the logarithmic growth phase. If cells reach $OD_{600} = 0.4–0.5$ before they are needed, cells can be arrested with formed pili for ~30 min by placing on ice.
6. The wash volume can be increased up to 1 mL when using Deep Well 96 Plates (Thermo Scientific). Further, the number of wash plates per selection cycle can be freely changed or washing solutions can be varied (see Sect. 18.4).
7. Incubation times, speed of up-and-down movement, and incubation temperature can be varied. If incubation times exceed 30 min, some types of beads can settle at the bottom of the plate. To circumvent this, recollect beads every 20–30 min, during the incubation, with magnet.
8. If titration of bound phages during the panning protocol is desired, take 10 μL of magnetic bead suspension from release plate and titer according to Sect. 18.3.4.
9. Once the *E. coli culture plate* is infected with phage particles (on magnetic beads), we refer to it as *selection stock plate*.
10. Dedicated microplate incubator shakers, such as iEMS (Thermo Scientific) or PST-60HL-4, Lab4You, Berlin, Germany) are able to shake >1,200 rpm, ensuring best possible aeration of the cultures in combination with breathable sealing tapes. This is of highest importance during phage particle production but also beneficial during soluble antibody fragment production.
11. Prepare a fresh overnight culture from *selection stock plate* glycerol stock (see Sects. 18.3.2 and 18.3.3) by inoculating fresh 96-well U-bottom PP microtiter plate containing 180 μL 2YT-AG-2 with 20 μL of glycerol stock and incubate overnight at 37°C and 1,400 rpm. Next day, inoculate fresh 96-well U-bottom PP microtiter plate containing 200 μL 2YT-AG-0.1 with 20 μL of the overnight culture and incubate plate for 2 h at 37°C and 1,400 rpm. Transfer culture into 96-well filtration plate and continue with step 2 of Sect. 18.3.3.
12. Filter tips should be used throughout all experimental steps involving phage particles.
13. Microtiter plates can be centrifuged in Eppendorf 5810 R with swing out rotor A-4-62 and microplate holders. As an alternative to centrifugation, a

MultiScreen$_{HTS}$ Vacuum Manifold (Millipore) can be used to separate phage particle containing media from bacterial cell mass.

14. In addition to the phage dilutions, all used solutions should be assayed for phage contamination (mock-infections) and should be plated out together with the noninfected *E. coli* on selective agar plates.
15. To avoid magnetic beads sticking to polystyrene surface of Matrix 96-well plate, block wells by completely filling them with PTM and incubate for 1 h at RT prior use.
16. If function is available, set reader to shake/mix plate before reading. This will ensure proper dispersion of the developed color due to enzymatic breakdown of the substrate.
17. Host strain switching is advantageous for high level expression of antibody fragments from the Tomlinson libraries I and J, since an amber stop codon is inserted between the antibody fragment and the gIII. In the *E. coli* strain HB2151, this amber stop is not suppressed, and therefore, only antibody fragments without pIII fusion are produced. Furthermore, expression of the phage coat protein pIII can be toxic for the host at higher concentrations.
18. If available, microtiter plates can be washed using an ELISA washer (e.g., TECAN Columbus Plus, Crailsheim, Germany). Otherwise, microtiter plates can be washed manually by either filling the plates with a multichannel pipette or by submerging plates in wash solution filled plastic tanks (2–5 L volume). Plates are emptied by simply shaking out the plates after each washing step into a waste tank and tapped dry on a clean linen-free towel.
19. Recombinant Protein L binds only to human V-Kappa light chains. In cases other than where the Tomlinson I and J antibody phage display libraries are used, the recombinant Protein L-HRP needs to be substituted with an appropriate, tag-dependent detection antibody, e.g. a mouse anti-myc-tag monoclonal antibody (9E10, SIGMA-ALDIRCH).

Acknowledgments This work was supported by the German Federal Ministry for Education and Research (BMBF) through the National Genome Research Network (NGFN-II) project "Antibody Factory" (Grant No. 01GR0427) and the Max Planck Society. ZK acknowledges additional support from EU-FP6 CA "Proteome Binders" (RICA. 026008). TSL gratefully acknowledges financial support from the Ministry of Higher Education Malaysia and Institute for Research in Molecular Medicine, University Science Malaysia.

References

Barbas CF III, Kang AS, Lerner RA, Benkovic SJ (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci USA 88:7987–7992

Boder ET, Wittrup KD (1997) Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol 15:533–557

Breitling F, Dübel S, Seehaus T, Klewinghaus I, Little M (1991) A surface expression vector for antibody screening. Gene 104:147–153

Buckler DR, Park A, Viswanathan M, Hoet RM, Ladner RC (2008) Screening isplates from antibody phage display libraries. Drug Discov Today 13:318–324

Cohen S, Trinka RF (2002) Fully automated screening systems. Methods Mol Biol 190:213–228

de Wildt RM, Mundy CR, Gorick BD, Tomlinson IM (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. Nat Biotechnol 18:989–994

Dübel S (2007) Handbook of therapeutic antibodies. Wiley-VCH, Weinheim

Fukuda I, Kojoh K, Tabata N, Doi N, Takashima H, Miyamoto-Sato E, Yanagawa H (2006) In vitro evolution of single-chain antibodies using mRNA display. Nucleic Acids Res 34:e127

Hallborn J, Carlsson R (2002) Automated screening procedure for high-throughput generation of antibody fragments. Biotechniques Suppl: 30–37

Hamilton S (2002) Introduction to screening automation. Methods Mol Biol 190:169–193

Hanes J, Plückthun A (1997) In vitro selection and evolution of functional proteins by using ribosome display. Proc Natl Acad Sci USA 94:4937–4942

Hawlisch H, Müller M, Frank R, Bautsch W, Klos A, Köhl J (2001) Site-specific anti-C3a receptor single-chain antibodies selected by differential panning on cellulose sheets. Anal Biochem 293:142–145

Hust M, Maiss E, Jacobsen HJ, Reinard T (2002) The production of a genus specific recombinant antibody (scFv) using a recombinant potyvirus protease. J Virol Methods 106:225–233

Kala M, Bajaj K, Sinha S (1997) Magnetic bead enzyme-linked immunosorbent assay (ELISA) detects antigen-specific binding by phage-displayed scFv antibodies that are not detected with conventional ELISA. Anal Biochem 254:263–266

Konthur Z (2007) Automation of Selection and Engineering. In: Dübel S (ed) Handbook of therapeutic antibodies. Wiley-VCH, Weinheim, pp 413–431

Konthur Z, Crameri R (2003) High-throughput application of phage display in proteomic analyses. Targets 2:261–270

Konthur Z, Walter G (2002) Automation of phage display for high-throughput antibody development. Targets 1:30–36

Konthur Z, Hust M, Dübel S (2005) Perspectives for systematic in vitro antibody generation. Gene 364:19–29

Krebs B, Rauchenberger R, Reiffert S, Rothe C, Tesar M, Thomassen E, Cao M, Dreier T, Fischer D, Höss A, Inge L, Knappik A, Marget M, Pack P, Meng XQ, Schier R, Söhlemann P, Winter J, Wölle J, Kretzschmar T (2001) High-throughput generation and engineering of recombinant human antibodies. J Immunol Methods 254:67–84

Li M (2000) Applications of display technology in protein analysis. Nat Biotechnol 18:1251–1256

McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348:552–554

McConnell SJ, Dinh T, Le MH, Spinella DG (1999) Biopanning phage display libraries using magnetic beads vs. polystyrene plates. Biotechniques 26(208–10):214

Menke KC (2002) Unit automation in high throughput screening. Methods Mol Biol 190: 195–212

Mondon P, Dubreuil O, Bouayadi K, Kharrat H (2008) Human antibody libraries: a race to engineer and explore a larger diversity. Front Biosci 13:1117–1129

Noppe W, Plieva F, Galaev IY, Pottel H, Deckmyn H, Mattiasson B (2009) Chromato-panning: an efficient new mode of identifying suitable ligands from phage display libraries. BMC Biotechnol 9:21

Parmley SF, Smith GP (1988) Antibody selectable filamentous fd phage vectors: affinity purification of target genes. Gene 73:305–318

Rhyner C, Konthur Z, Blaser K, Crameri R (2003) High-throughput isolation of recombinant antibodies against recombinant allergens. Biotechniques 35:672–674

Sambrook T, Fritsch FF, Maniatis T (1989) Molecular cloning – A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Soltes G, Hust M, Ng KK, Bansal A, Field J, Stewart DI, Dübel S, Cha S, Wiersma EJ (2007) On the influence of vector design on antibody phage display. J Biotechnol 127:626–637

Taussig MJ, Stoevesandt O, Borrebaeck CA, Bradbury AR, Cahill D, Cambillau C, de Daruvar A, Dübel S, Eichler J, Frank R, Gibson TJ, Gloriam D, Gold L, Herberg FW, Hermjakob H, Hoheisel JD, Joos TO, Kallioniemi O, Koegl M, Konthur Z, Korn B, Kremmer E, Krobitsch S, Landegren U, van der Maarel S, McCafferty J, Muyldermans S, Nygren PA, Palcy S, Plückthun A, Polic B, Przybylski M, Saviranta P, Sawyer A, Sherman DJ, Skerra A, Templin M, Ueffing M, Uhlen M (2007) ProteomeBinders: planning a European resource of affinity reagents for analysis of the human proteome. Nat Methods 4:13–17

Thie H, Meyer T, Schirrmann T, Hust M, Dübel S (2008) Phage display derived therapeutic antibodies. Curr Pharm Biotechnol 9:439–445

Turunen L, Takkinen K, Söderlund H, Pulli T (2009) Automated panning and screening procedure on micorplates for antibody generation from phage display libraries. J Biomol Screen 14: 282–293

Walter G, Konthur Z, Lehrach H (2001) High-throughput screening of surface displayed gene products. Comb Chem High Throughput Screen 4:193–205

Part IV
Engineering and Production of Immunoglobulins

Chapter 19
Aspects of Isotype Selection

Zehra Kaymakcalan, Alexander Ibraghimov, Andrew G. Goodearl, and Jochen G. Salfeld

19.1 Introduction

Advances in recombinant antibody technology allow for the design of novel therapeutic drugs that were not even considered possible 10 years ago. However, the widespread use of therapeutic antibodies has also highlighted the need for a more refined mechanistic understanding of their function *in vivo*. It is noteworthy that the exploration of the true *in vivo* mechanism of action of one the most successful antibodies, rituximab, has spanned nearly a decade, and the debate goes on unabated (Uchida et al. 2004; Wang and Weiner 2008). As therapeutic antibodies come of age, second- and third-generation antibodies for the same target are being considered and, in fact, are in development. However, optimization of a second- or third-generation antibody can only be as good as the understanding of the mechanism one wishes to improve upon, be it epitope, affinity, effector functions, etc.

Most therapeutic antibodies have two functional domains, the antigen-binding domain (Fv domain) and the constant (Fc) domain. Both play critical functions in therapeutic outcomes. The Fv domain determines not only the specificity of the antibody, but also the detailed binding characteristics, including epitope, overall affinity, on and off rates, etc. The Fc domain plays a role in structural integrity, half-life, tissue distribution, effector functions, and antigen–antibody complex removal.

There are five classes of immunoglobulins (Ig): IgG, IgA, IgM, IgD, and IgE. Most therapeutic antibodies are of the IgG class, which is subdivided into the IgG_1, IgG_2, IgG_3, and IgG_4 subclasses, or isotypes, and engineered variants thereof. These four subclasses have evolved as part of the adaptive immune response to

Z. Kaymakcalan (✉), A. Ibraghimov, A.G. Goodearl, and J.G. Salfeld
Abbott Bioresearch Center, Department of Biologics, 100 Research Drive, Worcester, MA 01605, USA
e-mail: zehra.kaymakcalan@abbott.com

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_19,

play specific biologic roles and, therefore, are not functionally interchangeable (Salfeld 2007). Although we have known about the four human IgG isotypes since 1967 (Prahl 1967), new biologic features of IgG4 and IgG2 are still being discovered 40 years later (Yoo et al. 2003; van der Neut Kolfschoten et al. 2007).

The discovery and development of small molecule and biologic medicines are converging on similar drug design themes, albeit using completely different structural platforms (Blaich et al. 2007). However, the ability to truly design the drug-like properties for a biologic is still in its infancy, and may at times conflict with the desire to preserve human-like IgG sequences. Therefore, the screening of multiple candidates for meeting predefined criteria (including half-life, tissue distribution, behavior in a set of preformulation screening assays, etc.) is often preferred over excessive engineering.

The following discussion focuses on the generation of unmodified or naked therapeutic antibodies (i.e., those that mediate the full therapeutic efficacy through binding and antibody-specific downstream effects). Different rules apply to antibodies that are designed to work as conjugates with radioisotopes or toxins. For example, the cancer antibody gemtuzumab (Mylotarg®; Wyeth Pharmaceuticals, Inc., Philadelphia, PA) uses the inactive IgG4 isotype, but mediates clinical efficacy via the tethered immunotoxin calicheamicin. The choice of an inactive isotype in the humanization of the original p67.6 mouse CD33-specific antibody may have been driven by the desire to focus the mechanism on the calicheamicin-mediated effects on CD33+ leukemia cells while reducing bystander killing mediated by Fc-gamma receptor (FcγR) binding.

In this context, current research explores the use of antibodies lacking the ability to bind the neonatal FcR (FcR_n) for cancer imaging, and possibly targeting. Although antibody binding-site–mediated cancer targeting remains unaffected, rapid clearance from the body facilitates the differential accumulation of drug in the target tissue (Wu and Senter 2005).

The creation of a novel, unmodified therapeutic biologic, therefore goes through the following steps:

1. Target validation (understanding the target biology and confirming the role of the target in a relevant model of the intended human disease)
2. Development of a therapeutic strategy, including the proposed mechanism of action (e.g., antagonistic, agonistic, cell-lysing/eliminating antibody)
3. Development of necessary reagents and screening assays (at least one assay for each aspect of the intended mechanism of action; must include reagents and assays from the planned nonhuman toxicity study species [e.g., cynomolgus monkey, mouse, dog])
4. Generation of candidate antibodies, or binding portions thereof, using any of the available methods (see Sects. 19.1.1–19.1.5)
5. Generation of the full-length IgG antibodies (application of recombinant DNA-cloning techniques to fuse the preferred antibody binding regions to the antibody-constant region that is compatible with the intended mechanism of action)
6. Expression and purification of the antibody (see Sects. 19.1.6 and 19.1.7)

7. Testing of the antibody candidates in screening funnel assays (see Sects. 19.1.8–19.1.10); additional tests include rat pharmacokinetic studies and a series of preformulation studies
8. Selection of a development candidate that meets all predefined criteria for the human proteins and assays and demonstrates good cross-reactivity with target proteins from the planned toxicity species

The best possible match between Step 2 (the intended mechanism of action) and Step 5 (the selection of the appropriate constant region) requires a good understanding of the biologic effects mediated by each IgG subclass or variant thereof. Table 19.1 summarizes some key features of each IgG subclass.

Importantly, recent data regarding the *in vivo* structure of IgG4, and to some extent IgG2 have heightened the need for careful selection of isotypes. Briefly, nonstabilized IgG4 tends to form monovalent half molecules *in vivo* and can form mixed IgG4 forms with unrelated antibodies. Incidentally, a postmarketing commitment for natalizumab includes an assay for bispecific natalizumab (Summary of Tysabri Post-Marketing Commitments 2009; Yednock 2007). In contrast, the unique IgG2 hinge region of nonstabilized IgG2 leads to a sequential cysteine-linkage exchange (IgG2A→IgG2A/B→IgG2B) that, in some cases, may lead to dimer formation (Yoo et al. 2003).

After a full-length antibody has been created with a specific isotype constant region, the resulting effector functions need to be confirmed in relevant assays (see Sect. 19.1.10). Unexpected behavior of therapeutic antibodies, including a depleting IgG4 version of Campath-1 (Isaacs et al. 1996) and an unexpected C1q binding by huOKT3γ1(A^{318}) (Xu et al. 2000), serves as a reminder that experimental confirmation of expected effector functions is critical.

The choices of constant regions for full-length antibody generation are becoming more complex by the availability of engineered variants and, in some cases, mixed isotypes. Key references for engineered constant regions are shown in Table 19.2. It should be noted that some technologic solutions may require licenses from patent holders (see also Sect. 19.1.12.5).

19.2 History of Use

Today, the most common isotype of successful therapeutic chimeric, humanized, or human antibodies is IgG1 (14/17), followed by IgG4 (2/17) and, lastly, IgG2 (1/17) (Reichert 2008).

Two main factors may have contributed to the therapeutic success of IgG1. First, human IgG1 isotype is the dominant isotype in human plasma; thus, IgG1 was better understood at the time that initial isotype selection decisions were made. Second, most early therapeutic antibodies (e.g., Campath-1 [CD52]; anti-Tac [interleukin-2 (IL-2) receptor α/CD25]) were designed for use against cell surface targets in indications requiring cellular depletion (e.g., transplantation,

Table 19.1 Key information for each IgG isotype

	IgG1	IgG2	IgG3	IgG4
Structure				
Accession number, allele name, allotypes (International Immunogenetics Information System 2009)	J00228, IGHG1*01, G1m(z,a)	J00230, (IGHG2*01, G2m(n)	X03604, IGHG3*01, G3m(5*) or G3m(b*) where 5*=5,10,11,13,14 and b*=b0,b1,b3,b4,b5)	K01316, IGHG4*01
Number of allotypes	4	1	13	1
Protein structure *in vivo*	Monomeric, bivalent	Monomeric, bivalent or dimeric, tetravalent; undergoes cysteine bridge transition from form A→A/B→B	Monomeric	Monomeric, bivalent and half-Ig, monovalent
Effector functions and FcR_n binding				
C1	++	–	+++	–
FcγRI	+++	–	+++	++
FcγRII	+	+/–	+	?
FcγRIII a/b	+	–	+	+/–
FcR_n binding	+	+	+	+
Therapeutic use				
Use in therapeutic antibodies	Frequent	Some	Rare	Some
Engineered variants	see Table 19.2			
Stabilizing mutants published	NR	NR	NR	Yes (see Table 19.2)

?, unknown; NR, none reported

Table 19.2 Key references for engineered IgG variants[a]

	Reduced effector function[b]	Enhanced effector function	Altered FcR_n binding	Stabilization
IgG1	Duncan and Winter (1988)	Idusogie et al. (2001)	Shields et al. (2001)	–
	Shields et al. (2001)	Shields et al. (2001)	Chamberlain et al. (2006)	
	Dall'Acqua et al. (2006)	Guler-Gane et al. (2008)	Hinton et al. (2008)	
	Guler-Gane et al. (2008)	Lazar et al. (2008)	Lazar et al. (2008)	
	Lazar et al. (2008)	Natsume et al. (2008)	Presta (2008)	
	Presta (2008)	Presta (2008)		
	Stavenhagen et al. (2008)	Stavenhagen et al. (2008)		
IgG2		Idusogie et al. (2001)	Chamberlain et al. (2006)	–
		Presta (2008)	Hinton et al. (2008)	
IgG3	Duncan and Winter (1988)[c]	Natsume et al. (2008)	Hinton et al. (2008)	–
	Canfield and Morrison (1991)			
	Jefferis et al. (1995)[c]			
IgG4	Canfield and Morrison (1991)	Presta (2008)[c]	Hinton et al. (2008)	Reddy et al. (2000)
	Jolliffe (1993)			van de Winkel et al. (2008)
	Reddy et al. (2000)			

[a]All engineered antibody variants should be tested for expected *in vitro* and *in vivo* characteristics
[b]References include those with FcR or C1q binding data only, without functional assay follow-up
[c]Reference contains no experimental data for this particular isotype but suggests extension of data from other isotypes or discusses data from additional references

graft-versus-host disease, cancer) and were selected for optimal effector functions (i.e., antibody-dependent cellular cytotoxicity [ADCC] and complement-dependent cytotoxicity [CDC]). IgG3 is rarely, if ever, used because of stability issues owing to the extended hinge regions and therefore IgG1 became the active isotype of choice for any depleting application.

In addition to cell surface targets, IgG1 has also been successfully used for soluble targets, including vascular endothelial growth factor (VEGF; bevacizumab [Avastin®; Genentech, Inc., South San Francisco, CA]), tumor necrosis factor (TNF; infliximab [Remicade®; Centocor, Inc., Malvern, PA] and adalimumab [HUMIRA®; Abbott Laboratories, Abbott Park, IL]), and IgE (omalizumab [Xolair®; Genentech, Inc.]).

It may be correct to say that initially, IgG1 was the isotype of choice for any application, except in cases where effector functions were thought, or demonstrated to be detrimental. A case in point is the humanization of mouse anti-CD3 antibody OKT3, or muromonab, first reported in 1979 (Kung et al. 1979) and approved in 1986 by the United States Food and Drug Administration (FDA) for

use in kidney transplantation. Although muromonab was an effective therapeutic, a serious adverse effect was the first-dose effect, which included fever, hyperdynamia, hypertension, and tachycardia experienced by patients within 1–2 h of administration. This adverse effect was shown to be related to cytokine release (primarily TNF and interferon-γ [IFN-γ]) that could be modulated by concomitant use of corticosteroids (Chatenoud et al. 1990). Further investigation led to the conclusion that first-dose responses required OKT3 target binding and additional engagement of FcRs on T cells. Consequently, the isotype selected for humanized OKT3 was the IgG4 isotype (Woodle et al. 1992), and in 1993, a variant with the additional Glu235 to Leu mutation, originally described by Duncan and Winter (1988) as being involved in FcγR binding, was shown to reduce T-cell activation *in vitro* (Jolliffe 1993). A similar variant with the L234A, L235A double mutation, HuOKT3γ1(Ala-Ala), demonstrated reduced first-dose responses in patients (Woodle et al. 1999). A humanized OKT3 was never developed nor approved for human use, possibly owing to ethical problems in withholding effective medicines in placebo-controlled, blinded Phase II or Phase III trials for the second-generation molecule.

Another instructive example for an isotype choice other than IgG1 is the cancer antibody gemtuzumab (Mylotarg), approved by the FDA in 2000 for use in acute myeloid leukemia. As previously explained, this antibody was designed as an immune conjugate to mediate clinical efficacy not by antibody effector functions, but rather by action of the tethered immunotoxin calicheamicin. The choice of the IgG4 isotype in the humanization of the mouse CD33-specific antibody p67.6 may have been driven by the desire to focus the therapeutic effect on CD33+ leukemia cells while reducing the probability of additional and widespread interaction of the antibody with FcγRs on other cells and resulting bystander killing.

However, a very instructive recent side-by-side analysis of different isotype variants of an anti-CD70 antibody found, that while the FcR nonbinding mutant IgG1 E233P L243V L235A demonstrated the best therapeutic index, the correlation of FcγR binding and therapeutic efficacy was limited (McDonagh et al. 2008).

The consistent selection of the IgG1 isotype for unmodified cancer antibodies is similarly instructive. Campath-1 was described first in 1983 (Hale et al. 1983), and clinical data with a humanized IgG1 version were reported in 1988 (Hale et al. 1988). The choice of IgG1 versus IgG4 was documented experimentally, as the IgG4 variants demonstrated reduced ADCC function as expected (Greenwood et al. 1993).

However, effector functions mediated by the IgG1 constant region are not the only mechanism of action of some cancer antibodies. The humanized anti-human epidermal growth factor receptor (EGFR)-2 antibody, trastuzumab (Herceptin; Genentech, Inc.) with the IgG1 isotype was first reported in 1992 (Carter et al. 1992), but additional signaling mechanisms were discovered and reported years later (Yakes et al. 2002). The variety of reported mechanisms of action of Herceptin include ADCC, inhibition of HER2 shedding, inhibition of the PI3K-Akt pathway,

attenuation of cell signaling, and inhibition of tumor angiogenesis (Valabrega et al. 2007). The authors stated that, "considering that ~10 years have elapsed since the initial evidence of its clinical activity in patients, it is surprising to note that relevant questions concerning the mechanism of action of trastuzumab *in vivo* are still uncertain." However, it is not uncommon that the mechanism of action of an antibody with a particular isotype may not be fully understood until years after initial development.

Recent use of IgG2 isotype in antibodies such as panitumumab (Vectibix®; Amgen, Inc., Thousand Oaks, CA) originally stemmed from the fact that the transgenic mice used for the generation of the antibody, contained only the IgG2 heavy-chain constant region gene. However, the data to consider when selecting this isotype have become more complex. Recently, it was discovered that recombinant human IgG2 antibodies form three major disulfide-mediated isoforms (Wypych et al. 2008). Surprisingly, these different isoforms of some IgG2 antibodies exhibited different neutralization activities, namely, different IC50 values for neutralization of IL-1 (Dillon et al. 2008). These major isoforms were not an artifact of recombinant antibody manufacturing, as similar isoforms were isolated from normal human serum (Wypych et al. 2008). A time-dependent conversion from one isoform to another was observed *in vitro* and was also demonstrated when an IgG2 antibody therapeutic was administered into normal human subjects (Liu et al. 2008).

The history of isotype selection for therapeutic antibodies clearly points to the need for good understanding of the underlying target and disease biology for optimal match of antibody characteristics with desired therapeutic outcomes. In addition, especially considering the emergence of engineered isotype variants, full characterization of each antibody variant *in vitro* and, if feasible, *in vivo*, is essential.

19.3 Selection Principles

Depending on the nature of the target molecule and anticipated mechanism of action, therapeutic antibodies can be classified into four major groups based on application:

1. Agonistic antibodies that mimic endogenous ligands by binding to cell receptors with the purpose of:
   (a) Activating a target cell (e.g., TGN1412, humanized anti-CD28 IgG4)
   (b) Delivering a death signal (e.g., rituximab, chimeric anti-CD20 IgG1)
2. Blocking antibodies such as:
   (a) Neutralizing antibodies that bind to soluble ligands (e.g., adalimumab, human anti-TNF IgG1)

(b) Antagonistic antibodies that bind to cell receptors to prevent the action of endogenous ligand (e.g., panitumumab, human anti-EGFR IgG2) or cell trafficking/adhesion (e.g., natilizumab, humanized anti-CD49d IgG4)
(c) Modulation of cell receptor (e.g., huOKT3γ1, humanized anti-CD3 IgG1)

3. Antibodies that recruit immune effector functions (e.g., alemtuzumab, humanized anti-CD52 IgG1).
4. Conjugated antibodies used for:

(a) Delivery of cytotoxic substances (e.g., gemtuzumab, humanized anti-CD33 IgG4, calicheamicin-conjugated)
(b) Delivery of immunostimulatory substances (e.g., IL-2 in anti-cancer therapy, anchored by anti-ED-B (Wagner et al. 2008) or anti-CD20 (Gillies et al. 2005); both in preclinical stage)
(c) Tumor imaging (preclinical research)

Some of these categories are not mutually exclusive; thus, an antibody can engage in several modes of action. For example, rituximab is able to kill CD20+ human B-lymphoma cells by a combination of natural killer (NK) cell recruitment (Reff et al. 1994), complement activation (Reff et al. 1994), CD20-mediated signaling (Shan et al. 1998), and FcγRII-mediated phagocytosis (Manches et al. 2003). Humanized muromonab-CD3 antibody could both deplete T cells and induce T-cell receptor internalization (Xu et al. 2000).

The molecular requirements for each mode of action differ, and IgG properties beneficial for one mechanism might be disadvantageous for another. Thus, for antibodies designed to execute effector functions, IgG1 is the isotype of choice because it is the most efficient isotype in binding to FcγRs to recruit and activate NK cells (demonstrated for rituximab, trastuzumab, and apolizumab (Bowles and Weiner 2005)) and fixing complement (rituximab (Reff et al. 1994)) to mediate ADCC and CDC, respectively.

In contrast, for neutralizing antibodies as well as for some agonistic and conjugated antibodies, the presence of an effector function-capable Fc domain can lead to dangerous adverse effects, including unwanted cytotoxicity (Newman et al. 2001), unintended agonism through cross-linking with participation of FcγR (Chatenoud et al. 1990), cytokine release with associated toxic effects (Wing et al. 1996), or platelet aggregation (Langer et al. 2005). With this in mind, isotypes with diminished ADCC and CDC capability, namely IgG2 and IgG4, could be considered. This principle of isotype selection was demonstrated in a side-by-side study of 5 Fc variants of the same humanized antibody targeting OKT3, including wild-type IgG1, wild-type IgG4, and 3 Fc-mutated variants devoid of FcγR and C1q binding ability (Xu et al. 2000).

For some antibodies or antibody-based therapeutics, the ability to increase or decrease the half-life could result in enhanced therapeutic efficacy. Interaction with FcR_n is the major (though probably not the only) factor that defines the half-life of therapeutic antibodies. All four major isotypes of human IgG appear to have equal ability to bind to FcR_n. Although tremendous effort has been applied to enhance

antibody half-life and numerous mutations in CH2 and CH3 domains have been reported to achieve this goal, a diminished half-life of a therapeutic antibody seems advantageous in some instances (Pop et al. 2005). This is particularly true for toxin-bearing antibody conjugates. Diminished half-life may be achieved either by mutating the Fc region to undermine its FcR_n recognition (Pop et al. 2005) or by eliminating the Fc region altogether and using antibody Fab fragment instead, resulting in a significant increase in maximum tolerated dose (Vitetta et al. 1991). In some instances, cross-linking (potentially with FcγR engagement) could induce internalization and be advantageous when combined with an intracellularly activated conjugate, thus limiting nonspecific toxicity (Wu and Senter 2005).

19.4 Selection Case Studies

A brief review of some case studies in which experimental testing of different isotypes with respect to efficacy and safety outcomes was conducted may be useful to appreciate the difficulty of choosing a "correct" antibody isotype. These examples ultimately show that each antibody is unique, and even when the proper preclinical studies were conducted before progressing into clinical trials, the results may sometimes be unexpected and may require second-generation antibody work.

An intuitive prediction is that selection of the correct isotype for an antibody directed against a cell-surface antigen/target is more critical than for an antibody against a soluble target. Thus, examples of antibodies to a soluble cytokine, TNF, are a good reminder that widely held dogmas are not always correct and that different antibodies for the same target could perform differently. In the early development stage of a generation of neutralizing antibodies for human TNF, two mouse/human chimeric antibodies, differing only in the isotype, were tested in a rabbit pyrexia model (Suitters et al. 1994). It was shown that the antibody with the inactive, human IgG4 isotype prevented human TNF-induced pyrexia in rabbits in a dose-dependent manner. The pyrexia induced by human TNF peaked at 60 min and subsided at 120 min. On the other hand, the same antibody with the active, human IgG1 isotype had no effect on pyrexia; at the highest administered dose of antibody, the pyrexia was sustained at 300 min after TNF challenge, the last observation time point in the study. Administration of preformed immune complexes of the same antibodies with human TNF showed no pyrexia with IgG4 complex, but an ever-increasing body temperature with the IgG1 complex. Both antibodies were equally effective in reducing the concentrations of administered human TNF in a dose-dependent manner.

In the same study (Suitters et al. 1994), another anti-TNF antibody (a hamster/mouse chimeric antibody against mouse TNF) protected mice from lipopolysaccharide-induced lethality in a dose-dependent manner only if it contained the inactive mouse IgG1 isotype; the antibody had little effect on lethality if it contained the active mouse IgG2a isotype. Clearance of both antibodies in mice was similar.

Based on these preclinical results, an inactive human IgG4 isotype was selected for the human therapeutic anti-TNF antibody CDP570. Later, an identical study of human TNF-induced rabbit pyrexia was repeated with a human anti-human TNF antibody, D2E7 (HUMIRA Biologic License Application 125057). D2E7 with human IgG1 and IgG4 isotypes were prepared and tested in human TNF-challenged rabbits. Both isotypes of D2E7 prevented pyrexia in a dose-dependent manner. Preformed immune complexes of both isotypes of D2E7 with human TNF were not pyretic when administered to rabbits. Because no adverse effects were observed with the IgG1 isotype, D2E7 with the IgG1 isotype was chosen for clinical development.

Passive immunization with specific antibodies against ever-increasing drug-resistant pathogens is currently underutilized. Examples in this area are mainly restricted to mouse studies. *Cryptococcus neoformans* is a fungal infection affecting patients with AIDS. Antibodies to one of its polysaccharide capsule constituents, glucuronoxylomannan (GXM) have provided some very interesting results with respect to isotype selection. One mouse IgG1 antibody, 18B7, was entered into clinical trials (Casadevall et al. 1998). Later, mouse/human IgG1 chimeric versions of 18B7 and another mouse antibody were prepared and shown to be efficacious in mouse models (Zebedee et al. 1994). Preclinical studies with mouse anti–*C neoformans* antibodies also showed that between isotype switch variants of a particular antibody, the IgG1 variant caused acute lethal toxicity (Lendvai et al. 2000). The authors concluded that GXM–IgG1 immune complexes induced greater levels of platelet-activating factor, causing lethality.

In continuing their work with GXM antibodies, the same group prepared human IgM, IgG1, IgG2, IgG3, IgG4, and IgA isotypes of the 18B7 antibody and observed that isotype had an effect on fine binding characteristics (McLean et al. 2002). All isotypes except IgG3 and IgM produced the protective annular binding pattern to GXM on yeast particles. Chimeric IgG3 and IgM produced a punctate pattern shown to be nonprotective in previous studies (Nussbaum et al. 1997). Interestingly, when the authors prepared a mouse IgM variant of the 18B7 antibody, it too showed the punctate binding pattern, indicating that the structural element of the IgM constant region – not the human sequence – was responsible for the change in fine specificity of the binding. It is usually assumed that the mechanism of antibody-mediated protection against cellular pathogens encompasses complement fixation, FcR binding, and phagocytosis. In order to verify this hypothesis, the variable region of a mouse antibody, 3E5, against GXM was joined to human IgG1, IgG2, IgG3, and IgG4 constant regions by another group (Beenhouwer et al. 2007). All four mouse/human chimeric antibodies bound to GXM with similar affinities. In contrast to the previously described study, these isotype variants all showed the annular binding pattern to GXM on yeast particles. When tested *in vivo*, only IgG2 and IgG4 isotypes prolonged the survival times of mice with *C neoformans* infection. The IgG3 isotype had no effect on survival rates, and the IgG1 isotype shortened survival time.

Finally, antibodies to surface antigens expressed on the body's own cells constitute a very important and broad class of therapeutics. Obviously, engagement of

effector functions when these therapeutic antibodies are bound to their respective target cells creates environments where cell lysis, cytokine release, and effector cell activation could lead to unwanted inflammation and toxicities. Thus, choosing the correct isotype is very important for this class of antibodies. Experience with anti-CD4 antibodies provides some interesting additional examples.

Blocking activation of T cells by anti-CD4 antibodies demonstrated clinical efficacy in autoimmune diseases, albeit with the undesirable effect of T-cell depletion (Herzog et al. 1989; van der Lubbe et al. 1993). Subsequent efforts searched for antibodies that did not cause cell depletion. An antihuman CD4 macaque/human chimeric IgG1 lambda antibody, keliximab, was found to cause very little CD4 T-cell depletion in chimpanzees after repeated administrations and did not modulate complement-mediated lysis (Anderson et al. 1997). Based on these desired features, keliximab was entered into clinical trials for rheumatoid arthritis (RA) and asthma. Unlike the results in chimpanzees, cell depletion was observed in humans (Kon et al. 1998). To improve upon keliximab, its isotype was changed to IgG4 with two amino acid modifications: Leu to Glu at residue 235 to completely abolish its capacity to bind to FcRs and Ser to Pro at residue 228 in the hinge region to stabilize the disulfide bond between the two heavy chains (Reddy et al. 2000). The resulting antibody, clenoliximab, was shown to retain all the binding and immunomodulatory characteristics of keliximab for CD4, but exhibited highly reduced binding to FcRs on human cells *in vitro*. A single-dose study of clenoliximab in patients with RA showed dose-dependent binding of CD4+ cells with no cell depletion (Mould et al. 1999).

A subsequent study in patients with RA tested multiple dosing regimens (4 weekly doses of 150 mg or 350 mg and two weekly doses of 350 mg or 700 mg), in addition to single doses (0.05, 0.2, 1, 5, 10, and 15 mg/kg). Total CD4 counts were measured using an anti-CD4 antibody that did not interfere with the binding of clenoliximab. Throughout the study, total CD4 T-cell counts remained stable and no depletion was observed. Coating of CD4 molecules with clenoliximab and down-modulation was dose-dependent. Interestingly, the concentration of clenoliximab-bound soluble CD4 was much higher than clenoliximab-bound membrane CD4, a phenomenon that the authors referred to as antibody-mediated stripping of CD4 molecules from T-lymphocyte surfaces (Hepburn et al. 2003).

These studies demonstrated that greatly reducing the FcR binding capacity of a therapeutic antibody directed against a cell-surface molecule, eliminated the undesired effect of cell depletion without interfering with the intended mechanism of action of the antibody, namely, immunomodulation. An *in vivo* study conducted later in a human CD4 transgenic mouse by the makers of keliximab and clenoliximab demonstrated that at high doses (25 or 125 mg/kg), clenoliximab also caused depletion of CD4 T cells, but at a lower dose (5 mg/kg), no cell depletion was observed (Sharma et al. 2000). This finding indicates that although antibody engineering had eliminated the undesired cell depletion effect at clinically relevant doses, other mechanisms may account for cell depletion at very high doses.

19.5 Future Considerations

When administered as drugs, antibodies possess the intrinsic property of interacting with many proteins, including not only the target(s) but also Fcγ receptors, FcR_n, and complement proteins, interactions that can occur serially or sometimes simultaneously. As such, antibodies can be regarded as functional integrators of the diverse activities represented by these different interactions. The diversity of antibody isotypes, both from native IgG diversity as well as engineered derivatives, serves to tune or modulate this integrated state, ultimately defining the functional profile of the drug from both an efficacy and toxicity standpoint. Thus, isotype selection and modification is emerging as a critical aspect in the design of therapeutic antibodies. Importantly, rigorous testing of antibodies in relevant assays (see Sect. 19.1.9 and 19.1.10) is required to confirm the consequence of isotype selection.

As noted above, an understanding of the clinical mechanism of action of some of the most successful biologics drugs, particularly those specific for cell surface targets, is at best, only partially understood. Looking forward, there are two general areas of research that promise to inform and clarify isotype selection. First, our understanding of the biology of effector systems and their interplay with different target types continues to advance, impacting our understanding of the role of isotypes on therapeutic antibody function. The recent interest in Vg9Vd2 gamma/delta T cells is an example of an important effector cell, with the potential to mediate antibody drug efficacy in cancer, whose properties are only beginning to be understood (Puan et al. 2007). Secondly, we are currently at an interesting and exciting stage in pharmaceutical and clinical science, in which the clinical testing of second- and third-generation versions of established therapeutic antibodies is permitting the comparative analysis of differences in isotype and Fc structure on the relative performance of these drugs in humans; the publication of the findings from such studies, both positive and negative, promises to advance the science of isotype selection and engineering during the design and optimization of future novel antibody drugs.

Acknowledgements Editorial support was provided by Robin L. Stromberg, PhD, of Arbor Communications, Inc. (Ann Arbor, MI, USA) and funded by Abbott Laboratories.

References

Anderson D, Chambers K, Hanna N, Leonard J, Reff M, Newman R, Baldoni J, Dunleavy D, Reddy M, Sweet R, Truneh A (1997) A primatized MAb to human CD4 causes receptor modulation without marked reduction in CD4+ T cells in chimpanzees: in vitro and in vivo characterization of a MAb (IDEC-CE9.1) to human CD4. Clin Immunol Immunopathol 84:73–84

Beenhouwer DO, Yoo EM, Lai CW, Rocha MA, Morrison SL (2007) Human immunoglobulin G2 (IgG2) and IgG4, but not IgG1 or IgG3, protect mice against *Cryptococcus neoformans* infection. Infect Immun 75:1424–1435

Blaich G, Janssen B, Roth G, Salfeld J (2007) Overview: differentiating issues in the development of macromolecules compared with small molecules. In: Gad SC (ed) Handbook of pharmaceutical biotechnology. John Wiley & Sons, Hoboken, pp 89–123

Bowles JA, Weiner GJ (2005) CD16 polymorphisms and NK activation induced by monoclonal antibody-coated target cells. J Immunol Methods 304:88–99

Canfield SM, Morrison SL (1991) The binding affinity of human IgG for its high affinity Fc receptor is determined by multiple amino acids in the CH2 domain and is modulated by the hinge region. J Exp Med 173:1483–1491

Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci USA 89:4285–4289

Casadevall A, Cleare W, Feldmesser M, Glatman-Freedman A, Goldman DL, Kozel TR, Lendvai N, Mukherjee J, Pirofski LA, Rivera J, Rosas AL, Scharff MD, Valadon P, Westin K, Zhong Z (1998) Characterization of a murine monoclonal antibody to *Cryptococcus neoformans* polysaccharide that is a candidate for human therapeutic studies. Antimicrob Agents Chemother 42:1437–1446

Chamberlain AK, Desjarlais JR, Karki SB, Lazar GA, Vielmetter J, Yoder SC (2006) inventors; Xencor, Inc, assignee. Fc variants with altered binding to FcRn. US patent application 20,070,135,620

Chatenoud L, Ferran C, Legendre C, Thouard I, Merite S, Reuter A, Gevaert Y, Kreis H, Franchimont P, Bach JF (1990) In vivo cell activation following OKT3 administration. Systemic cytokine release and modulation by corticosteroids. Transplantation 49:697–702

Dall'Acqua WF, Cook KE, Damschroder MM, Woods RM, Wu H (2006) Modulation of the effector functions of a human IgG1 through engineering of its hinge region. J Immunol 177:1129–1138

Dillon TM, Ricci MS, Vezina C, Flynn GC, Liu YD, Rehder DS, Plant M, Henkle B, Li Y, Deechongkit S, Varnum B, Wypych J, Balland A, Bondarenko PV (2008) Structural and functional characterization of disulfide isoforms of the human IgG2 subclass. J Biol Chem 283:16206–16215

Duncan AR, Winter G (1988) The binding site for C1q on IgG. Nature 332:738–740

Gillies SD, Lan Y, Williams S, Carr F, Forman S, Raubitschek A, Lo KM (2005) An anti-CD20-IL-2 immunocytokine is highly efficacious in a SCID mouse model of established human B lymphoma. Blood 105:3972–3978

Greenwood J, Clark M, Waldmann H (1993) Structural motifs involved in human IgG antibody effector functions. Eur J Immunol 23:1098–1104

Guler-Gane G, Holgate R, Jermutus L, Levens J, Lund J, Stewart R, Thom A, Webster C (2008) inventors; MedImmune, assignee. Fc polypeptide variants obtained by ribosome display methodology. International patent application WO/2008/114011

Hale G, Bright S, Chumbley G, Hoang T, Metcalf D, Munro AJ, Waldmann H (1983) Removal of T cells from bone marrow for transplantation: a monoclonal antilymphocyte antibody that fixes human complement. Blood 62:873–882

Hale G, Dyer MJ, Clark MR, Phillips JM, Marcus R, Riechmann L, Winter G, Waldmann H (1988) Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. Lancet 2:1394–1399

Hepburn TW, Totoritis MC, Davis CB (2003) Antibody-mediated stripping of CD4 from lymphocyte cell surface in patients with rheumatoid arthritis. Rheumatology (Oxford) 42:54–61

Herzog C, Walker C, Müller W, Rieber P, Reiter C, Riethmüller G, Wassmer P, Stockinger H, Madic O, Pichler WJ (1989) Anti-CD4 antibody treatment of patients with rheumatoid arthritis: I. Effect on clinical course and circulating T cells. J Autoimmun 2:627–642

Hinton PR, Tsurushita N, Tso JY, Vasquez M, inventors; PDL BioPharma (2008) Inc, assignee. Alteration of FcRn binding affinities or serum half-lives of antibodies by mutagenesis. US patent 7,361,740

HUMIRA Biologic License Application (BLA 125057). Abbott Laboratories, Abbott Park, http://www.fda.gov/cder/biologics/review/BLA125057_S000_HUMIRA_PHARMR.pdf. Accessed 11 Feb 2009

Idusogie EE, Presta LG, Mulkerrin MG (2001) inventors; Genentech, Inc, assignee. Polypeptide variants. US patent 6194551

International Immunogenetics Information System. Gene table: Human (*Homo sapiens*) IGHC. http://www.imgt.org/textes/IMGTrepertoire/LocusGenes/tabgenes/human/IGH/IGHC/Hu_IGHC.html. Cited 22 Jan 2009

Isaacs JD, Wing MG, Greenwood JD, Hazleman BL, Hale G, Waldmann H (1996) A therapeutic human IgG4 monoclonal antibody that depletes target cells in humans. Clin Exp Immunol 106:427–433

Jefferis R, Lund J, Goodall M (1995) Recognition sites on human IgG for Fc gamma receptors: the role of glycosylation. Immunol Lett 44:111–117

Jolliffe LK (1993) Humanized antibodies: enhancing therapeutic utility through antibody engineering. Int Rev Immunol 10:241–250

Kon OM, Sihra BS, Compton CH, Leonard TB, Kay AB, Barnes NC (1998) Randomised, dose-ranging, placebo-controlled study of chimeric antibody to CD4 (keliximab) in chronic severe asthma. Lancet 352:1109–1113

Kung P, Goldstein G, Reinherz EL, Schlossman SF (1979) Monoclonal antibodies defining distinctive human T cell surface antigens. Science 206:347–349

Langer F, Ingersoll SB, Amirkhosravi A, Meyer T, Siddiqui FA, Ahmad S, Walker JM, Amaya M, Desai H, Francis JL (2005) The role of CD40 in CD40L- and antibody-mediated platelet activation. Thromb Haemost 93:1137–1146

Lazar GA, Chirino AJ, Wei D, Desjarlais JR, Doberstein SK, Hayes RJ, Karki SB, Vafa O (2008) inventors; Xencor, Inc, assignee. Optimized Fc variants. US patent 7317091

Lendvai N, Qu XW, Hsueh W, Casadevall A (2000) Mechanism for the isotype dependence of antibody-mediated toxicity in *Cryptococcus neoformans*-infected mice. J Immunol 164:4367–4374

Liu YD, Chen X, Enk JZ, Plant M, Dillon TM, Flynn GC (2008) Human IgG2 antibody disulfide rearrangement in vivo. J Biol Chem 283:29266–29272

Manches O, Lui G, Chaperot L, Gressin R, Molens JP, Jacob MC, Sotto JJ, Leroux D, Bensa JC, Plumas J (2003) In vitro mechanisms of action of rituximab on primary non-Hodgkin lymphomas. Blood 101:949–954

McDonagh CF, Kim KM, Turcott E, Brown LL, Westendorf L, Feist T, Sussman D, Stone I, Anderson M, Miyamoto J, Lyon R, Alley SC, Gerber HP, Carter PJ (2008) Engineered anti-CD70 antibody-drug conjugate with increased therapeutic index. Mol Cancer Ther 7:2913–2923

McLean GR, Torres M, Elguezabal N, Nakouzi A, Casadevall A (2002) Isotype can affect the fine specificity of an antibody for a polysaccharide antigen. J Immunol 169:1379–1386

Mould DR, Davis CB, Minthorn EA, Kwok DC, Elliott MJ, Luggen ME, Totoritis MC (1999) A population pharmacokinetic-pharmacodynamic analysis of single doses of clenoliximab in patients with rheumatoid arthritis. Clin Pharmacol Ther 66:246–257

Natsume A, In M, Takamura H, Nakagawa T, Shimizu Y, Kitajima K, Wakitani M, Ohta S, Satoh M, Shitara K, Niwa R (2008) Engineered antibodies of IgG1/IgG3 mixed isotype with enhanced cytotoxic activities. Cancer Res 68:3863–3872

Newman R, Hariharan K, Reff M, Anderson DR, Braslawsky G, Santoro D, Hanna N, Bugelski PJ, Brigham-Burke M, Crysler C, Gagnon RC, Dal Monte P, Doyle ML, Hensley PC, Reddy MP, Sweet RW, Truneh A (2001) Modification of the Fc region of a primatized IgG antibody to human CD4 retains its ability to modulate CD4 receptors but does not deplete CD4(+) T cells in chimpanzees. Clin Immunol 98:164–174

Nussbaum G, Cleare W, Casadevall A, Scharff MD, Valadon P (1997) Epitope location in the *Cryptococcus neoformans* capsule is a determinant of antibody efficacy. J Exp Med 185:685–694

Pop LM, Liu X, Ghetie V, Vitetta ES (2005) The generation of immunotoxins using chimeric anti-CD22 antibodies containing mutations which alter their serum half-life. Int Immunopharmacol 5:1279–1290

Prahl JW (1967) The C-terminal sequences of the heavy chains of human immunoglobulin G myeloma proteins of differing isotopes and allotypes. Biochem J 105:1019–1028

Presta L (2008) inventor; Genentech, Inc, assignee. Polypeptide variants with altered effector functions. US patent 7371826

Puan KJ, Jin C, Wang H, Sarikonda G, Raker AM, Lee HK, Samuelson MI, Märker-Hermann E, Pasa-Tolic L, Nieves E, Giner JL, Kuzuyama T, Morita CT (2007) Preferential recognition of a microbial metabolite by human Vgamma2Vdelta2 T cells. Int Immunol 19:657–673

Reddy MP, Kinney CA, Chaikin MA, Payne A, Fishman-Lobell J, Tsui P, Dal Monte PR, Doyle ML, Brigham-Burke MR, Anderson D, Reff M, Newman R, Hanna N, Sweet RW, Truneh A (2000) Elimination of Fc receptor-dependent effector functions of a modified IgG4 monoclonal antibody to human CD4. J Immunol 164:1925–1933

Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR (1994) Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood 83:435–445

Reichert JM (2008) Monoclonal antibodies as innovative therapeutics. Curr Pharm Biotechnol 9:423–430

Salfeld JG (2007) Isotype selection in antibody engineering. Nat Biotechnol 25:1369–1372

Shan D, Ledbetter JA, Press OW (1998) Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. Blood 91:1644–1652

Sharma A, Davis CB, Tobia LA, Kwok DC, Tucci MG, Gore ER, Herzyk DJ, Hart TK (2000) Comparative pharmacodynamics of keliximab and clenoliximab in transgenic mice bearing human CD4. J Pharmacol Exp Ther 293:33–41

Shields RL, Namenuk AK, Hong K, Meng YG, Rae J, Briggs J, Xie D, Lai J, Stadlen A, Li B, Fox JA, Presta LG (2001) High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. J Biol Chem 276:6591–6604

Stavenhagen J, Vijh S, Rankin C, Gorlatov S, Huang L (2008) inventors; Macrogenics, Inc, assignee. Identification and engineering of antibodies with variant Fc regions and methods of using same. US patent 7,355,008

Suitters AJ, Foulkes R, Opal SM, Palardy JE, Emtage JS, Rolfe M, Stephens S, Morgan A, Holt AR, Chaplin LC, Shaw NE, Nesbitt AM, Bodmer MW (1994) Differential effect of isotype on efficacy of anti-tumor necrosis factor alpha chimeric antibodies in experimental septic shock. J Exp Med 179:849–856

Summary of Tysabri Post-Marketing Commitments. http://www.investorvillage.com/smbd.asp?mb=160&mn=76891&pt=msg&mid=1383292. Cited 22 Jan 2009

Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM, Tedder TF (2004) The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. J Exp Med 199:1659–1669

Valabrega G, Montemurro F, Aglietta M (2007) Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. Ann Oncol 18:977–984

van de Winkel J, Vink T, Schuurman J, Parren P, Aalberse R, van der Neut Kolfshoten M (2008) inventors; Genmab A/S, assignee. Stable IgG4 antibodies. International patent application WO/2008/145142

van der Lubbe PA, Reiter C, Breedveld FC, Krüger K, Schattenkirchner M, Sanders ME, Riethmüller G (1993) Chimeric CD4 monoclonal antibody cM-T412 as a therapeutic approach to rheumatoid arthritis. Arthritis Rheum 36:1375–1379

van der Neut Kolfschoten M, Schuurman J, Losen M, Bleeker WK, Martínez-Martínez P, Vermeulen E, den Bleker TH, Wiegman L, Vink T, Aarden LA, De Baets MH, van de Winkel JG, Aalberse RC, Parren PW (2007) Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. Science 317:1554–1557

Vitetta ES, Stone M, Amlot P, Fay J, May R, Till M, Newman J, Clark P, Collins R, Cunningham D, Ghetie V, Uhr JW, Thorpe PE (1991) Phase I immunotoxin trial in patients with B-cell lymphoma. Cancer Res 51:4052–4058

Wagner K, Schulz P, Scholz A, Wiedenmann B, Menrad A (2008) The targeted immunocytokine L19-IL2 efficiently inhibits the growth of orthotopic pancreatic cancer. Clin Cancer Res 14:4951–4960

Wang SY, Weiner G (2008) Complement and cellular cytotoxicity in antibody therapy of cancer. Expert Opin Biol Ther 8:759–768

Wing MG, Moreau T, Greenwood J, Smith RM, Hale G, Isaacs J, Waldmann H, Lachmann PJ, Compston A (1996) Mechanism of first-dose cytokine-release syndrome by CAMPATH 1-H: involvement of CD16 (FcgammaRIII) and CD11a/CD18 (LFA-1) on NK cells. J Clin Invest 98:2819–2826

Woodle ES, Thistlethwaite JR, Jolliffe LK, Zivin RA, Collins A, Adair JR, Bodmer M, Athwal D, Alegre ML, Bluestone JA (1992) Humanized OKT3 antibodies: successful transfer of immune modulating properties and idiotype expression. J Immunol 148:2756–2763

Woodle ES, Xu D, Zivin RA, Auger J, Charette J, O'Laughlin R, Peace D, Jollife LK, Haverty T, Bluestone JA, Thistlethwaite JR Jr (1999) Phase I trial of a humanized, Fc receptor nonbinding OKT3 antibody, huOKT3gamma1(Ala-Ala) in the treatment of acute renal allograft rejection. Transplantation 68:608–616

Wu AM, Senter PD (2005) Arming antibodies: prospects and challenges for immunoconjugates. Nat Biotechnol 23:1137–1146

Wypych J, Li M, Guo A, Zhang Z, Martinez T, Allen MJ, Fodor S, Kelner DN, Flynn GC, Liu YD, Bondarenko PV, Ricci MS, Dillon TM, Balland A (2008) Human IgG2 antibodies display disulfide-mediated structural isoforms. J Biol Chem 283:16194–16205

Xu D, Alegre ML, Varga SS, Rothermel AL, Collins AM, Pulito VL, Hanna LS, Dolan KP, Parren PW, Bluestone JA, Jolliffe LK, Zivin RA (2000) In vitro characterization of five humanized OKT3 effector function variant antibodies. Cell Immunol 200:16–26

Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL (2002) Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer Res 62:4132–4141

Yednock T (2007) inventor; Elan Pharmaceuticals, Inc, assignee. Methods of treating inflammatory and autoimmune diseases with natalizumab. International patent application WO/2007/103112

Yoo EM, Wims LA, Chan LA, Morrison SL (2003) Human IgG2 can form covalent dimers. J Immunol 170:3134–3138

Zebedee SL, Koduri RK, Mukherjee J, Mukherjee S, Lee S, Sauer DF, Scharff MD, Casadevall A (1994) Mouse-human immunoglobulin G1 chimeric antibodies with activities against *Cryptococcus neoformans*. Antimicrob Agents Chemother 38:1507–1514

Chapter 20
Generation of Heavy and Light Chains (Chimeric Antibodies)

Kirstin A. Zettlitz

20.1 Introduction

The mouse hybridoma technology described in 1975 by Köhler and Milstein, paved the way for the emergence of therapeutic mouse monoclonal antibodies (Kohler and Milstein 1975). These antibodies represent a unique class of therapeutics because of their high specificity. But these mouse monoclonal antibodies were shown to have limited use as therapeutic agents because of their short serum half-life, their inability to trigger human effector functions, and the induction of a human anti-mouse antibody response (Shawler et al. 1985; Jones et al. 1986). In an attempt to reduce the immunogenicity of mouse antibodies, genetic engineering has been used to generate chimeric mouse human mAbs. These antibodies consist of human constant regions and mouse variable regions, and are approximately 75% human and 25% murine IgG (Morrison et al. 1984). At present, five chimeric antibodies are in clinical use. Although chimeric antibodies were perceived as less immunogenic than mouse antibodies, human anti-chimeric antibody response (HACA) have nonetheless been observed (Aarden et al. 2008).

The humanization of already existing and well-characterized nonhuman antibodies against an antigen to generate a therapeutic molecule may have promise as an alternative to the isolation of novel human mAbs (e.g., by transgenic animals or phage display). As a first approach toward a humanized antibody, a mouse-human chimeric antibody can be constructed. Because the variable domains that constitute the antigen-binding site are not altered, the chimeric antibody is expected to bind as well as the parental mouse antibody. If the chimeric antibody is constructed with

K.A. Zettlitz
Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, Stuttgart 70569, Germany
e-mail: kirstin.zettlitz@izi.uni-stuttgart.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_20,

the same human constant regions that will be used in a humanized antibody, it is a valuable positive control.

The Lonza Biologics' Glutamine Synthetase (GS) gene expression system in Chinese Hamster Ovary (CHO) cells is used for expression of the chimeric antibodies (Bebbington et al. 1992). The generation of "double-gene vectors" circumvents double transfections with two constructs containing heavy and light chain genes, respectively. This expression system facilitates the high-level gene expression of proteins using the GS gene as a selectable marker. Glutamine Synthetase is the enzyme responsible for the biosynthesis of glutamine and is selectively inhibited by methionine sulphoximine (MSX). For cell lines, such as CHO-K1, which produce endogenous GS glutamine-free medium, containing MSX provides the selection pressure.

20.2 Materials

20.2.1 Heavy and Light Chain Constant Regions

- NucleoBond® XtraMidi, (Cat. No. 740410.100) Macherey-Nagel, Düren, Germany
- nanodrop, ND-1000 Spectrophotometer, PEQLAB, Erlangen, Germany
- Restriction enzymes (AscI, EcoRI, XhoI), Fermentas, St.Leon-Rot, Germany
- Alkaline Phosphatase Calf Intestine (M182A) CIP, 1 u/µl, Promega, Madison, USA
- Ready Agarose Precast Gel Electrophoresis System, BioRad, Krefeld, Germany
- T4 DNA Ligase, Fermentas, St. Leon-Rot, Germany
- TG1, genotype: *supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5* (rK– mK–) [F´ *traD36 proAB lacI*qZΔ*M15*], Stratagene, La Jolla, USA
- LB, 1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.5% NaCl
- $LB_{amp,\ glc}$, LB-medium, 1.5% (w/v) agar-agar, 100 µg/ml ampicillin, 1% glucose
- REDTaq ReadyMix™, Sigma-Aldrich, St. Louis, USA
- pAB1 or pAB11 (see Chapter 4 ScFv by two-step cloning)
- Primer LMB2 5′-GTA AAA CGA CGG CCA GT-3′ and LMB3 5′-CAG GAA ACA GCT ATG ACC-3′
- Ampicillin, 100 mg/ml in H_2O
- Primer:
  - XhoI-CH1-back 5′ ACC GTC TCG AGT GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA 3′
  - CH3-Stop-EcoRI-XbaI-for 5′ CT AGT CTA GAA TTC TTA TTT ACC CGG AGA CAG GGA GAG GCT 3′
  - AscI-CLλ-back 5′ ATT GGC GCG CCA TCG GTC ACT CTG TTC CCA CCC TCC 3′

- AscI-CLκ-back 5′ ATT GGC GCG CCA TCT GTC TTC ATC TTC CCG CCA TCT G 3′
- CLλ-Stop-EcoRI-for 5′ CCG GAA TTC TTA TGA ACA TTC TGT AGG GGC CAC TGT C 3′
- CLκ-Stop-EcoRI –for 5′CCG GAA TTC TTA ACA CTC TCC CCT GTT GAA GCT CTT TG 3′

20.2.2 Heavy and Light Chain Variable Regions

- RoboCycler 96, Stratagene
- Primer: HindIII-Igκ-leader-AgeI: 5′AAT CCC AAG CTT ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TCC ACC GGT GAG GTG CAG CTG GTC GAG AGC 3′
- Incubator, Infors HAT Multitron 2, Infors Ag, Basel Switzerland
- Primer:
  - VH-XhoI-for 5′ T ACC GCT CGA GAC GGT GAC CGT GGT CCC TTG 3′
  - VL-AscI-for 5′ T TGG CGC GCC CAC AGT CCG TTT CAG CTC CAG CTT GGT GCC 3′
  - SfiI-VL-back 5′CTC GCG GCC CAG CCG GCC GAC ATC GAG CTC ACC CAG TCT CCA 3′
- Herculase® Enhanced DNA Polymerase, Stratagene, La Jolla, USA
- NucleoSpin® Extract II Kit, Macherey-Nagel, Düren, Germany
- Restriction enzymes (HindIII), Fermentas, St. Leon-Rot, Germany

20.2.3 Construction of a "Double-Gene Vector"

- Primer: Lonza-F, Lonza-14.4-R
- Lonza pEE6.4, accessory vector, GS-System™pEE Expression vectors, Lonza Biologics, Berkshire, UK
- Lonza pEE14.4, GS-encoding expression vector, GS-System™pEE Expression vectors, Lonza Biologics, Berkshire, UK
- Restriction enzymes (NotI, BamHI), Fermentas, St. Leon-Rot, Germany

20.2.4 Transfection

- Cell line Lonza CHO-K1, Lonza Biologics, Berkshire, UK
- RPMI 1640 (-glutamine); 10% dFCS dialyzed, Invitrogen, Karlsruhe, Germany

– Opti-MEM®, Invitrogen, Karlsruhe, Germany
– Lipofectamine 2000, Invitrogen, Karlsruhe, Germany

20.2.5 Selection of GS-CHO Transfectants

– MSX, L-methionine sulfoximine, Sigma-Aldrich, M 5379 St. Louis, USA

20.3 Methods

20.3.1 Heavy and Light Chain Constant Regions

1. Design heavy and light chain constant region genes with appropriate restriction sites: e.g., *AscI*-C_L-*EcoRI* and *XhoI*-C_H1-C_H3-*EcoRI*. (see Note 2, Fig. 20.1.)
2. Order codon-optimized (for your expression system) DNA encoding the two constant regions from custom synthesis, (e.g., GeneArt, Regensburg, Germany). (see Note 3)
3. Prepare plasmid DNA according to manufacturer's protocol and isolate plasmid DNA using the NucleoBond®XtraMidi
4. Determine DNA concentration photometrically (e.g., using a nanodrop)
5. Digest about 10 µg of insert DNA and about 5 µg of vector pAB1 or pAB11 (see Sect. 20.2.2.2) with respective restriction enzymes (*AscI*, *EcoRI* for the light chain, *XhoI*, *EcoRI* in case of the heavy chain). Incubate at 37°C for at least 2 h. Add 1 µl of CIP to the vector preparations for dephosphorylation and incubate 1 h at 37°C. (see Note 1)
6. Separate your digested vector- and insert-fragments by agarose gel electrophoresis and purify the excised DNA using the NucleoSpin®Extract II Kit (Macherey-Nagel) according to the manufacturer's protocol. Air-dry DNA and dissolve in 30 µl H_2O
7. Determine DNA concentration photometrically

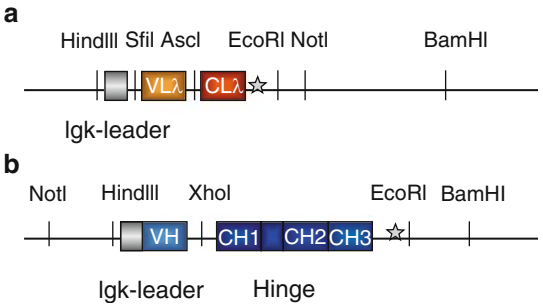


Fig. 20.1 Construction of a "double-gene vector" encoding chimeric IgG. (**a**) The light chain gene cloned into vector pEE.14.4 as HindIII-EcoRI fragment. (**b**) The heavy chain gene cloned into vector pEE6.4 as HindIII-EcoRI fragment. Subsequently, incorporate both vectors via NotI and BamHI

8. Use approximately 100–200 ng vector DNA for ligation and a molar ratio of insert to vector of 5:1. Carry out ligation using 2 µl T4 DNA Ligase (Fermentas) for 1 h at room temperature
9. Mix 10 µl of the ligation mix with 100 µl chemical competent *E. coli* TG1 cells, incubate 15 min on ice, and transform by heat shock at 42°C for 45 s
10. Resuspend transformed bacteria in 1 ml LB-medium, harvest cells by centrifugation and plate on $LB_{amp,\ glc}$-plates. (see Note 4) Incubate overnight at 37°C
11. Pick up to ten clones from the plates and use them as template for an analytical colony PCR using REDTaq ReadyMix™ (Sigma-Aldrich) and a primer set amplifying the inserted fragments. For vector pAB1, use primers LMB2 and LMB3 and 1 µl of undigested vector as control. At the same time, plate the picked clones onto a fresh $LB_{amp,\ glc}$-plate
12. Analyze PCR samples by agarose gel electrophoresis and identify positive clones showing a band of the predicted size (approximately 500 bp for C_L and 1,000 bp for C_H1-C_H3)
13. Use one of the positive clones to inoculate an overnight culture designated for plasmid isolation

20.3.2 Heavy and Light Chain Variable Regions

1. Design primers suitable for your antibody which contain an appropriate leader sequence for secretion and restriction sites. For the light chain variable region, e.g., HindIII-Igκ-leader-AgeI-V_L-AscI, for the heavy chain variable region, e.g., HindIII-Igκ-leader-AgeI-V_H-XhoI. (see Note 7)
2. Use 10 ng plasmid DNA (e.g., of scFv DNA) as input template for PCR amplification (50 µl reaction volume) of the variable domains using Herculase® Enhanced DNA Polymerase according to the manufacturer's protocol
3. Separate the PCR product by agarose gel electrophoresis and purify the excised DNA using the NucleoSpin®Extract II Kit (Macherey-Nagel) according to the manufacturer's protocol. Air-dry DNA and dissolve in 30 µl H_2O
4. Digest the purified PCR product and 5 µg of vector DNA (pAB1-C_H1-C_H3 and pAB1-$C_L\lambda$/$C_L\kappa$, respectively) with suitable restriction enzymes (e.g., *HindIII*, *AscI* for the light chain and *HindIII*, *XhoI* for the heavy chain) for at least 2 h at 37°C. Add 1 µl of CIP to the vector preparations for dephosphorylation and incubate 1 h at 37°C
5. Perform ligation and transformation as described for cloning of the constant region genes (see Sect. 20.3.1 (Note 8–11))
6. For the analytical colony-PCR expect a band of approximately 800 bp for the light chain and 1,500 bp for the heavy chain, respectively
7. Use one of the positive clones to inoculate an overnight culture designated for plasmid isolation

20.3.3 Construction of a "Double-Gene Vector"

(see Note 8)

1. Insert the light chain encoding DNA into Lonza vector pEE14.4 via *HindIII* and *EcoRI*
2. Insert the heavy chain encoding DNA into Lonza vector pEE6.4 via *HindIII* and *EcoRI*. The vector pEE6.4 is used to place the heavy chain gene within its own transcription cassette, before inserting the complete cassette into vector pEE14.4
3. Perform restriction digest, ligation, and transformation as described above
4. For the analytical colony-PCR use primers Lonza-F and Lonza-14.4-R
5. Digest both vectors with *NotI* and *BamHI* restriction enzymes. Add 1 μl of CIP to the pEE6.4 preparation for dephosphorylation and incubate 1 h at 37°C
6. Separate your digested fragments by agarose gel electrophoresis and isolate the complete hCMV-MIE promoter-*heavy chain*-SV40 transcription unit from pEE6.4, which is usually the larger fragment (approx. 3,650 bp). (see Note 8) Isolate the larger fragment of the digested vector pEE14.4, containing the GS transcription unit and the hCMV-MIE promoter-*light chain*-expression unit (approx. 10,350 bp)
7. Ligate the heavy chain fragment into vector pEE14.4. The heavy chain gene is cloned downstream of the light chain gene
8. Perform transformation as described above
9. Pick up to ten clones and use them as template for an analytical colony-PCR using REDTaq ReadyMix™ (Sigma-Aldrich) and both a primer set amplifying the heavy chain insert and the light chain insert. (see Note 10, 11)
10. Use one of the positive clones to inoculate an overnight culture designated for plasmid isolation using NucleoBond®XtraMidi
11. Precipitate the DNA in ethanol and store at −20°C until required
12. Prior to transfection, pellet the DNA by centrifugation, wash twice with 70% ethanol, air-dry for approximately 10 min and dissolve DNA in 100 μl sterile H_2O. (see Note 12)

20.3.4 Transfection

1. Trypsinise CHO-K1 cells and seed 1×10^7 cells in 10 ml of RPMI 1640 medium supplemented with 5% dFCS and 2 mM glutamine into a 10 cm-dish. Seed at least two dishes for your construct to be transfected and one dish each for the mock (H_2O instead of DNA) and the positive control (the expression vector used, i.e., pEE14.4). (see Note 13) Incubate overnight at 37° in a humidified CO_2 incubator

2. Replace medium with 10 ml Opti-MEM. Transfect 16 μg DNA per dish by lipofection using Lipofectamine2000 according to the manufacturer's protocol. (see Note 15)
3. Incubate the cells with the transfection mix for 6 h in the incubator
4. Aspirate and replace with 10 ml RPMI 1640, 2 mM glutamine containing 5% dFCS. Incubate at 37° in the incubator for 24 h

20.3.5 Selection of GS-CHO Transfectants

1. Twenty-four hours after transfection, replace the medium in each dish with 10 ml selective medium (RPMI 1640 (-gln, 5% dFCS) and MSX at a final concentration of 25 μM
2. After 2–5 days trypsinise the cells and dilute them to a concentration between 1×10^4 and 2×10^5 cells/ml in selective medium containing 25 μM MSX. Use about three cell concentrations within that range. (see Note 16)
3. Distribute the diluted cells over 96-well plates at 200 μl/well and place all plates in the humidified CO_2 incubator set at 37°C. (see Note 17)
4. Between two and three weeks after transfection, colonies of MSX-resistant transfectants should appear and may continue to appear up to 6 weeks
5. Take samples from the supernatant of confluent colonies (medium turns yellow) and replace with fresh selective medium. (see Note 18)
6. Expand positive transfectants once they reach approx. 50% confluence. The original well may be re-fed with selective medium as a backup
7. Maintain MSX selection throughout the expansion. Stock of cells should be cryopreserved. (see Note 19)
8. Screen the supernatants of transfectants for production of functional antibody by ELISA and for amount and assembly by Western blot analysis
9. Measure the specific production rate (SPR) in static culture: (see Note 20)

$$\text{SPR}\ (\mu\text{g}/10^6\text{cells/day}) = \frac{\text{antibody concentration}\ (\mu\text{g/ml})}{\text{viable cell concentration}\ (10^6\ \text{cells/ml}) * \text{incubation time}\ (\text{days})}.$$

20.3.6 Selection in the Presence of Increased MSX Concentrations

1. Select the highest producing cell lines of your transfectants (at least five cell lines) and dilute each of them to 1×10^5 cells/ml
2. For each MSX concentration to be used, inoculate a 24-well plate with 0.5 ml of cell suspension per well and incubate overnight
3. Prepare dilutions of MSX in selective medium (RPMI 1640, glutamine-free, 5% dFCS) in the range of 50–500 μM

4. Remove medium from each 24-well plate and replace with 0.5 ml of MSX dilution (one entire 24-well plate for each concentration), place in the incubator for 1 week
5. Replace medium every week with fresh selective medium containing MSX and re-incubate
6. Resistant colonies will take up to 3 weeks to appear. Expand colonies that grow in the two or three highest MSX concentrations and maintain these cells in the MSX concentration from which they were isolated. (see Note 21)

20.4 Notes

1. Both heavy and light chain encoding genes are assembled individually in pAB1 because of easy handling
2. Alternatively, isolate mRNA from human PBMCs, perform RT-PCR using the First Strand cDNA Synthesis Kit (Fermentas) and amplify constant region genes with appropriate primers
3. Check codon-optimized DNA for unwanted restriction sites
4. In case you get insufficient numbers of clones after transformation, incubate the resuspended bacteria for up to 1 h at 37°C with 225 rpm
5. Leader-sequences: Igκ-leader, CD5-leader. Both the heavy and the light chain gene need an own leader sequence
6. Primers for amplification of V_L should comprise the intersection between V_L and C_L. (see Sect. 20.5.1.4)
7. You can just as well clone a vector containing the Igκ-leader and amplify the variable domains as *AgeI*-V_L-*AscI*- or *AgeI*-V_H-*XhoI*-fragments
8. Adapted from the manufacturer's protocol "GS-System™ pEE GS Expression Vectors"
9. Over-digest the Lonza vectors to avoid remaining uncut vector. Check excised and purified pEE6.4 larger fragment on an agarose gel for contamination of the smaller fragment (approx. 2,800 bp)
10. The Lonza-F and Lonza-14.4-R primers anneal two times in the double-gene vector, and are for that reason not suitable for colony-PCR or sequencing of plasmid DNA. Alternatively, perform a control digestion with restriction enzymes cutting within the inserts; e.g., *HindIII*, *EcoRI*, *XhoI*, *AscI*, *AgeI*
11. The generated double gene vector is similar to that described by (Bebbington et al. 1992)
12. Perform DNA preparation for transfection in the sterile bench. You will need about 32 μg of purified DNA per transfection
13. The controls allow checking for transfection efficiency. Use water substituted for DNA for mock transfection and control the background of "false positive" clones. Use the original expression vector for positive control of your transfection technique. If your vector yields low numbers of clones, this may be due to the DNA

14. Serum-free, glutamine-free medium
15. Different transfection methods such as electroporation or calcium phosphate precipitation may be used
16. In order to obtain a high-producing cell line Lonza recommends screening a large number of transfectants (at least 100–200)
17. Keep the delay between plating the cells and placing the 96-well plates in the incubator as short as possible. Ensure that the cell suspension is mixed well and distributed regularly. The plates should not be disturbed during tansfectant formation. In order to check your plates for contamination and the transfection progress, select one plate
18. Prefer colonies which originate from a single transfectant to obtain monoclonal cell lines
19. Cultures in flasks should not exceed densities of 1×10^5–1×10^6 cells/ml (not reaching more than 90% confluence). Doubling times should be between 20 and 40 h, although they may vary between transfectants
20. Cell lines may be expanded to suspension culture or adapted to production in serum-free medium for higher yields of product
21. Selection in a higher MSX concentration can be an alternative method to obtain high-producing cell lines. The highest MSX concentration may vary between different cell lines

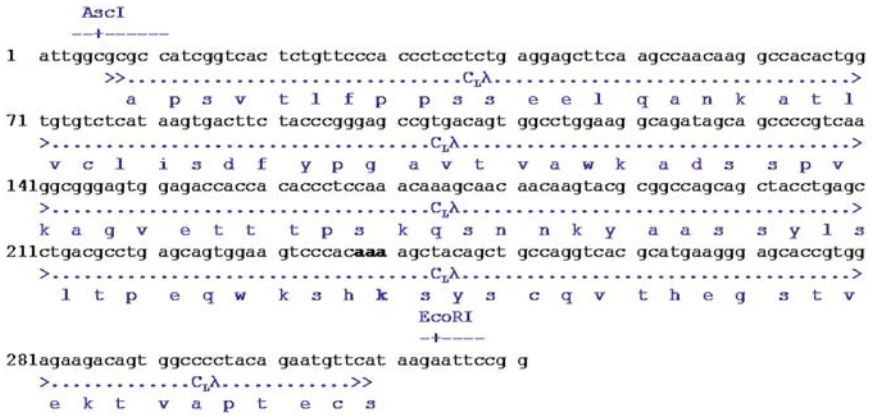
20.5 Sequences

(Letters in bold correspond to amino acids which are polymorphic in the other alleles; N-glycosylation sites are underlined)

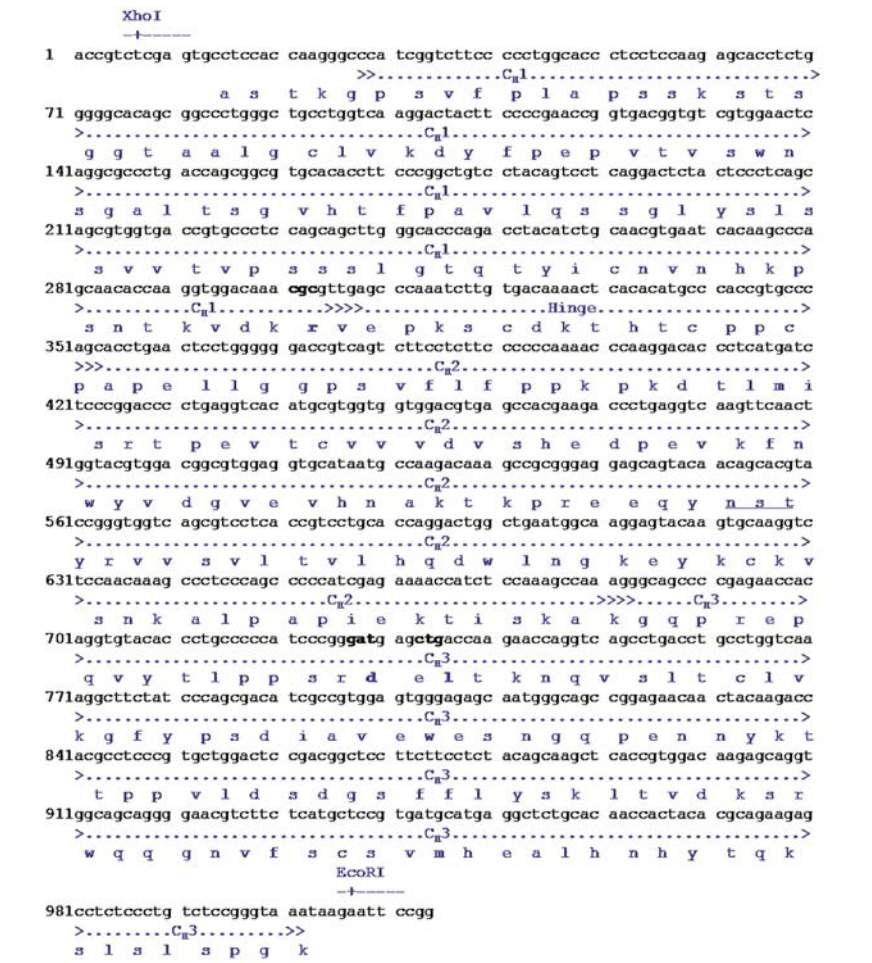
20.5.1 IGKC, J00241

```
      AscI
     --+------
1  attggcgcgc catctgtctt catcttcccg ccatctgatg agcagttgaa atctggaact gcctctgttg
       >>.................................CLK.................................>
         a   p   s   v   f   i   f   p   p   s   d   e   q   l   k   s   g   t   a   s   v
71 tgtgcctgct gaataacttc tatcccagag aggccaaagt acagtggaag gtggataacg ccctccaatc
   >.....................................CLK.....................................>
    v   c   l   l   n   n   f   y   p   r   e   a   k   v   q   w   k   v   d   n   a   l   q
141gggtaactcc caggagagtg tcacagagca ggacagcaag gacagcacct acagcctcag cagcaccctg
   >.....................................CLK.....................................>
   s   g   n   s   q   e   s   v   t   e   q   d   s   k   d   s   t   y   s   l   s   s   t   l
211acgctgagca aagcagacta cgagaaacac aaagtctacg cctgcgaagt cacccatcag ggcctgagct
   >.....................................CLK.....................................>
     t   l   s   k   a   d   y   e   k   h   k   v   y   a   c   e   v   t   h   q   g   l   s
                                                 EcoRI
                                               --+-----
281cgcccgtcac aaagagcttc aacaggggag agtgttaaga attccgg
   >..................CLK..................>>
    s   p   v   t   k   s   f   n   r   g   e   c
```

20.5.2 IGLC, X06876

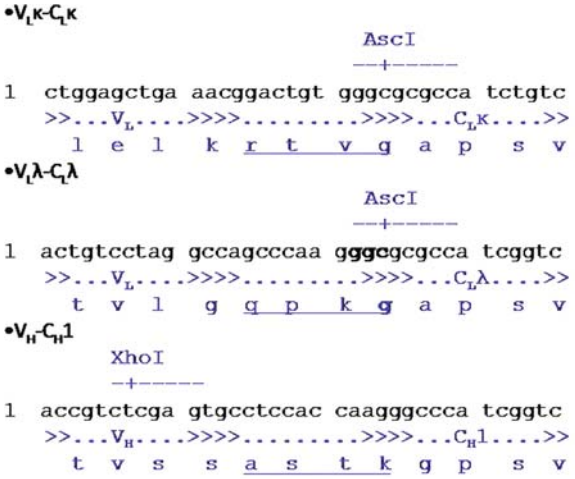


20.5.3 IGHG1, J00228



20.5.4 Intersections

(Letters in bold correspond to amino acids which were altered to generate the required restriction sites)



References

Aarden L, Ruuls SR et al (2008) Immunogenicity of anti-tumor necrosis factor antibodies-toward improved methods of anti-antibody measurement. Curr Opin Immunol 20(4):431–435

Bebbington CR, Renner G et al (1992) High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. Biotechnology (N Y) 10(2):169–175

Jones PT, Dear PH et al (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321(6069):522–525

Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256(5517):495–497

Morrison SL, Johnson MJ et al (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci USA 81(21): 6851–6855

Shawler DL, Bartholomew RM et al (1985) Human immune response to multiple injections of murine monoclonal IgG. J Immunol 135(2):1530–1535

Chapter 21
Humanising Antibodies by CDR Grafting

David Gareth Williams, David J. Matthews, and Tarran Jones

21.1 Introduction

Dramatic and positive change has taken place in the emphasis that large pharmaceutical companies have placed on antibody therapeutics. This has stemmed from the obvious success of those that have progressed to market and the observed lower risk of failure during development compared with small molecule therapeutics. The majority on the market originate from mouse hybridomas, but these have been engineered by the humanisation process known as complementarity determining region (CDR) grafting or reshaping, to overcome therapeutic deficiencies of mouse monoclonal antibodies, which include:

1. Short in vivo half-life
2. Weak effector functions mediated by the mouse heavy chain constant region
3. Patient sensitisation to the antibody, and generation of a human anti-mouse antibody (HAMA) response
4. Neutralisation of the mouse antibody by HAMA leading to loss of therapeutic efficacy.

Antibody chimerisation, the replacement of mouse heavy and light chain constant regions with equivalent human sequences, may alleviate the first two of these deficiencies. This imparts human Fc effector function on the antibody. Even though patient sensitisation and a HAMA response may still occur, chimerisation is responsible for several marketed therapeutics. In distinction, the CDRs and a small number of FR residues are the only mouse sequence remnants after antibody

D.G. Williams (✉), D.J. Matthews and T. Jones
Therapeutic Antibody Group, MRC Technology, 1-3 Burtonhole Lane, Mill Hill, London NW7 1AD, UK
e-mail: david.williams@tech.mrc.ac.uk

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_21,

humanisation by CDR grafting. In this way, all four deficiencies described above are minimised. This technique is responsible for the vast majority of humanised antibodies in the market and in the clinic.

CDR grafting technology was invented by Sir Greg Winter and colleagues (Jones et al. 1986; Riechmann et al. 1988) at the UK Medical Research Council's Laboratory of Molecular Biology and was subsequently patented. This patent, US5225539, is known as the Winter 1 patent, and is the broad dominant patent covering humanisation by CDR grafting. A "continuation in part" describes light chain FR mutations, and the set of Queen patents (e.g. US5585089) held by PDL Inc. describes FR mutations in both heavy and light chains to improve binding potency.

Although competing techniques have become available to reduce the immunogenicity of antibodies, CDR grafting remains the most successfully applied method. Transgenic mice (e.g. Xenomouse and UltiMAb-Mouse), containing large parts of the human immunoglobulin locus, can be a source of "fully human" antibodies, which need no further engineering. However, evidence that these antibodies display a therapeutic advantage over humanised antibodies remains elusive. Human antibodies derived from a bacteriophage library of human variable regions also need no humanisation, but frequently need further mutation in order to achieve high binding potency. A relatively small number of marketed antibodies have been derived using these platform technologies.

CDR grafting, from rodent antibodies into human antibody frameworks, is effective because the folding of the polypeptide backbone in the variable regions and the canonical structures are very similar between these species, despite sequence differences. Mouse B cell hybridomas are the origin of most monoclonal antibodies, and consequently our experience and understanding is predominantly founded on mouse antibody humanisation. Knowledge of the functional importance of critical residues (Al Lazikani et al. 1997; Chothia and Lesk 1987; Chothia et al. 1985, 1989, 1998; Morea et al. 1997, 1998; Tramontano et al. 1989, 1990) in the variable regions is vital in designing the humanised antibody. This knowledge is based on X-ray crystallographic data of free and antigen-complexed antibodies. The crystal structure of the mouse antibody to be humanised is rarely available. However, advances in molecular modelling have enabled homology models of the mouse antibody to be readily constructed based on other antibody structures. The homology model provides us with an indication of which FR residues may be in either direct or indirect (by supporting a CDR sequence) contact with the antigen, and which may therefore be of critical importance for antigen binding potency. A wide base of knowledge has been acquired through each humanisation project, demonstrating that certain FR residues can be critical. Nevertheless, accommodation of a wide variety of antigenic shapes and sizes in the antibody binding site infers that some of these FR residues are not always critical, and therefore can be replaced by a different natural human framework residue without compromising binding potency.

At least 118 antibodies have been humanised. More than 64 of these have been, or are presently undergoing clinical trials (data from IMGT). Of the 24 approved antibodies on the market, 13 are humanised, four murine, five chimeric and two human). These marketed, humanised antibodies were all generated by CDR grafting. Humanisation by CDR grafting has become a clinically proven technology, which continues to offer potential for novel therapeutics.

This chapter describes the CDR grafting method used by MRC Technology to humanise murine monoclonal antibodies.

21.2 Flowchart of Antibody Humanisation

The entire procedure followed by MRC Technology for the humanisation of murine antibodies by CDR grafting is broadly outlined in Fig. 21.1.

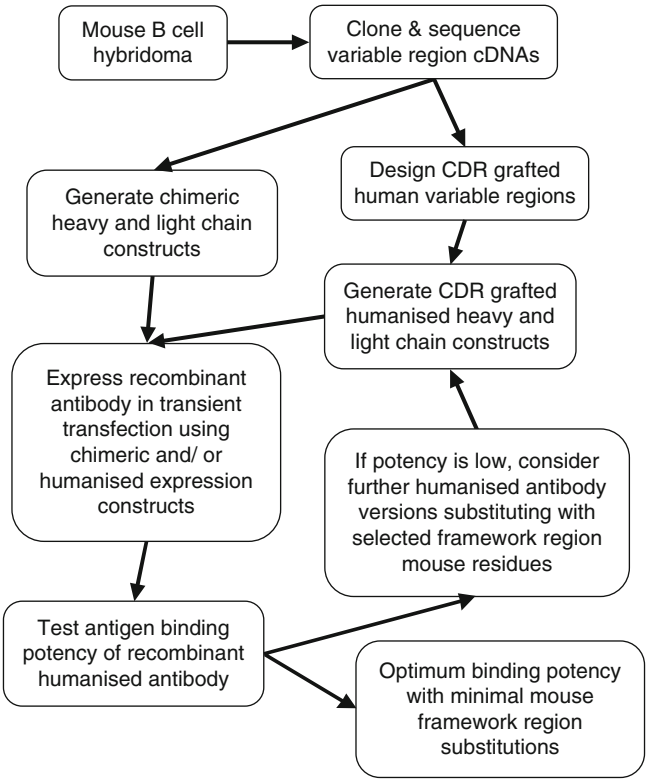


Fig. 21.1 Strategy for humanisation of mouse antibody by CDR grafting

21.3 Cloning and Sequencing Mouse Variable Region cDNA

21.3.1 Overview of Hybridoma DNA Sequencing

The initial task is to isolate cDNAs encoding the variable (V) regions of the mouse antibody to be humanised. V region cDNAs are amplified by polymerase chain reaction (PCR) using a set of primers complementary to the 5′-ends of the mouse constant regions and to the 5′ end of the leader sequence, upstream of the V region. These primers hybridise to sequences outside the V region cDNA, thereby avoiding primer-induced changes in this region. Amplified V region cDNAs are ligated into bacterial cloning vectors, cloned and sequenced. Cloned mouse variable region sequences are used in the construction of chimeric mouse-human light and heavy chains together with suitable leader sequences, and the sequence information is used to design the reshaped human light and heavy chains.

Most mouse myeloma lines used as a B cell fusion partner for generating hybridomas (e.g. SP2, NS0, NS1, P3X63Ag8) express a kappa light chain mRNA, which is not translated – sometimes known as the sterile transcript (Gen Bank accession no. M35669). This is efficiently amplified by the MKV2 + CK primer pair. In the event that this pair of PCR primers is the sole pair, which generates PCR product, it is likely that the functional kappa chain cDNA amplification product is present together with the sterile transcript PCR product. Isolating and sequencing a large number of clones may well not identify the functional light chain sequence. The sterile transcript mRNA can be selectively digested using a complementary ssDNA with RNAse H before 1st strand synthesis(Ostermeier and Michel 1996). An alternative method has recently been described (Cochet et al. 1999) using a poly-peptide-nucleic acid (PNA) specific to the sterile transcript to inhibit its PCR amplification, but allowing free amplification of the functional VK.

21.3.2 Method for RNA Isolation, RT-PCR and cDNA Cloning

21.3.2.1 Materials

- RNA isolation kit (QIAGEN Rneasy Midi Kit, catalogue number 75142)
- First strand cDNA synthesis kit (Pharmacia, 27-9261-01)
- TA Cloning Kit (Invitrogen, K2000-01)
- PCR primers for cloning of variable region genes (Tables 21.1 and 21.2)

21.3.2.2 Procedure

1. Grow the mouse hybridoma cell line in culture medium to provide a total viable cell count of at least 10^8 cells.

Table 21.1 PCR primers for cloning mouse kappa light chain variable regions

MKV1 (30mer)	ATGAAGATTGCCTGTTAGGCTGTTGGTGCTG
MKV2 (30mer)	ATGGAGWCAGACACACTCCTGYTATGGGTG
MKV3 (30mer)	ATGAGTGTGCTCACTCAGGTCCTGGSGTTG
MKV4 (33mer)	ATGAGGRCCCCTGCTCAGWTTYTTGGMWTCTTG
MKV5 (30mer)	ATGGATTTWCAGGTGCAGATTWTCAGCTTC
MKV6 (27mer)	ATGAGGTKCYYTGYTSAYCTYCTCTGRGG
MKV7 (31mer)	ATGGGCWTCAAAGATGGAGTCACAKWYYCWGG
MKV8 (25mer)	ATGTGGGGAYCTKTTTYCMMTTTTTCAATG
MKV9 (25mer)	ATGGTRTCCWCASCTCAGTTCCTTG
MKV10 (27mer)	ATGTATATATGTTTGTTGTCTATTTCT
MKV11 (28mer)	ATGGAAGCCCCAGCTCAGCTTCTCTTCC
MKC (20mer)	ACTGGATGGTGGGAAGATGG
CL12A	ATGRAGTYWCAGACCCAGGTCTTYRT
CL12B	ATGGAGACACATTCTCAGGTCTTTGT
CL13	ATGGATTCACAGGCCCAGGTTCTTAT
CL14	ATGATGAGTCCTGCCCAGTTCCTGTT
CL15	ATGAATTTGCCTGTTCATCTCTTGGTGCT
CL16	ATGGATTTTCAATTGGTCCTCATCTCCTT
CL17A	ATGAGGTGCCTARCTSAGTTCCTGRG
CL17B	ATGAAGTACTCTGCTCAGTTTCTAGG
CL17C	ATGAGGCATTCTCTTCAATTCTTGGG

MKV indicates primers that hybridise to leader sequences of mouse kappa chain V-genes. MKC indicates the primer that hybridises to the mouse kappa constant region gene. Primers CL12A to CL17C were described in US Patent 7186820 (Athwal D.S. et. al.)

Table 21.2 PCR primers for cloning mouse heavy chain variable regions

MHV1 (27mer)	ATGAAATGCAGCTGGGGCATSTTCTTC
MHV2 (26mer)	ATGGGATGGAGCTRTATCATSYTCTT
MHV3 (27mer)	ATGAAGWTGTGGTTAAACTGGGTTTTT
MHV4 (25mer)	ATGRACTTTGGGYTCAGCTTGRTTT
MHV5 (30mer)	ATGGGACTCCAGGCTTCAATTTAGTTTTCCTT
MHV6 (27mer)	ATGGCTTGTCYTTRGSGCTRCTCTTCTGC
MHV7 (26mer)	ATGGRATGGAGCKGGRGTCTTTMTCTT
MHV8 (23mer)	ATGAGAGTGCTGATTCTTTTGTG
MHV9 (30mer)	ATGGMTTGGGTGTGGAMCTTGCTTATTCCTG
MHV10 (27mer)	ATGGGCAGACTTACCATTCTCATTCCTG
MHV11 (28mer)	ATGGATTTTGGGCTGATTTTTTTTATTG
MHV12 (27mer)	ATGATGGTGTTAAGTCTTCTGTACCTG
MHCG1 (21mer)	CAGTGGATAGACAGATGGGGG
MHCG2a (21mer)	CAGTGGATAGACCGATGGGGG
MHCG2b (21mer)	CAGTGGATGAGCTGATGGGGG
MHCG3 (21mer)	CAAGGGATAGACAGATGGGGC

MHV indicates primers that hybridise to leader sequences of mouse heavy chain V-genes. MHCG indicates primers that hybridise to mouse gamma constant region genes

2. Sediment the cells in a centrifuge (250 *g*, 5 min) and resuspend in 20 ml of PBS.
3. Add 100 μl of cells to 200 μl of PBS and 200 μl of trypan blue solution, then mix. Pipette 10 μl of this mixture into a haemocytometer. Count the cells per

square and calculate the number of cells/ml according the manufacturer's instructions.

4. Pellet approximately 10^8 cells (250 g, 5 min).
5. Use the RNA isolation kit QIAGEN Rneasy Midi Kit (catalogue number 75142) as described by the manufacturer to purify total RNA from the cell pellet.
6. Determine the quantity and quality of the total RNA by measuring the OD_{260} and OD_{280} The concentration of RNA = $OD_{260} \times 40$ μg/ml. The quality is satisfactory if OD_{260}:$OD_{280} > 1.9$.
7. Following the manufacturer's instructions, use the GE Life Science 1st strand synthesis kit (Catalogue number 27-9261-01) with the NotI-$(dT)_{18}$ primer, to produce a single-stranded DNA transcript of the hybridoma mRNA. Use 5 μg of total RNA in a 33 μl final reaction volume.
8. Following the reaction, heat at 90°C for 5 min to denature the RNA-cDNA duplex template and to inactivate the reverse transcriptase. Then, chill on ice.
9. Label eleven GeneAmp™ PCR reaction tubes MKV1-11, and twelve tubes with MHV1-12.
10. Prepare a 20 μl reaction in each tube containing:

Kappa chain	Heavy chain
17 μl sterile water	17 μl sterile water
1.7 μl 10× HF2 PCR buffer	1.7 μl 10× HF2 PCR buffer
0.5 μl MKC primer (10 μM)	0.5 μl one MHC primer (10 μM) e.g. MHCG1
0.5 μl of one MKV primer (10 μM, $n = 11$)	0.5 μl of one MHV primer (10 μM, $n = 12$)
0.1 μl 1st strand reaction template.	0.1 μl 1st strand reaction template.
0.14 μl Advantage®-HF 2 DNA polymerase.	0.14 μl Advantage®-HF 2 DNA polymerase.

11. Load the reaction tubes into a DNA thermal cycler and cycle as follows:

Temperature	Time	Number of cycles
94°C	60 sec	1
94°C	30 sec	25
68°C	240 sec	
68°C	180 sec	1

12. Electrophorese 10 μl from each PCR-reaction on a 1% (w/v) agarose/1× TBE (or TAE) gel containing SYBR® Safe DNA stain, to determine which of the leader primers produces a PCR-product. Positive PCR-clones will be about 420–500 bp in size.
13. Directly clone a 4 μl sample of all PCR-products of the correct size into the pCR2.1®-TOPO® vector provided in the TOPO-TA cloning® kit, as described in the manufacturer's instructions [a]. Spread 10%, 1% and 0.1% of the transformed *E.coli* cells onto individual 90 mm diameter LB agar plates containing ampicillin (50 μg/ml) and overlaid with 25 μl of X-Gal (40 mg/ml in DMF).
14. Incubate overnight at 37°C (or over the weekend, at RT).

15. Positive colonies (white) are identified by PCR-screening of the colonies
16. Plasmid DNA minipreps are generated from overnight 3 ml cultures using the manufacturer's instructions.
17. Determine the DNA sequence of 3 minipreps from each PCR product.
18. Repeat those PCR-reactions that appear to amplify full-length variable region cDNA, using the other two 1st strand reaction preps. Three independent clones of each variable region cDNA are sequenced in order to eliminate PCR-errors[b] and RT errors.
19. If no functional V-kappa are detectable, use additional primers CL12A- CL17C (Table 21.1).

Notes:

[a]This kit allows the direct cloning of PCR products without any prior purification and takes advantage of the 3′ overhanging Thymidine at each end of a PCR-product inserted by Advantage®-HF 2 DNA polymerase.

[b]It should be noted that reverse transcriptase itself has an error frequency, approximately 10% of Advantage®-HF 2 DNA polymerase. Consequently, triplicate RT reactions should be set up to eliminate DNA sequence errors. Then, duplicate PCR-reactions from each RT template should be prepared to minimise the incorporation of PCR-based errors.

21.3.3 Method for Identifying Insert-Positive Bacterial Colonies

1. Prepare a bulk solution of the PCR-reaction master mix (sufficient for 20 samples):

sterile water	300.3 µl
10× PCR Buffer	42 µl
25 mM $MgCl_2$	25.2 µl
dNTP 10 mM	8.4 µl
1212 primer 10 µM (Table 21.3)	21 µl
1233 primer 10 µM (Table 21.3)	21 µl
AmpliTAQ/ Thermoprime DNA polymerase.	2.1 µl
Total volume	420 µl

Dispense the master mix (20 µl aliquots) into 20 PCR reaction tubes.

2. Dispense 5 ml of LB/ampicillin (50 µg/ml) into twenty 30 ml universal containers.
3. Using an inoculating needle, stab an individual colony from the transformation mix grown overnight on selective agar plates. Then, stab[a] the needle into one PCR master mix tube, ensuring that the base of the tube is gently touched.
4. With the same needle, inoculate LB in the universal container and shake (300 rpm) overnight at 37°C.

Table 21.3 PCR screening primers

1212 (17mer)	GTTTTCCCAGTCACGAC
1233 (24mer)	AGCGGATAACAATTTCACACAGGA

5. Load the PCR tubes (with negative and positive[b] controls) into a thermocycler and cycle:

94°C × 5 min	×1
94°C × 1 min	×25
50°C × 1 min	×25
72°C × 1 min	×25
72°C × 10 min	×1
hold at 4°C.	
Use a ramp time of 30 sec between each extension step.	

6. Electrophorese 20 μl from each PCR-reaction on a 1% ($^{w}/_{v}$) agarose/ TBE buffer gel containing SYBR® safe dye, to determine the size of any PCR-products.
7. Sequence the DNA from at least three separate clones of correct[c] size. Bands of 520–600 bp will be seen using the 1212 forward and 1233 reverse primers on pCR™2.1 vector containing a V region cDNA. A negative result (i.e. no insert) will produce an approximately 100 bp band.

21.4 Generating Chimeric Antibody

21.4.1 Design and Construction of Chimeric Antibody cDNA

Before embarking on generating humanised versions of the mouse antibody, a chimeric version is constructed and tested for its ability to bind to antigen. There are two main reasons for this. Firstly, binding to antigen confirms that the chimeric antibody is composed of the correct mouse variable regions rather than other cDNA transcripts, which may also be generated from the hybridoma. Secondly, the chimeric antibody is a comparator, which can be used in all binding assays during the humanisation procedure for evaluating each version of the humanised antibody.

In a chimeric antibody, unaltered mouse variable regions are functionally attached to full-length human immunoglobulin constant regions. The chimeric antibody is always constructed with the same human IgG1 kappa constant regions that will be used in the reshaped human antibody versions generated during the humanisation process (change to the ultimate preferred isotype is done at the end of the humanisation phase). It is therefore possible to compare directly the chimeric and reshaped human antibodies in antigen-binding assays that employ anti-human constant region antibody-enzyme conjugates for detection. In the vast majority of cases, chimeric antibody will bind antigen as well as its parent mouse antibody, notable exceptions being the mouse IgG3 isotype, which self-associates, leading to an anomalously high avidity compared with the chimeric antibody.

As a first step in the construction of chimeric light and heavy chains, the cloned mouse variable regions are modified by PCR: at the 5′- end to add restriction enzyme sites for convenient insertion into the expression vectors, Kozak sequence (Kozak 1987) for efficient eukaryotic translation and an appropriate leader sequence; and at the 3′ end, to incorporate kappa intron or heavy chain constant

region up to a natural restriction site. The adapted mouse light and heavy chain variable regions are then inserted into vectors designed to express chimeric or reshaped human light and heavy chains in mammalian cells (Kettleborough et al. 1991). These vectors contain the human cytomegalovirus (HCMV) enhancer and promoter for transcription, an appropriate human light or heavy chain constant region, a gene such as neomycin resistance (neo) for selection of transformed cells, and the SV40 origin of replication for DNA replication in cos cells (Maeda et al. 1991).

21.4.2 Expressing Recombinant Antibody by Transient Transfection

In our original method, the DNA (10 μg each of the kappa and heavy chain vectors) was electroporated into mammalian cells such as COS-7 cells. However, we now use FuGENE® 6 transfection following the manufacturer's procedure (Roche), with 0.5 microgram each of heavy and light chain expression plasmids transfected into mammalian cells, such as HEK293 cells seeded the previous day at 2×10^5 cells per well in a 6-well tissue culture plate.

21.5 Method

Grow cells in 10% FCS Penicillin/Streptomycin in DMEM GlutaMax in a T75 flask in a CO_2-gassed cell culture incubator. Passage the cells 1:3 every 2 days or 1:4 to 1:5 every 3–4 days. The cells adhere weakly to the flasks and only a light trypsinisation is necessary to detach cells during passaging

The day before transfection:

1. Trypsinise the cells, wash 1× in DMEM/10% FCS and count the cells.
2. Add 2 ml of culture media to each well
3. Seed cells at 2×10^5 cells/well.
4. Next day, check cells are at least 80% confluent and replace the medium with a fresh 2 ml per well.
5. 1 μg of total DNA is needed for each transfection and better results are obtained if the DNA concentration is at or above 90 ng/μl.
6. Aliquot 96 μl of Opti-MEM® into a sterile 1.5 ml tube and add 6 μl of FuGENE® 6 directly into the media, avoiding touching the sides of the tube with the FuGENE® 6
7. Mix and leave at RT for 5 min.
8. Add 1 μg of DNA (0.5 μg of heavy and light chain vector).
9. Mix and incubate at RT for 15 min.
10. Add FuGENE® 6 mixture dropwise around the well.
11. Incubate the 6-well plate for 3–4 days.

12. Harvest the conditioned medium, centrifuge and store the supernatant.
13. Perform IgG quantitation (expect 1–4 μg/ml antibody) and antigen binding assays on the supernatant.

The vectors will replicate in these mammalian cells through the action of host cell-encoded large T antigen on the SV40 replication origin on the plasmid. For at least 4 days, transfected cells will continue to survive, express and secrete recombinant antibody. Conditioned medium containing this secreted antibody is harvested after 3–4 days and its antibody content is measured.

21.6 Designing the Humanised Antibody

21.6.1 Overview of the Design Phase

Humanisation involves intercalating the mouse CDRs from each immunoglobulin chain within the FRs of the most appropriate human variable region. Selection of these human variable regions is of critical importance. There are over 9,000 heavy and over 2,500 kappa antibodies in the public databases. These include Kabat, GenBank and IMGT® databases. By aligning these databases with the Kabat numbering system and introducing gaps where necessary, it is possible to score each human variable region for identity to the mouse sequence. We determine the residue identity at FR, canonical, VH-VK interface residues and residues identified from the homology model as of potential importance. We identify human sequences that possess similar canonical structures, such as identical CDR lengths and canonical residues. In this way, we generate a limited set of potential human acceptor sequences. This is further refined by excluding sequences possessing potential N-glycosylation sites, unpaired cysteine residues and unusual proline residues, those with incomplete sequence and those with unusual FR4 amino acid sequences. The resulting shortlist of human variable region sequences is refined by maximising sequence identity and homology to the mouse antibody until an overall lead human sequence is established and selected as the human acceptor sequence for CDR grafting. The goal is to achieve antigen binding potency similar to the original antibody by introducing the minimum number of mouse residues into the FRs of the humanised antibody.

21.6.1.1 Databases and Software

The following databases and software packages have been found to be useful when designing a humanised antibody.

- Accelrys™ Modeler homology modelling software
- DNAStar Lasergene molecular biology software

- Kabat 1998 database of human and mouse immunoglobulin sequences: ftp://ftp.ncbi.nih.gov/genbank/repository/kabat/fasta_format
- IMGT® database of human and mouse immunoglobulin sequences http://www.IMGT®.org/
- V-base database of human germline immunoglobulin gene sequences: http://vbase.mrc-cpe.cam.ac.uk/
- A homology model of the mouse variable regions (unless an X-ray crystal structure is available)
- RCSB database of protein structures: http://www.rcsb.org
- Deep View, a pdb structure viewer: http://spdbv.vital-it.ch/

21.6.2 Selecting the Most Appropriate Human Frameworks for CDR Grafting

1. Create two files (using e.g. Editseq in Lasergene) containing the amino acid sequences of the heavy and light chain variable regions, of the mouse antibody in Kabat aligned format.
2. Generate human VH and VK databases, combining information from the Kabat 1998 database and the current IMGT® database in alignment using the Kabat numbering system.
3. Calculate the number of framework residues in each member of the human database, identical to the mouse antibody.
4. Calculate the number of canonical FR residues and VH-VL interface residues in each member of the human database, identical to the mouse antibody.
5. Identify a set of human frameworks that are most identical to the mouse frameworks at canonical residue positions.
6. The canonical loop structure is associated with a particular CDR length and certain variable region canonical positions. For a given loop structure, individual canonical positions (Table 21.4) are restricted to a group of alternative amino acids. A human FR is selected with canonical residues preferably consistent with the canonical structure of the mouse antibody. This will tend to ensure that the human FR supports the mouse CDR canonical structures.
7. Usually, the most appropriate human frameworks will come from different antibodies. This could result in misalignment of the unnaturally paired human VH and VL. Additionally, the mouse interface may be atypical, which will affect the CDR loop alignment. Usually, however, the interface residues are well conserved and the interface residues are included in the selection criteria for human FRs. An atypical interface residue in the mouse antibody may need to be conserved in the humanised antibody.
8. Arrange the human sequences in order of sequence identity to the mouse antibody, using canonical residue and total FR identity.
9. Discard from this list, human sequences in which VH CDR 1/2 and VK CDR1/2/3 lengths do not match the mouse antibody. However, do not discard

Table 21.4 Conserved residues found at the V_L/V_H interface[a]

Variable Region	Residue Position[b]	Number of sequences analysed	Number of different amino acids observed	Principal amino acids at this position (Number of occurrences[c])
V_L	34	1,365	16	A(326), H(306), N(280)
	36	1,324	7	Y(1057), F(143)
	38	1,312	11	Q(1158)
	44[d]	1,244	14	P(1060)
	46	1,252	17	L(827)
	87	1,222	8	Y(874), F319)
	89	1,238	16	Q(654)
	91	1,234	17	W(275), Y(216), G(209), S(169)
	96[d]	1,034	20	L(220), Y(203), W(196), R(121)
	98[d]	1,066	6	F(1058)
V_H	35	1,459	19	H(378), N(356), S(287)
	37	1,398	10	V(1212), I(151)
	39	1,397	13	Q(1315)
	45[d]	1,397	10	L(1362)
	47	1,357	14	W(1252)
	91	1,689	9	Y(1332), F(340)
	93	1,683	16	A(1426)
	95	1,451	20	D(285), G(212), S(187)
	100–100K[d, e]	1,211	19	F(707), M(224)
	103[d]	1,276	10	W(1251)

[a]The positions of interdomain residues were as defined by Chothia et al. 1985. The immunoglobulin sequences analysed were from the database of Kabat (Kabat et al. 1991)
[b]Numbering is according to Kabat (Kabat et al. 1991). The residue numbers printed in bold are located within the FRs of the variable region
[c]Only those residues that displayed a frequency of occurrence of >10% are shown
[d]One of six residues that constitute the core of the V_L/V_H interface (Chothia C, Novotny J et al. 1985)
[e]The residue that is immediately *N*-terminal to residue 101 in CDR3 is the amino acid that is part of the core of the V_L/V_H interface. The numbering of this residue varies

nonmatching human sequences if the hybridoma CDR length is not represented in the human germline gene repertoire. In this case, consider using human sequences with CDR similar in length to the mouse antibody.

10. Exclude those human sequences containing: (a) unusual proline (introduces rigidity into the polypeptide chain); (b) cysteine (introduces potential for oxidative damage) residues; and c) potential N-glycosylation sites.
11. Analyse for N-glycosylation motifs. To be an efficient acceptor for N-glycosylation, asparagine must be part of the consensus amino acid sequence N-X-(S/T)-Z, except where X and Z are prolines[e] (Gooley et al. 1991). No consensus sequence has yet been identified for O-glycosylation (Calvete and Sanz 2008). Following these rules will identify **possible** sites of glycosylation that should be treated with caution. N- or O-glycosylation within the human donor FR may cause steric hindrance of antigen binding by the reshaped antibody. Potential

glycosylation sites in the candidate human variable regions should therefore be avoided.

12. If there is a potential N-glycosylation site in the mouse FR, then this must be de-activated in the chimeric version by mutagenesis from Asn to Gln in order to determine whether it contributes to antigen binding. If it does, then this glycosylation motif must be retained, and preferably included in the search criteria for the best human acceptor FR. If it does not, then the motif can safely be ignored in the FR selection and back mutation of the humanised antibody.
13. Discard "human" sequences, which turn out to be of mouse origin, or humanised sequences or scFv.
14. By collating all of the above data and comparing the best candidates for conservative change at each mismatched residue, make a decision on the most appropriate human FR sequences.

21.6.3 Generating a Homology Model of the Mouse Antibody

It is important to identify amino acid residues that may contribute towards forming the antigen-binding site either through direct contact, or indirectly, by supporting structures that do make contact with antigen. The identification of these potentially critical residues is done by analysis of the primary structure of the mouse variable regions, and of the homology model of the 3-D molecular structure of the mouse antibody. Various homology-modelling programmes have been written. WAM, an internet-based resource from University of Bath (http://antibody.bath.ac.uk/), is specific for modelling antibody structures and utilises information about the antibody canonical loop structures. Commercially available homology modelling programmes are not specific to antibodies, but some can incorporate antibody-specific loop information. We have used Modeler (Accelrys) to generate homology models. All programmes use antibody X-ray crystallographic structures publicly available at the RSCB protein data bank (http://www.rcsb.org/pdb) As a first step in the modelling exercise, a BLAST search, using the complete variable region sequences of the hybridoma, is done against sequences in the structure database to identify the most appropriate structure templates to be used for homology modelling. Generally, the best heavy and light chain templates arise from different antibody structures. These templates must then be combined into a single antibody structure before homology modelling. Alternatively, the single best compromise antibody template is used. The homology model, based on the combined or single compromise template, can be further refined by loop remodelling, based on the best matching CDR loop structure identified by a BLAST search, but without remodelling the framework region.

The homology model is used to identify residues that may bind antigen directly, and also to identify residues that, by contacting the CDRs, support the hypervariable loops. These critical sets of residues are a priority for conservation during framework selection, and, if not conserved, are a priority for subsequent framework mutagenesis, to optimise the potency of the humanised antibody.

It is important to establish whether the FR contains N-glycosylation motifs, which may lead to glyosylation-dependent effects on antibody binding. If found, the functional significance of the motif must be established by its removal by mutagenesis. Antigen binding assays with and without the mutation are then made. If both forms are of equal binding potency, the motif can be safely ignored, otherwise its retention must be considered.

1. Using BLAST software, identify antibody structures with highest overall variable region identity to the mouse antibody heavy or light chains.
2. Combine these high identity heavy and light chain structures into a single template structure
3. Derive (using e.g. Modeler software) a homology model of the mouse antibody with this combined template, using the DOPE score to identify the best model.
4. Identify the loop structures with highest identity to the mouse antibody.
5. Remodel the loop structures without remodelling the framework structure.
6. Identify the best model, again using DOPE score.

21.6.4 Identifying Critical Framework Residues in the Homology Model

1. Identify all FR residues that are close to a CDR atom and/or make hydrogen bonds with a CDR residue. These residues are a high priority for retention in the human FR selection process.
2. Identify FR residues, which by virtue of location and orientation, may be able to bind antigen directly. Reserve these for possible back-mutation.
3. There are no canonical residues identified for the CDR H3 loop. Therefore, FR residues bordering the CDR H3 should be subject to analysis to determine whether their replacement by residues from the human framework may be detrimental to the CDR loop structure.
4. Identify exposed surface amino acids, which differ between the mouse and the human frameworks and are unlikely to have a role in antigen binding. These can be ignored during the human FR selection process.
5. Target atypical VH-VL interface residues (Table 21.4) for investigation. Using a pdb molecular visualisation programme (e.g. Deep View), replace the nonconserved mouse residue with the human equivalent. Determine whether that in silico mutation introduces no molecular stress, in which case, that residue may be considered for back mutation to the mouse residue in the humanised version. Alternatively, if known, try to reproduce the VH-VL interface residue pairing in the original human antibody from which the human acceptor was chosen; or reproduce the original interface residue pairing in the mouse antibody by introducing back mutations in the humanised versions.
6. Determine whether any potential glycosylation sites are located on the surface of the variable regions since these will need to be accessible if they are to be relevant.

21.6.5 Selecting Human FR Residues for Replacement with the Mouse Equivalent

1. If the chosen human FR is incomplete at the FR4 end, or contains unusual or inconvenient residues, then it may be replaced by the human germline JH sequence most identical to the mouse FR4 (this was done with Campath 1H VK: the human REI light chain has an unusual FR4).
2. Identify canonical residues, which do not conform to the canonical structure set, for priority back mutation.
3. Identify unconserved FR residues, in the van der Waals envelope around the CDRs, for priority back mutation.
4. Identify FR residues from the mouse antibody homology model, which may interact directly with antigen for lower priority back mutation.
5. Identify unusual VH-VL interface residues for lowest priority mutation, e.g. by substitution with the interface residue found in the normal human VK partner of the human antibody VH; or consider introducing mouse interface residues.

21.6.6 Design the Protein and DNA Sequences of the Initial Versions of the Humanised Antibody

1. Select the most appropriate human VH and VK in which to graft the mouse CDRs by considering all the evidence gathered as previously described.
2. Replace the amino acid sequences of the human Kabat CDRs with the residues of the mouse Kabat CDRs. This is the basic CDR grafted version.
3. Design a second version in which all unconserved canonical residues, which are not in the appropriate canonical group, in the basic CDR grafted version, are replaced by the mouse version (backmutation).
4. Generate the DNA sequences of the humanised chains using the natural mouse CDR and human FR DNA sequences.
5. Identify the human germline gene from which the human FR is derived, e.g. using IMGT® gene-quest or V-base.
6. Extract the leader DNA sequence from that germline gene and append it to FR1 of the humanised antibody construct.
7. Check that the leader sequence is predicted to cut appropriately by signal peptidase, using programs such as SignalP (http://www.cbs.dtu.dk/services/SignalP/). If correct cutting is not predicted, choose a different leader sequence.
8. Using DNA sequence editing software (e.g. DNAstar Lasergene) identify cryptic splice sites. Modify the DNA sequence to inactivate these sites without changing the amino acid sequence, using codons with similar human expression rates.
9. Append other sequences necessary for cloning and expression as described above for the chimeric antibody.

21.7 Generating and Testing the Humanised Antibody

21.7.1 Generating the DNA Sequences and Linking Them to Human Constant Regions

1. Once the amino acid sequences of the reshaped human variable regions have been designed, it is necessary to decide how DNA sequences coding for these amino acid sequences will be constructed. Advances in nucleic acid chemistry have made it relatively straightforward to generate complete variable regions by gene synthesis in a very short period. A five-day process is now commercially available (e.g. GENEART AG, Germany). This has greatly simplified and speeded up the humanisation process, giving us more flexibility to investigate the effect of alternative frameworks on binding potency, expression yield and biophysical stability.
2. Synthesise the basic CDR grafted version and the backmutated version of the humanised antibody VH and VL cDNAs.
3. Ligate the reshaped human variable regions, together with their leader sequences, into mammalian cell vectors that already contain a human constant region. These expression vectors are the same as those used for the construction of chimeric light and heavy chains (Kettleborough et al. 1991; Maeda et al. 1991).
4. Express humanised antibody by co-transfection of a heavy and a light chain expression plasmid into mammalian cells (Section. 21.4.2). Generate both fully humanised and chimeric + humanised, mixed chain combinations, as well as the fully chimeric antibody.

21.7.2 Expressing Initial Versions of Humanised Antibodies

The two mammalian cell expression vectors, encoding reshaped human and heavy chains, are co-transfected into mammalian cells, such as COS7 and HEK293T cells by either electroporation using the method of Kettleborough et al. (Kettleborough et al. 1991), or chemical transfection using, e.g. FuGENE® 6 (Roche) as described for the chimeric antibody.

Measure the recombinant antibody concentration in the conditioned medium harvested after 3–4 days culture.

21.7.3 Quantitating Recombinant Antibody in Transfected Cell Conditioned Medium

21.7.3.1 Materials

- Nunc-Immuno Plate MaxiSorp (Life Technologies, 43945A)
- Goat anti-human IgG antibody, Fc_{γ} fragment-specific (Jackson Immuno-Research Laboratories Inc. 109-005-098)

– Human IgG1/kappa antibody (Sigma, I-3889)
– Goat anti-human kappa light chain peroxidase conjugate (Sigma, A-7164)
– K-BLUE substrate (Sky Bio, KB176)
– RED STOP solution (Sky Bio, RS20)
– Sample enzyme conjugate buffer (SEC buffer)

SEC buffer recipe: 0.02% (v/v) TWEEN 20; 0.2% (w/v) BSA; diluted in 1× PBS

21.7.3.2 Procedure

1. Coat each well of a 96-well immunoplate with 100 µl aliquots of 0.4 mg/ml goat anti-human IgG antibody, diluted in SEC buffer, incubate overnight at 4°C.
2. Remove the excess coating solution and wash the plate three times with 200 µl/well of washing buffer (1× PBS, 0.1% TWEEN).
3. Dispense 100 µl of SEC buffer into all wells except the wells in column 2, rows B to G.
4. Prepare a 1 mg/ml solution of the human IgG1/kappa antibody in SEC buffer to serve as a standard. Pipette 200 µl/well into the wells in column 2, rows B and C.
5. Centrifuge the medium from transfected cos cells (250 *g*, 5 min) and save the supernatant.
6. Pipette 200 µl of the supernatant from the "no DNA" control (where cos cells were transfected in the absence of DNA) into the well in column 2, row D.
7. Pipette 200 µl/well of experimental supernatants into the wells in column 2, rows E, F, and G.
8. Mix the 200 µl aliquots in the wells of column 2, rows B to G, and then transfer 100 ml to the neighbouring wells in column 3. Continue to column 11 with a series of twofold dilutions of the standard, control, and experimental samples.
9. Incubate at 37°C for 1 hr. Rinse all the wells six times with 200 µl aliquots of washing buffer.
10. Dilute the goat anti-human kappa light chain peroxidase conjugate 5,000-fold in SEC buffer and add 100 µl to each well. Repeat the incubation and washing steps (step 9).
11. Add 150 µl of K-BLUE substrate to each well, incubate in the dark at room temperature for 10 min.
12. Stop the reaction by adding 50 µl of RED STOP solution to each well. Read the optical density at 655 nm.

21.7.3.3 Comments

- Coated immunoplates (step 1) may be stored for 1 month at 4°C.
- To avoid possible edge effects, the outer wells of the immunoplate are not used.
- The optimal dilution of any antibody or antibody-enzyme conjugate used should be determined for each lot.

21.7.4 Comparing the Antigen-Binding Potency of Humanised Antibody Versions

1. Chimeric and reshaped human antibodies are produced in the same way. These antibodies can then be tested and their relative antigen binding potencies compared. When purified antigen is available, the simplest potency assay is an ELISA in which antibody binding to an antigen-coated immunoplate is detected using an anti-human antibody-enzyme conjugate. This is sufficient to rank the relative potencies of each humanised version in order to determine the importance of individual FR mutations. More accurate equilibrium and kinetic constants can be determined using biosensors, which can monitor antibody-antigen interaction in real time, one example of this technology being surface plasmon resonance. However, a purified antigen is often not available for a binding assay. This may be because the antigen is a membrane protein that has not or cannot be expressed in a soluble form. Antigen expression on the surface of an adherent line, such as CHO, transfected with an expression plasmid, facilitates the design of a cell-based ELISA. Other methods exist for attaching nonadherent cells to immunoplates suitable for ELISA. Such cell-based ELISAs tend to have a higher well-to-well variability than those using purified antigen, and may suffer from binding to other cell surface components. Alternatives to the cell ELISA include quantitative flow cytometry and the kinetic exclusion assay (Rathanaswami et al. 2008).
2. Analyse the antigen binding by these versions and rank these versions in comparison to the chimeric antibody.

21.7.5 Designing Further Humanised Antibody Versions

1. Where the basic CDR graft, with no FR mutations, exhibits a binding affinity as good as the chimeric antibody, no further versions are made.
2. Where this version exhibits poor binding affinity, ascertain whether the heavy or light chain reshaped variable region is the cause. Express the individual humanised heavy and light chains in combination with the complementary chain from the chimeric antibody (e.g. co-transfection with humanised heavy plus chimeric light chains) and measure the binding affinity of the expressed antibody.
3. In the situation that the version with back mutations at all mismatched canonicals residues exhibit binding as good as the chimeric, but the basic CDR grafted version does not, then each back mutation should be separately reversed in a series of reverse back mutations to define the set of necessary and critical back mutations. However, if neither of these versions binds antigen as well as does the chimeric antibody, then define further back mutations to additionally introduce.
4. If a potential N-glycosylation acceptor site exists within one of the CDRs, consider removing it. Conversely, if a glycosylation motif is located within the mouse FR, consider introducing it into the reshaped antibody.

Table 21.5 Important residues for the maintenance of CDR loop conformation[a]

CDR Loop	Canonical structure (Loop size[b])	Residues important for loop conformation[c] (Most common amino acids)
L1	1 (10)[d]	2(I), 25(A), 29(V, I, L), 33(M, L), 71(F, Y)
	2 (11)	2(I), 25(A), 29(V, I, L), 33(V, L), 71(F, Y)
	3 (17)	2(I), 25(S), 29(V, I, L), 33(L), 71(Y, F)
	4 (16)	25(G), 30(I), 33(V), 71(A)
	5 (13)	25(G), 30(I), 33(V), 71(A)
	7 (14)	25(G), 30(I), 33(V), 71(A)
	8 (12)	2(N), 25(A), 29(V, I), 33(L), 71(Y)
L2	1 (7)	48(I, V), 64(G)
L3	1 (9)	90(Q, N, H), 95(P)
	2 (9)[d]	94(P), 95(L)
	3 (8)	90(Q), 96(P)
	4 (9)	90 (L, S), 94 (N, S), 95(H, L)
	5 (11)	94 (D, N, G)
H1	1 (5)	24(T, A,V, G, S), 26(G), 27(F, Y, T, G), 29(F, L, I, V), 34(M, I, V, L, T), 94(R, K, T, A)
	1′ (5)	26(G), 27(S, D), 29(F, I), 34(Y, W), 94(R, N)
	2 (6)	24(V, F), 26(G), 27(F, Y, G), 29(I, L), 35(W, C), 94(R, H)
	3 (7)	24(V, G, F), 26(G), 27(G, F, D), 29(L, I, V), 34(W, V), 94(R, H)
H2	1 (16)	55(G, D), 71(V, K, R, I)
	2 (17)	52a(P, T, A), 55(G, S), 71(A, T, L)
	3 (17)	52a(P, D), 54(G, S, N, D), 55(G, S), 71(R)
	4 (19)	54(K, S), 55(Y), 71(R)

[a]This table summarises information presented in a number of papers by Chothia et. al. (Al Lazikani et al. 1997; Chothia and Lesk 1987; Chothia et al. 1989, 1992; Tramontano et al. 1989, 1990)
[b]Loop size is the number of residues in the CDR loop as defined by Kabat et al. 1991
[c]Numbering is according to Kabat et al. 1991. Residues 26–30 of the heavy chain variable region are defined as FR residues by Kabat et al. 1991, however, structurally they are part of the H1 loop
[d]These canonical structures have been observed only in mouse antibodies and not in human antibodies

5. Synthesise and retest any new versions as before. Continue introducing back-mutations until optimum binding potency is achieved, then remove each in a separate version to define the minimum set of FR back mutations necessary. The more mutagenesis that is done, the closer the antibody approaches the mouse variable region sequence, thereby increasing potential immunogenicity and defeating the whole object of the humanisation strategy. The approach made here depends on the binding affinity of the first reshaped version. A reasonable expectation is that a reshaped antibody will exhibit a binding affinity of more than 30%, and preferably, at least 90%, of the chimeric antibody as measured either in ELISA or surface plasmon resonance assay Table 21.5.

Acknowledgements The authors wish to acknowledge the valuable contributions of all previous workers in the Therapeutic Antibody Group (and its earlier incarnations, including AERES Biomedical Ltd.) at MRC Technology in developing and testing the methods outlined in this chapter, especially MRC Technology scientists (past and present) Mary Bendig PhD, Alex Brown MSc, Jon Chappel PhD, Margaret Cronin BSc, Dolores Crowley BSc, Eilish Cullen BSc, Vicky

Heath BSc, Simon Keen BSc, Katy Kettleborough PhD, Olivier Léger PhD Alison Levy BSc, Charlotte Morrison BSc, Grant Munroe PhD, Siobhan O'Brien PhD, Sue Potts BSc, José Saldanha PhD, Alicia Sedo, BSc and Linda Smith BSc, as well as visiting scientists Dr. Hiroshi Maeda of Kaketsuken, Dr. Frank Kolbinger of Novartis, and Dr. Masa Tsuchiya and Dr. Koh Sato of Chugai Pharmaceuticals.4 to 5

References

Al Lazikani B, Lesk AM, Chothia C (1997) Standard conformations for the canonical structures of immunoglobulins. J Mol Biol 273(4):927–948

Calvete JJ, Sanz L (2008) Analysis of O-glycosylation. Methods Mol Biol 446:281–292

Chothia C, Gelfand I, Kister A (1998) Structural determinants in the sequences of immunoglobulin variable domain. J Mol Biol 278(2):457–479

Chothia C, Lesk AM (1987) Canonical structures for the hypervariable regions of immunoglobulins. J Mol Biol 196(4):901–917

Chothia C, Lesk AM, Gherardi E, Tomlinson IM, Walter G, Marks JD et al (1992) Structural repertoire of the human VH segments. J Mol Biol 227(3):799–817

Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, Air G et al (1989) Conformations of immunoglobulin hypervariable regions. Nature 342(6252):877–883

Chothia C, Novotny J, Bruccoleri R, Karplus M (1985) Domain association in immunoglobulin molecules. The packing of variable domains. J Mol Biol 186(3):651–663

Cochet O, Martin E, Fridman WH, Teillaud JL Selective PCR (1999) Amplification of functional immunoglobulin light chain from hybridoma containing the aberrant MOPC 21-derived V kappa by PNA-mediated PCR clamping. Biotechniques 26(5):818–820, 822

Gooley AA, Classon BJ, Marschalek R, Williams KL (1991) Glycosylation sites identified by detection of glycosylated amino acids released from Edman degradation: the identification of Xaa-Pro-Xaa-Xaa as a motif for Thr-O-glycosylation. Biochem Biophys Res Commun 178(3):1194–1201

Jones PT, Dear PH, Foote J, Neuberger MS, Winter G (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321(6069): 522–525

Kabat EA, Wu TT, Perry HM, Gottesman KS, and Foeller C (1991) Sequences of Proteins of Immunological Interest. 5, 1-3242. NIH National Technical Information Service

Kettleborough CA, Saldanha J, Heath VJ, Morrison CJ, Bendig MM (1991) Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation. Protein Eng 4(7):773–783

Kozak M (1987) At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J Mol Biol 196(4):947–950

Maeda H, Matsushita S, Eda Y, Kimachi K, Tokiyoshi S, Bendig MM (1991) Construction of reshaped human antibodies with HIV-neutralizing activity. Hum Antibodies Hybridomas 2(3):124–134

Morea V, Tramontano A, Rustici M, Chothia C, Lesk AM (1997) Antibody structure, prediction and redesign. Biophys Chem 68(1-3):9–16

Morea V, Tramontano A, Rustici M, Chothia C, Lesk AM (1998) Conformations of the third hypervariable region in the VH domain of immunoglobulins. J Mol Biol 275(2):269–294

Ostermeier C, Michel H (1996) Improved cloning of antibody variable regions from hybridomas by an antisense-directed RNase H digestion of the P3-X63-Ag8.653 derived pseudogene mRNA. Nucleic Acids Res 24(10):1979–1980

Rathanaswami P, Babcook J, Gallo M (2008) High-affinity binding measurements of antibodies to cell-surface-expressed antigens. Anal Biochem 373(1):52–60

Riechmann L, Clark M, Waldmann H, Winter G (1988) Reshaping human antibodies for therapy. Nature 332(6162):323–327

Tramontano A, Chothia C, Lesk AM (1989) Structural determinants of the conformations of medium-sized loops in proteins. Proteins 6(4):382–394

Tramontano A, Chothia C, Lesk AM (1990) Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins. J Mol Biol 215(1):175–182

Chapter 22
Humanization by Resurfacing

Johan Desmet, Karen Vanhoorelbeke, and Hans Deckmyn

22.1 Introduction

Different techniques have been developed to reduce the antigenicity of murine antibodies while preserving their affinity. Joining the variable domains of a murine antibody to the constant domains of a human antibody to construct a chimeric antibody was the first strategy to reduce antigenicity (Morrison et al. 1984; Boulianne et al. 1984). Although some successfully designed chimeric antibodies have been reported (Maloney 1999; Wagner et al. 2003; LoBuglio et al. 1989), the majority of them still induced pathologically relevant immune responses against the variable domains (Khazaeli et al. 1991). Since it has been shown that foreign framework regions (FRs) can evoke immune responses (Bruggemann et al. 1987), the next step in the development of humanized antibodies was the "grafting" of the complementarity determining regions (CDRs) on a human acceptor antibody (Jones et al. 1986; Verhoeyen et al. 1988). This procedure usually leads to a significant reduction or complete loss of binding affinity, because certain framework residues are important for maintaining the conformation of the CDRs (Foote and Winter 1992; Chothia et al. 1989) or are even directly involved in antigen binding (Mian et al. 1991). This problem could be solved by reintroducing murine residues into the human framework at positions that are deemed to be critical for CDR loop conformation (Co et al. 1991; Queen et al. 1989; Riechmann et al. 1988). Although Foote and Winter (1992) defined a "Vernier" zone of framework residues that are important for CDR loop conformation, the necessary mutations should nevertheless be determined experimentally for every antibody.

J. Desmet
AlgoNomics NV Gent, Belgium
K. Vanhoorelbeke and H. Deckmyn (✉)
Laboratory for Thrombosis Research, KU Leuven campus Kortrijk, E. Sabbelaan 53, 8500 Kortrijk, Belgium
e-mail: Hans.Deckmyn@kuleuven-kortrijk.be

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_22,

Some CDR-grafted antibodies have shown reduced antigenicity in clinical trials (Nussenblatt et al. 1999; Colnot et al. 2003), while others were still able to evoke immune responses (Ritter et al. 2001).

Next to CDR-grafting, monoclonal antibodies are frequently humanized by changing the murine framework surface residues that are not observed in human immunoglobulins (Igs). These residues are typically mutated into those observed in the most resembling human counterpart (Padlan 1991; Roguska et al. 1994, 1996). This technique of resurfacing an antibody, which was first described by Padlan (Padlan 1991), is based on the observation that the antigenicity of a protein is determined by the accessible and protruding residues (Novotny et al. 1986; Barlow et al. 1986; Benjamin et al. 1984). Since nothing is changed to the CDR-framework interactions, nor to the framework interior, and only subtle changes are made to the exterior, this usually has only minor effects on the conformation and the activity of the resurfaced antibody (Delagrave et al. 1999; Roguska et al. 1994, 1996).

22.2 Materials

It is clear that before any humanization can be envisaged, the sequence of the antibody must be available. Methods to determine the sequence can be found in Chap. 1 (Nina Strebe, Frank Breitling, Dieter Moosmayer, Bodo Brocks & Stefan Dübel) of this book.

22.2.1 Construction of 3-D Models

- A browser with web access
- Microsoft Excel. (See also *Note 1*.)
- A software package for molecular modeling of protein structures. (See also *Note 8*.)

22.2.2 Expression of Humanized Constructs

- QuickChange® Multi Site-Directed Mutagenesis Kit from Stratagene (catalog number 200514 and 200515)
- NZY^+ Broth: 10 g NZ amine, 5 g yeast extract, 5 g NaCl, add deionized water to a final volume of 1 L, adjust pH to 7.5 using NaOH, autoclave, add 12.5 mL of filter-sterilized 1 M $MgCl_2$, 12.5 mL of 1 M $MgSO_4$, 10 mL of 1 M glucose

22.3 Methods

22.3.1 Construction of a 3-D Model

1. Retrieve the amino acid sequences for V_L ("SEQ_VL") and V_H ("SEQ_VH") in FASTA format (http://www.ncbi.nlm.nih.gov/blast/fasta.shtml)
2. Access the web site http://blast.ncbi.nlm.nih.gov/Blast.cgi using a suitable browser
3. Select the BLAST program "protein blast"
4. Enter SEQ_VL in the "Enter accession number, gi, or FASTA sequence" field
5. Select "Protein Data Bank proteins" from the drop-down list in the "database" field
6. Click the "BLAST" button
7. Click "Formatting options"
8. Select "Flat query-anchored with dots for identities" from the drop-down list in the "Alignment View" field
9. Scroll down to the "Alignments" block
10. Select (highlight) and copy (Ctrl-C) all alignment data to the clipboard
11. Paste data as text (using "Paste Special") in the upper-left cell of a blank Excel sheet. (See also *Note 1*.)
12. Convert the data using the "Text to Columns" function with "Fixed width" option, and insert "Break lines" after each character of the sequence
13. Move each 60-character data block up to the right of the previous block in order to reconstruct each sequence on a single line
14. Delete all columns that do not contain sequence data (except the first column comprising the PDB codes)
15. Identify and mark (e.g., by shading) the three CDRs based on the Kabat definitions for residue numbering and CDR location (http://www.bioinf.org.uk/abs). (See also *Note 2*.)
16. Remove amino acid sequences comprising insertions/deletions in FR fragments, if any
17. Calculate for each sequence a value "score" reflecting the usefulness of the corresponding PDB structure as a template structure. A simple and most useful scoring metric is the percentage sequence identity of the FR segments (i.e., the number of dots in FR columns divided by the total number of FR positions * 100). (See also *Note 3*.)
18. Sort data in descending order of scoring values
19. Repeat steps 2–18 for SEQ_VH
20. Calculate the average of the highest V_L and V_H scores ("TOP-score")
21. For each PDB code in the V_L data sheet, check whether it also appears in the list of V_H hits (e.g., using the MATCH function). If it does, calculate the average V_L and V_H score. Search for the highest average value ("COMBI-score")

22. If TOP-score > COMBI-score + 10%, then proceed with the next step. If not, then retrieve the PDB file (e.g., from http://www.rcsb.org) with the highest COMBI-score as the working structure ("WORK") and skip steps 23–25. (See also *Note 4*.)
23. Retrieve each of the individual top-scoring PDB files ("TOP_VL" and "TOP_VH", respectively)
24. Structurally superimpose TOP_VL with TOP_VH by performing a least-squares fit on the main-chain atoms of the beta-barrel fragments. (See also *Note 5*)
25. Remove the V_H part of TOP_VL and the V_L part of TOP_VH. The resulting structure is further named "WORK"
26. Based on the alignments in the Excel sheets, make a list of substitutions to be performed in V_L and V_H in order to construct a FR structure with the correct amino acid sequence
27. Model the substitutions according to one of the following possibilities: (1) perform an energy-based rotameric search for each individual substitution in the context of the structure WORK, or (2) check for the most frequent conformation of each substitution in other PDB files, and mutate the structure WORK accordingly. (See also *Note 6*.)
28. Optionally, model the CDR loops using a suitable loop modeling/grafting method. (See also *Note 7*.)
29. Optionally perform 200 steps steepest descent energy minimization, followed by another 200 steps conjugate gradient minimization. Name this structure "MODEL_MOUSE"

22.3.2 Construction of a Humanized Fv Model

1. Access the web site http://www.ncbi.nlm.nih.gov/igblast using a suitable browser
2. Enter SEQ_VL in the "Enter sequence" field
3. Select "Blastp" from the drop-down list in the "Program" field
4. Select "Mouse" from the drop-down list in the "Origin of the query sequence" field
5. Select "nr" from the drop-down list in the "Database" field
6. Select "Human" from the drop-down list in the "Organism" field
7. Select "100" from the drop-down list in the "Maximal number of alignments to show" field
8. Click the "Search" button
9. After retrieving the results, scroll down to the alignments
10. Select (highlight) and copy (Ctrl-C) all alignment data to the clipboard
11. Paste data (Ctrl-V) in the upper-left cell of a blank Excel sheet
12. Select column B, Copy, and Paste-Special data as values in the upper-left cell of a blank sheet. Delete the previous sheet

13. Delete all lines relating to non-human sequences (typically, the top three hits referring to murine germline sequences). Also, delete all blank lines
14. Process the data in the same way as in Sect. 22.3.1, steps 12–16
15. Calculate for each amino acid of the input sequence, located within a FR fragment, the frequency "FREQ" at which it occurs in the human sequences below. Preferably, the FREQ-values are calculated using the COUNTIF function and are listed on a line inserted below the input sequence
16. Highlight (e.g., using conditional formatting) all FREQ values less than or equal to two. Such amino acid residues are referred to as "CANDIDATE MOUSE" or "CM" residues
17. For each CM residue, identify the most frequent amino acid in the human sequences. Such amino acid residues are referred to as "CANDIDATE HUMAN" or "CH" residues
18. Repeat steps 2–17 for SEQ_VH
19. Compile a list of candidate substitutions for both VL and VH, named "LIST_VL" and "LIST_VH," respectively
20. Load the structure MODEL_MOUSE, derived in Sect. 22.3.1, using a suitable protein modeling program. (See also *Note 8*.)
21. For each residue position in LIST_VL and LIST_VH, compute the solvent-accessible surface area (ASA) of the side chain of the wild-type (CM) residue (1) in the context of all atoms and (2) in the context of only the main-chain atoms. Divide the former by the latter value and multiply by 100 to obtain the degree of exposure to solvent ("%ASA"). Enter the %ASA data in the Excel sheets on a line inserted below the FREQ data
22. Model the CH mutations from LIST_CH_VL and LIST_CH_VH according to one of the following possibilities: (1) perform an energy-based rotameric search for each individual substitution in the context of the structure MODEL_MOUSE, or (2) check for the most frequent conformation of a given substitution in PDB structures, and mutate the structure MODEL_MOUSE accordingly
23. For each CH residue, calculate the closest distance in Ångström between its side chain and the CDR atoms ("DIST_CDR"). Enter the data in the Excel sheets on a line inserted below the %ASA data. (See also *Note 9*.)
24. For each CH residue, calculate the closest distance in Ångström between its side chain and any other substituted side chain ("DIST_CH"). Enter the data in the Excel sheets on a line inserted below the DIST_CDR data
25. Compile a final list of residues to be humanized ("LIST_HUM_VLVH") by selecting CH residues for which (1) %ASA $>25\%$, (2) "DIST_CDR" >5 Å, (3) DIST_CH <5 Å. (See also *Note 10*.)
26. Optionally, make a final 3-D model for the humanized Fv structure by modeling only the residues from LIST_HUM_VLVH. For this purpose, reload the MODEL_MOUSE structure and apply the same method as in step 22. Optionally, perform 200 steps steepest descent energy minimization, followed by another 200 steps conjugate gradient minimization. Name this structure "MODEL_HUMAN"

22.3.3 Humanization of the Murine Fv

The amino acid residues that need to be changed for the resurfacing according to the above protocol are introduced by either site-directed mutagenesis or by de novo DNA synthesis.

22.3.3.1 Site-directed Mutagenesis

Site-directed mutagenesis can, e.g., be performed using the QuickChange® Multi Site-Directed Mutagenesis Kit from Stratagene (catalog number 200514 and 200515) as described by the manufacturer:

1. Isolate double stranded (ds) plasmid DNA template encoding the murine scFv from a dam$^+$ *Escherichia coli* strain using standard miniprep protocols
2. Design primers to introduce the desired mutations using the Stratagene Web-Based QuickChange Primer Design Program at http://www.stratagene.com/qcprimerdesign
3. Add all primers in approximately equimolar amounts (see also *Note 11*).
   Prepare the mutant strand synthesis reaction for thermal cycling by mixing 2.5 μL 10× QuickChange Multi reaction buffer, and appropriate volumes of double-distilled water (to a final volume of 25 μL), ds-DNA template (50 ng), up to 0.75 μL QuickSolution if templates >5 kb are used, mutagenic primers (100 ng each primer for 1–3 primers; 50 ng each primer for 4–5 primers), 1 μL dNTP mix, 1 μL QuickChange Multi enzyme blend
4. Cycle the reaction using the following protocol: 1 cycle of 95°C for 1 min, 30 cycles of 95°C for 1 min, 55°C for 1 min and 65°C for 2 min per kb of plasmid length
5. Place the reaction mixture on ice for 2 min to cool it to 37°C
6. Add 1 μL of *Dpn*I restriction enzyme (10U/μL) to the reaction mixture
7. Carefully mix the reaction mixture by pipetting up and down, spin down at 13,000 rpm in a microcentrifuge for 1 min and immediately incubate the reactions at 37°C for 1 h to digest the nonmutated parental ds-DNA
8. Gently thaw the XL10-Gold ultra competent cells on ice and add 45 μL cells to a prechilled 14-mL BD Falcon polypropylene round-bottom tube
9. Add 2 μL of β-mercapto-ethanol, swirl the cells gently and incubate the cells for 10 min on ice. Gently swirl the cells every 2 min
10. Gently transfer 1.5 μL of the DpnI-treated DNA to the ultracompetent cells
11. Gently swirl the Falcon tubes and incubate the reaction on ice for 30 min
12. Heat-pulse the tube in a 42°C water bath for exactly 30 sec and immediately incubate on ice for 2 min
13. Add 0.5 mL of preheated NZY+ broth and incubate for 1 h at 37°C with shaking at 225–250 rpm
14. Plate 1 μL, 10 μL and 100 μL of the transformation reaction on agar plates containing the appropriate antibiotic for the plasmid vector
15. Incubate the transformation plates at 37°C for >16 h

16. Pick colonies and prepare ds plasmid DNA using standard miniprep protocols
17. Determine the DNA sequence of the humanized Fv fragment by standard sequencing reactions to determine successful mutagenesis
18. Express the humanized Fv in prokaryotic or eukaryotic cells, depending on the plasmid used, as described in Chap. 29

22.3.3.2 Mutagenesis by de novo DNA Synthesis

Changing the amino acids needed for the humanization of the light and heavy chains also can be done by de novo DNA synthesis, before insertion into a cloning vector such as pUC57 (Genscript corp, Piscataway, NJ, USA). An additional advantage of this approach is that at the same time, some of the original codons can be replaced by codons optimized for high expression levels in the expression cell line of choice (see Chap. 29).

22.4 Notes

1. Steps 11–22 may be executed using any other suitable data processing environment, e.g., MATLAB (http://www.mathworks.com/products/matlab).
2. Alternatively, the less widely used but structurally more relevant Chothia numbering scheme (Delagrave et al. 1999; Roguska et al. 1994, 1996) can be followed.
3. Alternatively, the percentage sequence similarity or a combination identity-similarity can be applied. More sophisticated weighed scoring schemes may include additional parameters such as CDR sequence similarity, the crystallographic quality of candidate structures (e.g., resolution, refinement, structural completeness, NMR/X-ray), and/or the biological origin of the antibody.
4. The proposed 10% tolerance for choosing between TOP- and COMBI-scores results from (unpublished) insights that it is usually advisable to build Fv structures from independent V_L and V_H templates if their average sequence identity with the target sequence is more than 10% higher than that of an already paired V_L/V_H complex.
5. Recommended fragments for fitting include (following Kabat numbering) V_L residues 35–38, 44–49, 84–88, 98–106 and V_H residues 36–39, 45–49, 88–94, 103–110. Alternatively, V_L and V_H domains may be assembled by structural superposition of TOP_VL and TOP_VH framework backbone fragments onto the PDB structure with the highest COMBI-score as identified in step 21. The latter method is formally the same as the method of Sivasubramanian et al. (2009).
6. For a recent benchmark comparison of different methods, see Lu et al. (2008).
7. Various methods exist for de novo loop modeling (Liwo et al. 2008; Olson et al. 2008), as well as specific methods for CDR grafting (Sivasubramanian et al. 2009).
8. Various commercial and free packages exist; see http://en.wikipedia.org/wiki/List_of_software_for_molecular_mechanics_modeling for an overview.

Table 22.1 Sequence of the oligonucleotide primers used for the construction of the humanized scFv of 82D6A3

Mutation(s)[a]	Oligonucleotide primers[b]
H16Glu->Ala, H17Thr->Ser	5′-CTG AAG AAG CCT GGA GCC TCC TCA AGA TCT CCT GC-3′
H43Lys->Gln	5′-CAG GCT CCA GGA CAG GGT TTA AAG TGG-3′
H68Ala->Val, H72Glu->Asp	5′-GGA CGG TTT GTC TTC TCT TTG GAC ACC TCT GTT AG-3′
H82BAsn->Ser, H84Asn->Ser	5′-CC TAT TTG CAG ATC AAC TCC CTC AAA GCC GAA GAC ACG GCT AC-3′
L18Lys->Arg	5′-CA TCT CTG GGA GGC CGG GTC ACC ATC AC-3′
L74Ser->Thr, L77Asn->Ser	5′-A GAT TAT TCC TTC ACC ATC AGC TCC CTG GAG CCT GAG-3′

[a]The first three-letter code of the listed mutations identifies the amino acid of 82D6A3 at the indicated position on the heavy (H) or the light (L) chain that is mutated to the consensus sequence of the selected human antibodies (last three-letter code)
[b]Only the primer hybridizing with the noncoding DNA-strand is shown. The underlined nucleotides introduce the desired mutations

9. Inherent to the method of resurfacing, there is no need to accurately model the CDR loops. If the latter have not been modeled precisely, it is advised to calculate the distances to the FR termini flanking the CDRs.
10. The criteria are heuristic and are basically intended to mutate only residues that are solvent-exposed, are sufficiently remote from the CDRs, and form nonhuman multi-residue patches at the surface. Another aspect that is difficult to capture as an objective rule is the long-range effect of particular substitutions. For example, mutations involving intricate H-bond networks, charge reversal, or beta-branching may exert perturbing effects that are transmitted through the scaffold toward the CDRs. The availability of an atomic model may be of high value in this respect, although structure-based assessment of potential risks can be complicated and subjective. Nevertheless, a rational "second opinion" can be of considerable aid in the final decision process.
11. For the construction of the humanized scFv of 82D6A3, four different primers were used, each introducing one or two mutations (Table 22.1). Mutations were introduced stepwise using one primer at a time. Constructs were always sequenced before introducing new mutations.

22.5 Results

22.5.1 Construction of 3-D Mouse and Humanized Models of 82D6A3 and 6B4 Fv

Worked examples of the proposed resurfacing protocol (Sects. 22.3.1 and 22.3.2) have been published in Staelens et al. (2006) and Fontayne et al. (2006). The former publication related to the humanization of the monoclonal antibody 82D6A3,

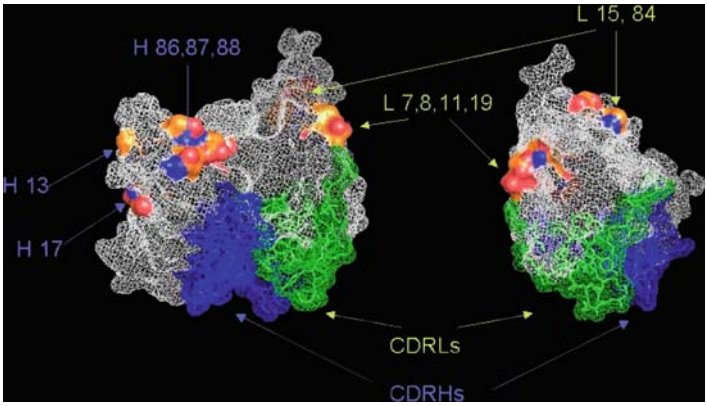


Fig. 22.1 3-D computer model of mFv-6B4 with candidate residues for humanization. The antigen binding region, constituted by the three CDR loops of the light chain (*green*) and the three CDR loops of the heavy chain (*blue*) are shown. The surface framework residues that are nonhuman-like in both VL and VH and that are therefore, preferably mutated to "human-like" amino acids are indicated

directed against the von Willebrand factor A3-domain, whereas the latter concerned the anti-GPIbα mAb 6B4 (Fig. 22.1). The resurfacing method was written out in full in Staelens et al. (2006); some key aspects are discussed hereinafter.

Various highly homologous PDB templates were found for the murine 82D6A3 V_L and V_H sequences, showing framework sequence identities exceeding 90%. The PDB structure 1FJ1 ranked first (93.8% FR identity) in the table comprising V_L alignments, and 17th (91.7% FR identity) in the table with V_H alignments (see Table 3 in Staelens et al. 2006). Since the top-ranked V_H homolog (1IAI) had only about 5% higher identity (96.4%), the criterion for selecting a single PDB template (Sect. 22.3.1, step 22) was fulfilled and the VL-VH pairing steps (steps 23–25) could be omitted. Moreover, the CDRs L1, L2, H1, and H2 were already of the proper length and were taken from the 1FJ1 template. CDRs L3 and H3 required a 1-residue deletion and a 5-residue insertion, respectively, which were carried out (step 28) by selecting and grafting corresponding fragments with high target sequence identity from the PDB structures 1a0Q and 1IGT, respectively. To complete the mouse Fv model, 5 and 18 substitutions were required in V_L and V_H, respectively. All modeling actions were performed using the Brugel package (Delhaise et al. 1988).

The selection of candidate humanization (CH) residues was performed based on evaluation (Sect. 22.3.2, steps 20–25) of significant differences in amino acid usage (steps 15–19) between 82D6A3 murine and homologous human sequences (steps 1–14) in the context of the structure MODEL_MOUSE (Sect. 22.3.1). The benefit of a concrete, case-specific structure became obvious from a comparison between our accessibility criterion (step 21) and the generalized distribution of accessible residues according to Pedersen et al. (1994), which showed several discrepancies (see Table 4 in Staelens et al. 2006). According to the rules of Pedersen, 1 and 5

substitutions should be performed in V_L and V_H, respectively, whereas we selected 5 and 9 based on our ASA criterion. Apart from this, the structure suggested riskful substitution of four residues (two in each chain), and these were omitted from the CH list (see also *Note 10*). The final three V_L mutations formed a spatial cluster, and five of the seven V_H mutations formed a second one. The two remaining V_H mutations, though isolated on the surface, and thus in discord with the DIST_CH rule of Sect. 22.3.2, step 25, were maintained in the final list (Table 22.1) because of their high mouse/human specificity and low-risk profile. A similar approach also resulted in a resurfacing proposal for 6B4 (Fig. 22.1).

22.5.2 *Humanization of the Murine anti-VWF scFv 82D6A3 and anti-GPIbα Fab 6B4*

The DNA sequence encoding the mscFv of 82D6A3 was cloned in the eukaryotic expression vector pSecTag/FRT/V5-His-TOPO® and the mscFv-82D6A3 was expressed in Flp-In™ CHO cells (Staelens et al. 2006). This expressed mscFv bound to VWF and inhibited the VWF binding to collagen, as did its $mIgG_{2a}$ counterpart. This expression plasmid pSecTag/FRT/V5-His-TOPO® encoding mscFv of 82D6A3 was used as a template to perform the site directed mutagenesis in order to create the humanized scFv of 82D6A3 (hscFv 82D6A3). Using the QuickChange® Multi Site-Directed Mutagenesis Kit and the primers listed in Table 22.1, the hscFv 82D6A3 was constructed.

Although the hscFv-82D6A3 bound well to its antigen VWF, expression yields were too low to thoroughly investigate and compare inhibition capacities between mscFv-82D6A3 and hscFv-82D6A3. Therefore, the DNA encoding for both mscFv-82D6A3 and hscFv-82D6A3 was recloned into the prokaryotic expression vector pCANTAB 5E. The mscFv-82D6A3 and hscFv-82D6A3 were expressed in both the medium and the periplasm and were purified to more than 90% purity. As determined by SPR, the affinities of hscFv-82D6A3 and mscFv-82D6A3 for VWF were comparable ($p = 0.08$) and were slightly lower (although not statistically significant: $p = 0.2$), than the affinity of the $mIgG_{2a}$ for VWF (Table 22.2). This was also reflected in the IC_{50}–values: both the mscFv and the hscFv were equally effective ($p = 0.83$) in inhibiting VWF binding to collagen, but were significantly less effective than the $mIgG_{2a}$ ($p < 0.05$) (Table 22.1). These data demonstrate that resurfacing of the mscFv-82D6A3 was successful (Staelens et al. 2006).

To produce the resurfaced humanized 6B4 Fab, the full coding sequence for h6B4-Fab was synthesized de novo (Fontayne et al. 2006). In addition, to allow for production of this molecule on a large scale, 64.2 and 53.7% of the codons were changed in the coding sequence of the V_L and V_H, respectively, for optimal expression by, ultimately, CHO cells. Both V_L and V_H sequences were then introduced into pKaneo-CM30-Lvar and into pKaneo-MCS50-Fabvar before inserting the light chain into pKaneo-MCS50-Fabvar.

Table 22.2 Summary of the activity of murine and humanized 82D6A3(-fragments)

	k_a ($M^{-1}s^{-1}$)[a]	k_d (s^{-1})[a]	K_A (M^{-1})[a]	IC_{50} (M)[b]
$mIgG_{2a}$	$1.7 \pm 0.1 \times 10^6$	$1.8 \pm 0.4 \times 10^{-4}$	$2.8 \pm 2.0 \times 10^{11}$	$2.5 \pm 0.3 \times 10^{-10}$
mscFv	$1.3 \pm 0.5 \times 10^5$	$5.1 \pm 2.1 \times 10^{-5}$	$4.1 \pm 1.5 \times 10^{10}$	$4.3 \pm 0.6 \times 10^{-9}$
hscFv	$2.3 \pm 4.1 \times 10^5$	$8.2 \pm 2.3 \times 10^{-5}$	$4.7 \pm 1.0 \times 10^{10}$	$4.1 \pm 0.2 \times 10^{-9}$
$hIgG_4$	$6.4 \pm 0.4 \times 10^5$	$4.3 \pm 1.2 \times 10^{-5}$	$7.5 \pm 0.6 \times 10^{10}$	$4.3 \pm 0.3 \times 10^{-10}$

[a]Kinetic (k_a, k_d) and equilibrium (K_A) constants were determined by SPR and calculated according to a simple 1:1 (Langmuir) binding model with the software of the Biacore™ 1,000 system. The data represent the mean ± SEM calculated from at least seven different antibody concentrations.
[b]The inhibition of the VWF binding to collagen by 82D6A3(-fragments) was determined in ELISA as described in Sect. 22.2. To calculate the IC_{50}, the absorbance obtained in the absence of 82D6A3(-fragments) was arbitrarily set as 0% inhibition. The calculated IC_{50}-data represent the mean ± SEM from three independent experiments.

As anticipated by the codon optimization, the expression yield of h6B4-Fab in HEK 293T/17 cells was doubled as compared to m6B4-Fab (from 15 to 31.6 mg/L).

Both Fab-fragments competed equally with the binding of the biotinylated 6B4-mIgG1 to rGPIbα (IC_{50} of m6B4-Fab was 5.4 ± 1.4 μg/mL; IC_{50} of h6B4-Fab was 1.8 ± 0.2 μg/mL). That h6B4-Fab was equivalent to m6B4-Fab was further shown by affinity measurements using SPR, inhibition tests in vitro and finally also by in vivo thrombosis experiments in baboons (Fontayne et al. 2008).

22.6 Conclusions

Resurfacing is an attractive alternative approach for the humanization of monoclonal antibodies, as a rather minimal number of amino acid residues need to be changed, resulting in maintenance of the framework structure and the correct presentation of the CDRs. This should, in principle, help avoid losses in affinity, which we could confirm by resurfacing two independent murine monoclonal antibodies. The ultimate goal obviously is to produce humanized antibodies with reduced immunogenicity when administered to patients. However, at present, no data are available as to whether resurfacing of antibodies indeed also fulfills this criterion and how then such antibodies would compare with the same antibody not humanized, or humanized by, e.g., CDR grafting (Sect. 22.1.5.1).

References

Barlow DJ, Edwards MS, Thornton JM (1986) Continuous and discontinuous protein antigenic determinants. Nature 322:747–748

Benjamin DC, Berzofsky JA, East IJ, Gurd FR, Hannum C, Leach SJ, Margoliash E, Michael JG, Miller A, Prager EM (1984) The antigenic structure of proteins: a reappraisal. Annu Rev Immunol 2:67–101

Boulianne GL, Hozumi N, Shulman MJ (1984) Production of functional chimaeric mouse human-antibody. Nature 312:643–646

Bruggemann M, Williams GT, Bindon CI, Clark MR, Walker MR, Jefferis R, Waldmann H, Neuberger MS (1987) Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. J Exp Med 166:1351–1361

Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, Air G, Sheriff S, Padlan EA, Davies D, Tulip WR (1989) Conformations of immunoglobulin hypervariable regions. Nature 342:877–883

Co MS, Deschamps M, Whitley RJ, Queen C (1991) Humanized antibodies for antiviral therapy. Proc Natl Acad Sci USA 88:2869–2873

Colnot DR, Roos JC, de Bree R, Wilhelm AJ, Kummer JA, Hanft G, Heider KH, Stehle G, Snow GB, van Dongen GA (2003) Safety, biodistribution, pharmacokinetics, and immunogenicity of 99mTc-labeled humanized monoclonal antibody BIWA 4 (bivatuzumab) in patients with squamous cell carcinoma of the head and neck. Cancer Immunol Immunother 52:576–582

Delagrave S, Catalan J, Sweet C, Drabik G, Henry A, Rees A, Monath TP, Guirakhoo F (1999) Effects of humanization by variable domain resurfacing on the antiviral activity of a single-chain antibody against respiratory syncytial virus. Protein Eng 12:357–362

Delhaise P, Bardiaux M, De Maeyer M, Prevost M, Vanbelle D, Donneux J, Lasters I, Vancustem E, Alard P, Wodak SJ (1988) The Brugel package – toward computer-aided design of macromolecules. J Mol Graph 6:219–219

Fontayne A, Vanhoorelbeke K, Pareyn I, Van Rompaey I, Meiring M, Lamprecht S, Roodt J, Desmet J, Deckmyn H (2006) Rational humanization of the powerful antithrombotic anti-GPIbα antibody: 6B4. Thromb Haemost 96:671–684

Fontayne A, Meiring M, Lamprecht S, Roodt J, Demarsin E, Barbeaux P, Deckmyn H (2008) The humanized anti-glycoprotein Ib monoclonal antibody h6B4-Fab is a potent and safe antithrombotic in a high shear arterial thrombosis model in baboons. Thromb Haemost 100:670–677

Foote J, Winter G (1992) Antibody framework residues affecting the conformation of the hypervariable loops. J Mol Biol 224:487–499

Jones PT, Dear PH, Foote J, Neuberger MS, Winter G (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321:522–525

Khazaeli MB, Saleh MN, Liu TP, Meredith RF, Wheeler RH, Baker TS, King D, Secher D, Allen L, Rogers K (1991) Pharmacokinetics and immune response of 131I-chimeric mouse/human B72.3 (human gamma 4) monoclonal antibody in humans. Cancer Res 51:5461–5466

Liwo A, Czaplewski C, Oldziej S, Scheraga HA (2008) Computational techniques for efficient conformational sampling of proteins. Curr Opin Struct Biol 18:134–139

LoBuglio AF, Wheeler RH, Trang J, Haynes A, Rogers K, Harvey EB, Sun L, Ghrayeb J, Khazaeli MB (1989) Mouse/human chimeric monoclonal antibody in man: kinetics and immune response. Proc Natl Acad Sci USA 86:4220–4224

Lu M, Dousis AD, Ma J (2008) OPUS-Rota: a fast and accurate method for side-chain modeling. Protein Sci 17:1576–1585

Maloney DG (1999) Preclinical and phase I and II trials of rituximab. Seminars Oncol 26:74–78

Mian IS, Bradwell AR, Olson AJ (1991) Structure, function, and properties of antibody binding sites. J Mol Biol 217:133–151

Morrison SL, Johnson MJ, Herzenberg LA, Oi VT (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci USA 81:6851–6855

Novotny J, Handschumacher M, Haber E, Bruccoleri RE, Carlson WB, Fanning DW, Smith JA, Rose GD (1986) Antigenic determinants in proteins coincide with surface regions accessible to large probes (antibody domains). Proc Natl Acad Sci USA 83:226–230

Nussenblatt RB, Fortin E, Schiffman R, Rizzo L, Smith J, Van Veldhuisen P, Sran P, Yaffe A, Goldman CK, Waldmann TA, Whitcup SM (1999) Treatment of noninfectious intermediate

and posterior uveitis with the humanized anti-Tac mAb: a phase I/II clinical trial. Proc Natl Acad Sci USA 96:7462–7466

Olson MA, Feig M, Brooks CL 3rd (2008) Prediction of protein loop conformations using multi-scale modeling methods with physical energy scoring functions. J Comput Chem 15:820–831

Padlan EA (1991) A possible procedure for reducing the immunogenicity of antibody variable domains while preserving their ligand-binding properties. Mol Immunol 28:489–498

Pedersen JT, Henry AH, Searle SJ, Guild BC, Roguska M, Rees AR (1994) Comparison of surface accessible residues in human and murine immunoglobulin Fv domains. Implication for humanization of murine antibodies. J Mol Biol 235:959–973

Queen C, Schneider WP, Selick HE, Payne PW, Landolfi NF, Duncan JF, Avdalovic NM, Levitt M, Junghans RP, Waldmann TA (1989) A humanized antibody that binds to the interleukin 2 receptor. Proc Natl Acad Sci USA 86:10029–10033

Riechmann L, Clark M, Waldmann H, Winter G (1988) Reshaping human antibodies for therapy. Nature 332:323–327

Ritter G, Cohen LS, Williams C Jr, Richards EC, Old LJ, Welt S (2001) Serological analysis of human anti-human antibody responses in colon cancer patients treated with repeated doses of humanized monoclonal antibody A33. Cancer Res 61:6851–6859

Roguska MA, Pedersen JT, Keddy CA, Henry AH, Searle SJ, Lambert JM, Goldmacher VS, Blattler WA, Rees AR, Guild BC (1994) Humanization of murine monoclonal antibodies through variable domain resurfacing. Proc Natl Acad Sci USA 91:969–973

Roguska MA, Pedersen JT, Henry AH, Searle SM, Roja CM, Avery B, Hoffee M, Cook S, Lambert JM, Blattler WA, Rees AR, Guild BC (1996) A comparison of two murine monoclonal antibodies humanized by CDR-grafting and variable domain resurfacing. Protein Eng 9:895–904

Sivasubramanian A, Sircar A, Chaudhury S, Gray JJ (2009) Toward high-resolution homology modeling of antibody F_v regions and application to antibody-antigen docking. Proteins 74:497–514

Staelens S, Desmet J, Ngo TH, Vauterin S, Pareyn I, Barbeaux P, Van Rompaey I, Stassen JM, Deckmyn H, Vanhoorelbeke K (2006) Humanization by variable domain resurfacing and grafting on a human IgG_4, using a new approach for determination of nonhuman like surface accessible framework residues based on homology modeling of variable domains. Mol Immunol 43:1243–1257

Verhoeyen M, Milstein C, Winter G (1988) Reshaping human antibodies: grafting an antilysozyme activity. Science 239:1534–1536

Wagner CL, Schantz A, Barnathan E, Olson A, Mascelli MA, Ford J, Damaraju L, Schaible T, Maini RN, Tcheng JE (2003) Consequences of immunogenicity to the therapeutic monoclonal antibodies ReoPro and Remicade. Dev Biol (Basel) 112:37–53

Chapter 23
Human Antibodies by Guided Selection

Sang Jick Kim, Insoo Park, and Hyo Jeong Hong

23.1 Introduction

Since the advent of hybridoma technology by Köhler and Milstein in 1975, monoclonal antibody (MAb) and its derivatives have found a variety of applications in research and human health care as a result of their exquisite specificity. However, most of the MAbs of murine origin have limited use as therapeutic agents. Repeated administrations of murine MAb cause unwanted induction of a human anti-mouse antibody response, which results in short serum half-life and reduced therapeutic efficacy, and most of them cannot trigger human effector functions (Shawler et al. 1985; Khazaeli et al. 1994). These limitations have driven diverse approaches to the humanization of murine Mabs, including the initial chimeric antibody approach (Morrison et al. 1984), and humanized antibody approaches such as Complementarity Determining Region (CDR) grafting (Jones et al. 1986; Winter and Harris 1993; Reichert 2001), humanization by resurfacing (Roguska et al. 1994), and deimmunization by the removal of T-cell epitope (Pendley et al. 2003). However, the resulting humanized antibodies still have the potential to provoke an immune response in humans.

Guided selection is an alternative method for humanization. This method has been developed to convert a murine antibody into a completely human antibody with similar binding characteristics, based on chain shuffling of V-genes by using phage display technology (Figini et al. 1994, 1998; Jespers et al. 1994; Watzka et al. 1998; Beiboer et al. 2000). Mouse VH and VL domains are used to guide the selection of a human antibody partner and are replaced sequentially or in parallel

S.J. Kim and I. Park
Korea Research Institute of Bioscience and Biotechnology, 111 Gwahangno, Yuseong-gu, Daejeon 305-806, South Korea
H.J. Hong (✉)
School of Biomedical Sciences, Kangwon National University, Chuncheon, Gangwon-do 200-701, South Korea
e-mail: hjhong@kribb.re.kr

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_23,

with human VH and VL domains, respectively, using phage selection to derive a human antibody. The resulting human antibodies therefore bind to epitopes that are at least overlapping with that of the original mouse MAb.

In sequential procedure (Figini et al. 1998; Watzka et al. 1998; Schmidt et al. 2001; Bao et al. 2005; Zhang et al. 2007), original mouse VH or VL domain is shuffled with human VL or VH repertoires, respectively, and the resulting semi-human antibody library is displayed on the phage surface, followed by selection against the target antigen. The human VLs or VHs selected are then shuffled with human VH or VL repertoires, respectively, to select a completely human antibody with the best affinity. Whether the VH or VL of mouse antibody would be shuffled first depends on how much the VH or VL contributes to the antigen-combining site. Some studies started with human light chain shuffling (Watzka et al. 1998; Schmidt et al. 2001; Zhang et al. 2007), while other studies started with human heavy chain shuffling (Figini et al. 1998; Bao et al. 2005). In a parallel procedure (Wang et al. 2000), mouse VH and VL are shuffled with human VL and VH repertoires, respectively, at the same time and the selected human VHs and VLs are recombined. In addition, retention of HCDR3 or both HCDR3 and LCDR3 of the original mouse antibody can be employed during the guided selection procedure (Rader et al. 1998; Beiboer et al. 2000; Klimka et al. 2000).

Guided selection has several advantages over other humanization technologies. The final product, after guided selection, can be completely of human origin unlike other humanization methods, and hence, better therapeutic efficacy can be expected by removing problems related to the immunogenicity of mouse residues. In addition, the guided selection method using chain shuffling can generate diverse human antibodies with better properties compared with the original mouse antibody. While it is common to select antibodies with related sequences to the original mouse antibody, it is also possible to select antibodies with distinct sequences, which show enhanced affinity, expression, and/or stability (Jespers et al. 1994; Kim and Hong 2007).

The utility of guided selection was clearly demonstrated by HUMIRA, the world's first human antibody approved for marketing (Osbourn et al. 2005). HUMIRA is approved for the treatment of rheumatoid arthritis and other autoimmune diseases (Kupper et al. 2007). Selection of HUMIRA with nonmouse components was guided by the anti-human TNF-α mouse MAb MAK 195, and they show similar affinities and neutralizing activities to TNF-α (Osbourn et al. 2005). Thus, guided selection provides a powerful tool for humanization of the preexisting nonhuman antibodies of which biological activities are demonstrated.

23.1.1 Outline of Guided Selection Procedure

The guided selection procedure can be performed sequentially or in parallel using either a scFv or Fab format. This chapter will describe the sequential guided selection procedure using a Fab format as an example, which is outlined in Fig. 23.1. The first step is to clone the variable regions of a mouse antibody to

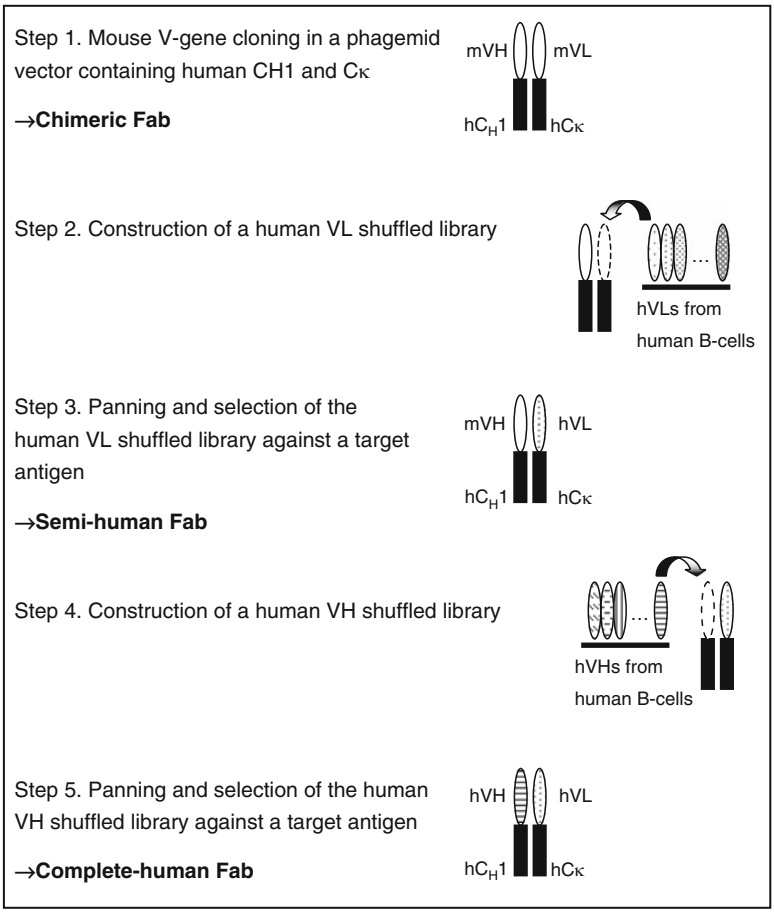


Fig. 23.1 Overview of the sequential guided selection procedure

a phagemid vector containing human constant domains, Cκ and C_H1, which results in a chimeric Fab (*see* Sect. 23.3.1). The next step is to construct a human VL-shuffled library by replacing the mouse VL with human VL repertoires (*see* Sect. 23.3.2). The third step is to select binders after panning against a target antigen (*see* Sect. 23.3.3), which results in the selection of a human VL paired with the mouse VH. The fourth step is to construct a human VH-shuffled library by replacing the mouse VH in the semi-human Fab with human VH repertoires, similar to the protocol for VL shuffling. The fifth step is to select complete-human Fab clones after panning, similar to the protocol for the selection of human VL. Finally, one should confirm the epitope specificity and the affinities of selected human Fabs using proper assays such as competition ELISAs (*see* Sect. 23.3.4) and Biacore analysis.

One of the critical steps to succeed the guided selection procedure is the construction of large human VH and VL-shuffled repertoires because high affinity antibodies can be isolated from the large size of repertoires (Griffiths et al. 1994; Vaughan et al. 1996).

23.2 Materials

- Agarose gel electrophoresis kit
- Centrifuge bottles (500 ml, Nalgene Ref:3120-9500)
- Electroporation apparatus (MicroPulser, Bio-Rad)
- ELISA reader
- GM/MP cuvettes (0.2 cm) for electroporation (BRL, Ref:118219)
- High-speed centrifuge (Sorvall or equivalent)
- Microcentrifuge
- Microtiter plate (96 well, Maxisorp, Nunc)
- Microtiter reader (molecular devices)
- PCR machine
- Shaking incubator
- UV transilluminator
- Vortex mixer
- Water bath
- Agarose (Invitrogen, Ref:15510-027)
- Acetic acid (Sigma, Ref:A9967)
- Gel DNA recovery kit (Zymoresearch, Ref:D4001)
- Taq DNA polymerase (Takara, Ref: RR001)
- *E.coli* TG1 (Stratagene, Ref:200123)
- Helper phage VCSM13 (Stratagene, Ref:200251)
- Isopropyl-β-D-galactopyranoside (IPTG, Sigma, Ref:I6758)
- Trizma base (Sigma, Ref:T1503)
- T4 DNA ligase (Roche Ref:10716359001)
- 3 M NaAc, pH 5.2 (Sigma, Ref:S2889)
- $MgCl_2$ (Fluka, Ref:63068)
- Glycogen (Roche, Ref:10901393001)
- Ethanol (Merck, Ref:1009831011)
- Glycerol (Amresco, Ref:0854)
- Dimethyl sulphoxide (Sigma, Ref:D2650)
- BstNI (Roche, Ref:11228075001)
- BstXI (Roche, Ref:1117785)
- SfiI (Roche, Ref:1288024)
- PEG 8000 (Sigma, Ref:P2139)
- NaCl (Sigma, Ref:S6191)
- Skim milk (Difco, Ref:232100)

- Ampicillin (Sigma, Ref:A9518)
- Kanamycin (Sigma, Ref:K4000)
- D-(+) Glucose (Sigma Ref:G8270)
- BSA (bovine serum albumin, Sigma)
- TMB substrate reagent set (BD, Ref:555214)
- Goat anti-mouse IgG(Fc specific)-HRP (Pierce, Ref:31437)
- Goat anti-human IgG $F(ab')_2$-HRP (Pierce, Ref:31482)
- Minimal agar plate:
- 10× M9 salt (64 g $Na_2HPO_4{\cdot}7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl per 500 ml H_2O). Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.
- Agar (7.5 g bacto-agar per 450 ml H_2O). Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle. Cool down to 50°C. Add 50 ml of 10× M9 salt, 2.5 ml of 1 M $MgCl_2$, 5.5 ml of 2 M glucose, 500 μl of 1 M thiamine hydrochloride, and 50 μl of 1 M $CaCl_2$
- 2×YT (16-g tryptone, 10-g yeast extract, 5-g NaCl per liter, pH 7.0 with 10 N NaOH)
- 2×YTA (2×YT containing 100 μg/ml ampicillin)
- 2×YTG (2×YT containing 2% glucose)
- SOBAG plate (20-g tryptone, 5-g yeast extract, 0.5-g NaCl, 15-g of Bacto-agar, 10 ml of 1 M $MgCl_2$, 55.6 ml of 2 M glucose, and 1 ml of 100 mg/ml ampicillin per liter).
- Coating buffer (1.59-g Na_2CO_3, 2.93-g $NaHCO_3$ per liter, pH 9.6)
- PBS (8-g NaCl, 0.2-g KCl, 1.44-g Na_2HPO_4, and 0.24-g KH_2PO_4 per liter, pH 7.4)
- PBST (PBS containing 0.05% Tween 20)

23.3 Protocols

23.3.1 Cloning of Mouse V-gene in a Phagemid Vector

The first step is to subclone the murine VH and VL genes into a phagemid vector (Fig. 23.2) harboring the human constant domains, C_H1 and Cκ. After transformation, the resulting chimeric Fab is expressed in *Escherichia coli,* and the culture supernatants or periplasmic extracts are analyzed for antigen-binding activity by ELISA.

23.3.1.1 A Phagemid Vector (KRIBB-Fab) Map

See Fig. 23.2

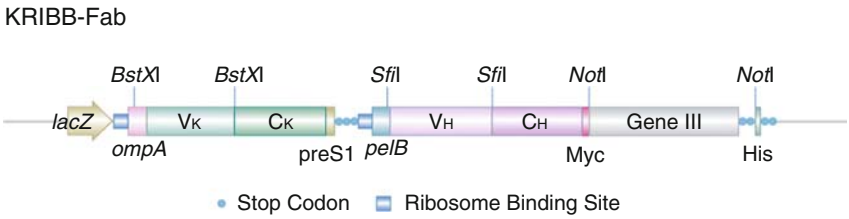


Fig. 23.2 A schematic presentation of the KRIBB-Fab vector. It harbors the *LacZ* promoter, two leader sequences (*ompA* and *PelB*, respectively), a human Ig Fab backbone, and the C-terminal part of M13 Gene III. It also contains three different tags, preS1, myc, and His. The mouse Vκ region can be cloned into the two incompatible *Bst*XI sites, while the mouse VH region into the two incompatible *Sfi*I sites. The Gene III portion can be deleted using the two *Not*I restriction sites while maintaining the His tag. Two stop codons are introduced in the VH region (not shown) to prevent the wild-type backbone contamination in the library. The *OmpA* leader sequence was derived from *E.coli*, while the *PelB* leader sequence from *Erwinia carotovora*

23.3.1.2 Expression of Chimeric Fab in *E. coli*

1. Transform *E. coli* TG1 with the phagemid DNA for the expression of a chimeric Fab.
2. Inoculate 5 ml of 2×YTA with a colony harboring the phagemid DNA.
3. Culture at 37°C until the OD_{600} reaches 0.7~0.8.
4. Add IPTG to 1 mM, then culture at 30°C overnight.
5. Transfer the culture in eppendorf tubes.
6. Centrifuge at 12,500 rpm for 10 min at 4°C.
7. Transfer the culture supernatant in new eppendorf tubes and store at −80°C.

23.3.1.3 ELISA for Antigen-Binding Activity

1. Coat a 96-well plate with a target antigen (100 ng/well) in a coating buffer at 4°C overnight.
2. Wash the plate twice with PBST.
3. Block the wells with 2% skim milk in PBST for 1 h at room temperature (RT).
4. Wash the plate twice with PBST.
5. Add 100 μl of the culture supernatant to each well.
6. Incubate the plate at RT for 30 min.
7. Wash the plate three times with PBST.
8. Add 100 μl of goat anti-human IgG $F(ab')_2$-HRP (1/2,000 dilution).
9. Incubate the plate at RT for 1 h.
10. Wash the plate four times with PBST.
11. Add 100 μl of mixed TMB substrate reagent.

12. Incubate the plate at RT for 5 min.
13. Stop the reaction by adding 50 µl of 2 M H_2SO_4
14. Measure the absorbance using an ELISA reader at 450 nm.

23.3.2 Construction of a Human VL- or VH-Shuffled Library

23.3.2.1 PCR Amplification and Purification of Human Heavy or Light Chain Genes

1. Prepare human VH or VL DNAs by PCR amplification of the cDNAs derived from human B cell sources using human antibody specific primer sets (*see* Sect. 1.2.2 in this book).

H_2O	37.8 µl
10× Taq buffer	5.0 µl
4 mM dNTP	4.0 µl
50 pM Backward primer	1.0 µl
50 pM Forward primer	1.0 µl
50 ng DNA Template	1.0 µl
Taq DNA polymerase	0.2 µl

PCR condition: 30 cycles of 94°C 30 s, 55°C 30 s, 72°C 30 s followed by incubation at 72°C for 10 min.

2. Purify the DNAs on an 1.5% agarose gel using the Gel DNA Recovery Kit, and resuspend the DNA in H_2O.
3. 2nd PCR for adding suitable restriction enzyme sites. Both the purified DNAs from Step 2 and V-genes from a preexisting antibody library can be used as PCR templates [PCR condition: 20 cycles of 94°C 30 s, 55°C 30 s, 72°C 30 s followed by incubation at 72°C for 10 min].
4. Purify the DNAs as described in Step 2 and resuspend in H_2O.
5. Digest the DNAs with restriction endonuclease.
6. Purify the DNA fragments as described in Step 2, and resuspend the DNAs in H_2O.

23.3.2.2 Ligation

1. Set up the following ligation reaction in an eppendorf tube:

Vector DNA (20 ng/µl)	50 µl
Insert DNA fragments (20–30 ng/µl)	10 µl
H_2O	20 µl
10× ligase buffer	10 µl
T4 DNA ligase (1 uint/µl)	10 µl

2. Incubate the tube at 16°C overnight.
3. Heat-kill the ligation mix for 10 min at 70°C.
4. Precipitate the ligation mix with ethanol (2.2 v/v) and 3 M NaAc pH 5.2 (0.1 v/v) in the presence of glycogen.
5. Centrifuge at 12,500 rpm for 15 min at 4°C.
6. Wash the pellet using 75% ethanol, and dry the pellet.
7. Resuspend the pellet in 100 μl of H_2O.

23.3.2.3 Preparation of Electrocompetent Cells and Electroporation

1. Inoculate a single colony of *E. coli* TG1 freshly grown on a minimal agar plate into 10 ml of 2×YT containing 2% glucose and 5 mM $MgCl_2$.
2. Incubate the culture in a shaking incubator at 37°C overnight.
3. Measure the optical density (OD) at 600 nm. To measure the OD, dilute the overnight culture to 1 in 10 with 2×YT.
4. Calculate the quantity of overnight culture to make the starting $OD_{600} = 0.05$ in a large erlenmeyer flask containing 700 ml of 2×YT with 2% glucose and 5-mM $MgCl_2$.
5. Add the calculated quantity of overnight culture to the medium and incubate at 37°C with shaking until the OD_{600} reaches 0.8. It takes about 2 h, as the bacterial doubling time of TG1 is approximately 25 min.
6. Chill the culture on ice for 15 min.
7. Divide the culture into two prechilled 500ml centrifuge bottles. From here on, keep everything on ice, and manipulate as quickly as possible. Use prechilled centrifuge bottles and plastic pipettes.
8. Centrifuge at 5,000 rpm for 15 min at 4°C.
9. Discard the supernatant, and resuspend the cells in 700 ml of prechilled 10% glycerol.
10. Centrifuge at 5,000 rpm for 15 min at 4°C.
11. Discard the supernatant and resuspend the cells in 350 ml of prechilled 10% glycerol.
12. Centrifuge at 5,000 rpm for 15 min at 4°C.
13. Discard the supernatant and resuspend the cells in 50 ml of prechilled 10% glycerol.
14. Centrifuge at 5,000 rpm for 15 min at 4°C.
15. Discard the supernatant and resuspend cells in 3 ml of prechilled 10% glycerol.
16. Transfer the electrocompetent cells to a prechilled 50-ml falcon tube and mix with 50 μl of ligated DNA.
17. Transfer each 300 μl of mixture into a prechilled 0.2-cm electroporation cuvette. Dry the cuvette with a paper towel and place inside the electroporation chamber of a MicroPurser (Biorad).
18. Pulse once (2.5 kV, 25 μF, 200 Ohm). Add immediately 1 ml of 2×YT containing 2% glucose. Remove the cuvette from the chamber and pulse the next cuvette.

19. Wash the cuvette four times (1 ml each) with 2×YT containing 2% glucose and 5 mM $MgCl_2$, and collect the cells in a 500-ml erlenmeyer flask.
20. Incubate the cells in a shaking incubator at 37°C for 1 h.
21. Take 100 µl of the culture, make serial dilutions, and plate 100 µl of each on 2×YTA plates to estimate the number of transformants.
22. Centrifuge the remaining cells at 3,500 rpm for 7 min at 20°C.
23. Discard the supernatant and resuspend the cells in 10 ml of 2×YT.
24. Plate the cells on 245 × 245 × 200-mm SOBAG agar square plates.
25. Incubate the plates overnight at 37°C.
26. Harvest library cells: Pour 10 ml of 2×YT containing 2% glucose and 5-mM $MgCl_2$ on the bacterial lawn, collect the cells by gently scraping the cells , and transfer them to a 50-ml Falcon tube.
27. Measure the OD at 600 nm.
28. Add glycerol to make a final glycerol concentration of 20% and store at −80°C. *Note*: At the best condition, greater than 1×10^8 colonies/µg of ligated DNA can be obtained from the above procedure. The quality and the number of electrocompetent cells significantly affect transformation efficiency and can be tested using intact circular plasmid DNA.

23.3.3 Panning and Selection

23.3.3.1 Rescuing Library Phage

1. Inoculate the library cells (starting $OD_{600} = 0.1$) into 2 liters of 2×YTA. The inoculation size should cover the diversity of the library. We usually use tenfold excess of the number of different clones in the library.
2. Incubate in a shaking incubator (200 rpm) at 37°C until the OD_{600} reaches 0.8.
3. Add VCSM13 helper phages. The ratio of helper phage:bacteria should be 20:1 to ensure complete infection of all clones in the library.
4. Incubate at 37°C without shaking for 30 min and with shaking for another 30 min.
5. Add kanamycin to a final concentration of 70 µg/ml.
6. Incubate at 30°C overnight with shaking (200 rpm).
7. Centrifuge at 5,000 rpm for 15 min at 4°C.
8. Transfer supernatant to a new bottle and add 1/5 volume of 25 mM PEG8000 (5 mM final) and 1/10 volume of 5 M NaCl.
9. Mix well and incubate on ice for 2 h.
10. Centrifuge at 10,000 rpm for 1 h at 4°C.
11. Discard the supernatant, and remove a maximum amount of liquid using paper towels.
12. Resuspend the phage pellet in 5 ml of PBS.
13. Centrifuge the phage suspension at 12,500 rpm for 10 min to remove cell debris.
14. Filter the phage suspension using a 0.22-µm disposable syringe filter and store at −80°C.

23.3.3.2 Panning

1. Prepare microtiter wells coated with a target antigen (100 ng/well) and a control antigen (BSA, 100 ng/well) in a coating buffer at 4°C overnight.
2. Block the wells with 2% skim milk in PBST at 37°C for 1 h.
3. Mix the freshly prepared library phage with an equal volume of 4% skim milk in PBST and add to the BSA-coated wells, then incubate for 30 min at 37°C.
4. Transfer the precleared phages to the antigen-coated wells and incubate at 37°C for 2 h.
5. Discard the phages from the wells, and wash the wells five times with PBST. The stringency of selection can be increased with each round of panning by increasing the number of washes.
6. Wash with PBS twice and elute the bound phages by incubating for 10 min with 0.1-M glycine (adjusted with HCl to pH 2.5) containing 0.1% BSA. Transfer the eluted phage to a new eppendorf tube and neutralize with 3 μl of 2 M Tris base per 50 μl of elution buffer used.
7. Infect freshly prepared TG1 cells at exponential growth phase (OD_{600} = 0.7~0.8) with the eluted phages. Incubate at 37°C for 30 min without shaking and for another 30 min with shaking.
8. Titer the infected TG1 cells by plating dilutions of infected cells.
9. Plate the entire infected cells onto 245 × 245 × 200 mm SOBAG plates and incubate at 30°C overnight.
10. Harvest cells: Pour 10 ml of 2×YT containing 2% glucose and 5-mM $MgCl_2$ on the bacterial lawn, and collect the cells by gently scraping the cells, and transfer them to a 50 ml Falcon tube.
11. Measure the OD at 600 nm.
12. Use the harvested cells for the next round of panning or store at −80°C after adding glycerol to 20%.
    Note: Panning is repeated to enrich binder clones. The performance of panning can be checked by input and output phage titers or by phage ELISA. Usually, an increase in the ratio of output–input phage titer represents successful enrichment.

23.3.3.3 Screening (phage ELISA)

1. Inoculate individual colonies from the final round of panning into a 96-deep well culture plate containing 100 μl of 2×YTA.
2. Incubate at 37°C in a plate shaker with shaking at 400 rpm.
3. After growing till the exponential growth phase, make a replica plate by seeding 10 μl of original culture and incubating at 37°C.
4. Make a glycerol cell stock for the original culture plate and store at −80°C.
5. Rescue phages from the replica plate culture by adding 10^8 helper phages per each well as described previously.

6. Harvest phage supernatant from overnight culture and perform phage ELISA as described below.
7. Mix and preincubate the phage supernatant (100 μl) with 2% skim milk for 15 min.
8. Incubate the phages in the microtiter wells coated with a target antigen or a control antigen (BSA) at 37°C for 2 h.
9. Wash the wells three times with PBST.
10. Add anti-M13-HRP (1/2000 dilution in PBS) and incubate for 1 h.
11. Wash the wells five times with PBST.
12. Add 100 μl of mixed TMB substrate reagent.
13. Incubate the plate at RT for 5 min.
14. Stop the reaction by adding 50 μl of 2 M H_2SO_4
15. Read using an ELISA reader at 450 nm.
16. Select positive clones with a high absorbance for the target antigen but not for BSA.
17. Determine the final unique clones by BstNI digestion and DNA sequencing of the positive clones.
    Note: Positive clones can be selected by ELISA of the soluble Fab-PIII fusion protein which is expressed using the same protocol as for the expression of chimeric Fab.

23.3.4 Characterization of Selected Human Fabs

Finally, selected human Fabs are expressed in a soluble form in *E. coli* after deletion of the gene III fused to the C-terminus of CH1 for characterization of their affinities and epitope specificity. We use competition binding assay to test the epitope specificity and competition ELISA to determine the affinity.

23.3.4.1 Competition Binding Assay for Epitope Specificity

1. Coat microtiter wells with a target antigen (100 ng/well) in a coating buffer at 4°C overnight.
2. Block the wells with 2% BSA in PBS at 37°C for 1 h.
3. Incubate the original mouse antibody or Fab in the wells at 37°C for 1 h, in the absence or presence of increasing concentrations (0.1~10 μM) of the selected Fabs or an unrelated control Fab. Predetermine the amount of mouse antibody used by an antigen-binding assay at the same condition, and the amount corresponding to A_{450} ~1 is usually used. Purify fabs using anti-Fab or protein G column as described elsewhere.
4. Wash the wells three times with PBST.
5. Add anti-mouse IgG-HRP (Fc specific, 1/2000 dilution in PBS) or anti-mouse IgG $F(ab')_2$-HRP and incubate for 1 h to detect bound mouse antibody.

6. Wash the wells four times with PBST.
7. Add 100 μl of mixed TMB substrate reagent.
8. Incubate the plate at RT for 5 min.
9. Stop the reaction by adding 50 μl of 2 M H_2SO_4.
10. Measure the absorbance using an ELISA reader at 450 nm.
11. Calculate % binding of the mouse antibody against the Fab concentration. Obtain the IC_{50} value, the Fab concentration required for 50% inhibition of the binding of mouse antibody, from the plot.
    Note: The fine epitope specificity of the selected Fab clone can be further characterized using a specific epitope such as the linear peptide epitope if the epitope information for the original mouse antibody is available.

23.3.4.2 Competition ELISA for Affinity Determination

1. Prepare a coated microtiter plate as described above with the antigen.
2. Prepare Fab solution in 0.02% BSA. Predetermine the Fab concentration required using an antigen-binding assay to be the lowest concentration that gives an absorbance of 1. The Fab concentration should be close to, or lower than the value of the dissociation constant (K_D) to get a reliable result.
3. Prepare serial dilutions of antigen from 10^{-5} to 10^{-11} M in 0.02% BSA.
4. Mix an equal volume of Fab solution and the antigen solution prepared at different concentrations.
5. Incubate at the desired temperature until equilibrium is reached (more than 2 h).
6. Add the mixed solution onto the antigen-coated microtiter wells and incubate at the same temperature for 30 min.
7. Wash three times with PBST.
8. Add anti-F(ab′)$_2$-HRP (1/2000 dilution in PBS) and incubate for 30 min to detect bound Fab.
9. Wash four times with PBST.
10. Add HRP substrate solution for color development and measure the absorbance using a microtiter plate reader.
11. Calculate % binding of Fab and plot against the concentration of antigen. Estimate the value of K_D from the curve directly by extrapolating the antigen concentration corresponding to 50% binding of Fab, or calculate it from a Scatchard plot (see Sect. 1.9.1 in this book).
    Note: Also, the affinity of the selected Fab clones can be determined using SPR analysis of purified Fab. SPR analysis gives precise information on the binding kinetics such as association and dissociation rate constants (k_{on} and k_{off}) (see Sect. 1.9.2 in this book). In addition, the Fabs are converted to whole IgG format (see Sect. 1.6.2 in this book) and subjected to the analyzes of epitope specificity, affinity, and in vitro and in vivo functional properties.

References

Bao GQ, Li Y, Ma QJ, He XL, Xing JL, Yang XM, Chen ZN (2005) Isolating human antibody against human hepatocellular carcinoma by guided-selection. Cancer Biol Ther 4:1374–1380

Beiboer SH, Reurs A, Roovers RC, Arends JW, Whitelegg NR, Rees AR, Hoogenboom HR (2000) Guided selection of a pan carcinoma specific antibody reveals similar binding characteristics yet structural divergence between the original murine antibody and its human equivalent. J Mol Biol 296:833–849

Figini M, Marks JD, Winter G, Griffiths AD (1994) In vitro assembly of repertoires of antibody chains on the surface of phage by renaturation. J Mol Biol 239:68–78

Figini M, Obici L, Mezzanzanica D, Griffiths A, Colnaghi MI, Winter G, Canevari S (1998) Panning phage antibody libraries on cells: isolation of human Fab fragments against ovarian carcinoma using guided selection. Cancer Res 58:991–996

Griffiths AD, Williams SC, Hartley O, Tomlinson IM, Waterhouse P, Crosby WL, Kontermann RE, Jones PT, Low NM, Allison TJ et al (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. Embo J 13:3245–3260

Jespers LS, Roberts A, Mahler SM, Winter G, Hoogenboom HR (1994) Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen. Biotechnology 12:899–903

Jones PT, Dear PH, Foote J, Neuberger MS, Winter G (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321: 522–525

Khazaeli MB, Conry RM, LoBuglio AF (1994) Human immune response to monoclonal antibodies. J Immunother Emphasis Tumor Immunol 15:42–52

Kim SJ, Hong HJ (2007) Guided selection of human antibody light chains against TAG-72 using a phage display chain shuffling approach. J Microbiol 45:572–577

Klimka A, Matthey B, Roovers RC, Barth S, Arends JW, Engert A, Hoogenboom HR (2000) Human anti-CD30 recombinant antibodies by guided phage antibody selection using cell panning. Br J Cancer 83:252–260

Kupper H, Salfeld J, Tracey D, Kalden JR (2007) Adalimumab (Humira). In: Dübel S (ed) Handbook of therapeutic antibodies. Wiley-VCH Verlag GmBH & Co, KGaA, Weinheim

Morrison SL, Johnson MJ, Herzenberg LA, Oi VT (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci USA 81:6851–6855

Osbourn J, Groves M, Vaughan T (2005) From rodent reagents to human therapeutics using antibody guided selection. Methods 36:61–68

Pendley C, Schantz A, Wagner C (2003) Immunogenicity of therapeutic monoclonal antibodies. Curr Opin Mol Ther 5:172–179

Rader C, Cheresh DA, Barbas CF 3 rd (1998) A phage display approach for rapid antibody humanization: designed combinatorial V gene libraries. Proc Natl Acad Sci USA 95: 8910–8915

Reichert JM (2001) Monoclonal antibodies in the clinic. Nat Biotechnol 19:819–822

Roguska MA, Pedersen JT, Keddy CA, Henry AH, Searle SJ, Lambert JM, Goldmacher VS, Blattler WA, Rees AR, Guild BC (1994) Humanization of murine monoclonal antibodies through variable domain resurfacing. Proc Natl Acad Sci USA 91:969–973

Schmidt A, Muller D, Mersmann M, Wuest T, Gerlach E, Garin-Chesa P, Rettig WJ, Pfizenmaier K, Moosmayer D (2001) Generation of human high-affinity antibodies specific for the fibroblast activation protein by guided selection. Eur J Biochem 268:1730–1738

Shawler DL, Bartholomew RM, Smith LM, Dillman RO (1985) Human immune response to multiple injections of murine monoclonal IgG. J Immunol 135:1530–1535

Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, McCafferty J, Hodits RA, Wilton J, Johnson KS (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat Biotechnol 14:309–314

Wang Z, Wang Y, Li Z, Li J, Dong Z (2000) Humanization of a mouse monoclonal antibody neutralizing TNF-alpha by guided selection. J Immunol Methods 241:171–184

Watzka H, Pfizenmaier K, Moosmayer D (1998) Guided selection of antibody fragments specific for human interferon gamma receptor 1 from a human VH- and VL-gene repertoire. Immunotechnology 3:279–291

Winter G, Harris WJ (1993) Humanized antibodies. Immunol Today 14:243–246

Zhang Q, Zhang SH, Su MQ, Bao GQ, Liu JY, Yi J, Shen JJ, Hao XK (2007) Guided selection of an anti-gamma-seminoprotein human Fab for antibody directed enzyme prodrug therapy of prostate cancer. Cancer Immunol Immunother 56:477–489

Chapter 24
In Silico De-Immunization

Philippe Alard, Johan Desmet, and Ignace Lasters

24.1 Introduction

Immunogenicity of therapeutic antibodies has been observed and may affect the drug efficacy (Van Walle et al. 2007; Stas et al. 2009). Different techniques have been developed to reduce the antigenicity of murine antibodies while preserving their affinity. Joining the variable domains of a murine antibody to the constant domains of a human antibody to construct a chimeric antibody was the first strategy to reduce antigenicity (Morrison et al. 1984; Boulianne et al. 1984). Although successfully designed chimeric antibodies have been reported (Maloney 1999; Wagner et al. 2003; LoBuglio et al. 1989), the majority of them still induced relevant immune responses against the variable domains (Khazaeli et al. 1991). As it has been shown that foreign framework regions (FRs) can evoke immune responses (Bruggemann et al. 1987), the next step in the development of humanized antibodies was the "grafting" of the complementarity determining regions (CDRs) on a human acceptor antibody (Jones et al. 1986; Verhoeyen et al. 1988). This procedure can lead to a significant reduction or complete loss of binding affinity because certain framework residues are important for maintaining the conformation of the CDRs (Foote and Winter 1992; Chothia et al. 1989) or are even directly involved in antigen binding (Mian et al. 1991).

Therefore, an alternative to lower the immunogenicity is to remove the T cell epitopes (de-immunization) in the murine Fv domain (Chamberlain 2002; Baker and Jones 2007). We focus on HLA class II because they are necessary for the

P. Alard (✉), J. Desmet, and I. Lasters
AlgoNomics NV, Technologiepark 4, 9052, Gent, Belgium
e-mail: philippe.stas@algonomics.com

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_24,

development of a long-lived cytotoxic or humoral immune response. This approach mainly consists of the following steps:

- Locate the T cell epitopes by in vitro measurement or in silico prediction.
- Design point mutations that eliminate these epitopes by decreasing their binding affinity for HLA class II.
- Test these mutated peptides to be sure that they are either not or less immunogenic.
- Test the engineered antibody for compatibility with the three dimensional structure of the antibody and the preservation of the binding affinity to the target.

The protocol presented uses only in silico approaches. Because most T cells are DRB1 restricted, we will first try to eliminate T cell epitopes binding to one or several allotypes of DRB1. We start to remove the epitopes with the strongest binding affinity because they have a higher chance to be presented first (Kotturi et al. 2008). In decreasing order of conservativeness, the routes to decrease the T-cell epitope content are as follows:

- Replace an epitope by the corresponding sequence in a human germline.
- Introduce substitutions to alter the sequence to the one found commonly in mouse germline sequences.
- Introduce substitutions designed specifically in the context of this particular protein.

In these three cases, the total epitope content must be reduced while preserving the structure and function of the protein.

24.2 Methods

24.2.1 Construction of a 3D Model of the Fv Domain

A three-dimensional model of the antibody needs to be built to test the substitutions that will be proposed in the section "Construction of a de-immunized Fv model" (See Sect. 24.2.4). The model will help to study where the predicted epitopes are located and to assess the effect of a substitution on the stability of the antibody or on the binding affinity for the antigen.

To build the model, we will use a slightly modified homology modeling procedure. The classical homology modeling procedure is composed of five major steps: (1) search of a closely related (homolog) protein in the PDB (databank of structures), (2) make the sequence alignment between the target protein and the homolog that we found in the PDB, (3) model the insertions and deletions in the structure, (4) substitute the side chains that are different between target and homolog protein, and (5) optimize the energy of the structure.

Because there are more than 700 crystals of antibodies with similar structures and different sequences, we can build the model with improved accuracy. Often, the sequence of a target single domain, VH or VL, can reach 90% sequence identity on the frameworks. We select separately VH and VL from different crystals by sequence similarity and superimpose them to create a new model of the Fv domain structure. The CDRs may follow the same procedure except CDR3 of VH which is more difficult to model because of high variability in sequence and in length.

1. Retrieve the amino acid sequences for V_L ("SEQ_VL") and V_H ("SEQ_VH") of the target antibody in FASTA format (REF http://www.ncbi.nlm.nih.gov/blast/fasta.shtml)
2. Access the web site http://blast.ncbi.nlm.nih.gov/Blast.cgi using a suitable browser
3. Select the BLAST program "protein blast"
4. Enter SEQ_VL in the "Enter accession number, gi, or FASTA sequence" field
5. Select "Protein Data Bank proteins" from the drop-down list in the "database" field
6. Click the "BLAST" button
7. Click "Formatting options"
8. Select "Flat query-anchored with dots for identities" from the drop-down list in the "Alignment View" field
9. Scroll down to the "Alignments" block
10. Select (highlight) and copy (Ctrl-C) all alignment data to the clipboard
11. Paste data as text (using "Paste Special") in the upper-left cell of a blank Excel sheet. (See also *Note 1*)
12. Convert the data using the "Text to Columns" function with "Fixed width" option, and insert "Break lines" after each character of the sequence
13. Move each 60-character data block up to the right of the previous block in order to reconstruct each sequence on a single line
14. Delete all columns that do not contain sequence data (except the first column comprising the PDB codes)
15. Identify and mark (e.g., by shading) the three CDRs on the basis of the Kabat definitions for residue numbering and CDR location (http://www.bioinf.org.uk/abs). (See also *Note 2*)
16. Remove amino acid sequences comprising insertions/deletions in FR fragments, if any
17. Calculate for each sequence a value "score" reflecting the usefulness of the corresponding PDB structure as a template structure. A simple and most useful scoring metric is the percentage sequence identity of the FR segments (i.e., the number of dots in FR columns divided by the total number of FR positions * 100). (See also *Note 3*)
18. Sort data in descending order of scoring values
19. Repeat steps 2–18 for SEQ_VH
20. Calculate the average of the highest V_L and V_H scores ("TOP-score")

21. For each PDB code in the V_L data sheet, check whether it also appears in the list of V_H hits (e.g., using the MATCH function). If it does, calculate the average V_L and V_H score. Search for the highest average value ("COMBI-score")
22. If TOP-score > COMBI-score + 10%, then proceed with the next step. If not, then retrieve the PDB file (e.g., from http://www.rcsb.org) with the highest COMBI-score as the working structure ("WORK") and skip steps 23–25. (See also *Note 4*)
23. Retrieve each of the individual top-scoring PDB files ("TOP_VL" and "TOP_VH," respectively)
24. Structurally superimpose TOP_VL with TOP_VH by performing a least-squares fit on the main-chain atoms of the beta-barrel fragments. (See also *Note 5*)
25. Remove the V_H part of TOP_VL and the V_L part of TOP_VH. The resulting structure is further named "WORK"
26. On the basis of the alignments in the Excel sheets, make a list of substitutions to be performed in V_L and V_H in order to construct a FR structure with the correct amino acid sequence
27. Model the substitutions according to one of the following possibilities: (1) perform an energy-based rotameric search for each individual substitution in the context of the structure WORK, or (2) check for the most frequent conformation of each substitution in other PDB files, and mutate the structure WORK accordingly. (See also *Note 6*)
28. Optionally model the CDR loops using an advanced loop modeling/grafting method. (See also *Note 7*)
29. Optionally perform 200 steps steepest descent energy minimization, followed by another 200 steps conjugate gradient minimization. Name this structure "MODEL_MOUSE"

24.2.2 Search the T Cell Epitopes of the Murine Fv Domain

1. Access the web site http://www.cbs.dtu.dk/services/NetMHCII/ using a suitable browser
2. Enter SEQ_VL in the "*Paste a single sequence or several sequences in FASTA format into the field below*" field
3. Select all HLA DR alleles from the list in the "MHC Allele" field. They represent most of the major allotypes in the Caucasian population. More information on allotype frequencies is available on http://www.pypop.org/popdata/
4. Type "9" in the "Peptide length" field
5. Check the "Sort by affinity" button
6. Click the "Submit" button
7. Save the page of results on your computer
8. Repeat steps 2–7 for SEQ_VH

24.2.3 Make a Multiple Sequence Alignment of Germline VH and VL

The multiple sequence alignments of human and murine germline are a source of inspiration for the substitutions that will remove the T cell epitopes.

1. Access the web site of the "IMGT reference directory in FASTA format" (germline sequences) http://www.imgt.org/textes/vquest/refseqh.html using a suitable browser
2. Under "IMGT/GENE-DB reference directory sets", click in the table "F + ORF + in-frame F" on the Human link in the IGHV line (VH germline) under "Amino acids"
3. Save the page on your computer
4. Remove everything from the file before the first sequence in FASTA format and after the last sequence; all sequences that are not functional must be eliminated, and so all sequences with something different from "F" in the fourth field of the title line
5. Add the VH sequence of the target antibody in FASTA format to the list of germline
6. Select and copy all the sequences
7. Access the web site http://www.ebi.ac.uk/Tools/clustalw2/index.html
8. Paste the sequences in the field "Enter or paste a set of sequences in any supported format"
9. Click the "Run" button
10. Save the results on your computer
11. Repeat steps 2–10 for IGKV (Vκ) and IGLV (Vλ)

24.2.4 Construction of a de-Immunized Fv Model

24.2.4.1 Summary

The following data will now be used to de-immunize the Fv domain of the antibody: the 3D model of the antibody, the T cell epitopes, and the germline alignments. We examine whether one or more epitopes can be removed by introducing substitutions preferably in the framework segments. To minimize the risk of altering the binding affinity of the antibody (in absence of a structural model of the antibody-antigen complex) we will modify the sequences of the CDRs as a second option. The proposed substitutions must (1) lower the epitope count, (2) not induce strain in the antibody scaffold, and (3) avoid loss of binding affinity for the antigen. For each epitope, the substitution protocol described below will select substitution(s) (see Sect. 24.2.4.2)in the following ways:

(a) By finding a human germline sequence that replaces the current epitope with a minimum of substitution(s) and with substitution(s) as conservative as possible.

Human germline sequences are considered not to be immunogenic as these sequences are found in the pool of circulating antibodies.

(b) In case (a) fails, find substitutions guided by the aligned mouse germline sequences. We expect that these substitutions are at least structurally acceptable.
(c) In case (b) fails, search for ad-hoc substitution(s). These substitution(s) have to be carefully validated because they are not coming from human or mouse antibodies.
(d) Assess if the selected substitution(s) lower the epitope content and are acceptable from the structural point of view (see Sect. 24.2.4.3).

In detail, the process consists of following phases: subsequent to Sect. 2.4.2, the process may reiterate to Sect. 2.4.1.

24.2.4.2 Selection of Substitutions

1. For all strong epitopes of VH and VL, the following steps have to be performed:
2. Find the epitope in the multiple alignment of human germline and search the closest sequence in terms of number of substitutions.
3. Follow the steps 6–8 of Sect. 24.2.4.3 to test this(these) substitution(s). After having exhausted the possibilities of human germline, continue with murine germline.
4. Find the epitope in the multiple alignment of murine germline.
5. Note the different amino acids found in each position of the epitope.
6. Follow the steps of Sect. 24.2.4.3 to test them. If no successful substitution was found, continue with step 7.
7. Finally, you may try ad-hoc substitution(s). They are substitutions that you may find convenient at a given position for a given epitope.
8. Again, follow the steps of Sect. 24.2.4.3 to test them (See also *Note 8*).

24.2.4.3 Test of Substitutions

1. Introduce the substitution(s) in the corresponding sequence (VH or VL).
2. Execute the steps under Sect. 24.2.2 with the modified sequence.
3. Check that the epitope disappeared for the allotype where it was a strong binder or that at least it significantly diminished in binding affinity (epitope became a weak binder).
4. Check that the epitope didn't become a strong binder for other allotype(s).
5. Check that overlapping peptide(s) were not transformed into epitope(s) by the substitution(s).
6. Model the substitutions by performing an energy-based rotameric search for each individual substitution in the context of the structure MODEL_MOUSE derived in Sect. 24.2.1.

7. Check that the substitution(s) is (are) energetically acceptable (See also *Note 9*).
8. If all conditions are met, accept the substitution(s) and exit the procedure, otherwise continue by selecting new substitutions in 24.2.4.2 at the step where 24.2.4.3 was called.

24.3 Notes

1. Steps 11–22 may be executed using any other suitable data processing environment, e.g., MATLAB (http://www.mathworks.com/products/matlab).
2. Alternatively, the less widely used but structurally more relevant Chothia numbering scheme (Kotturi et al. 2008) can be followed.
3. Alternatively, the percentage sequence similarity, or a combination identity-similarity, can be applied. More sophisticated weighed scoring schemes may include additional parameters such as CDR sequence similarity, the crystallographic quality of candidate structures (e.g., resolution, refinement, structural completeness, NMR/X-ray) and/or the biological origin of the antibody.
4. The proposed 10% tolerance for choosing between TOP- and COMBI-scores results from (unpublished) insights, so it is usually advisable to build Fv structures from independent V_L and V_H templates if their average sequence identity with the target sequence is higher than that of an already paired V_L/V_H complex by more than 10%.
5. Recommended fragments for fitting include (following Kabat numbering) V_L residues 35–38, 44–49, 84–88, 98–106, and V_H residues 36–39, 45–49, 88–94, 103–110. Alternatively, V_L and V_H domains may be assembled by structural superposition of TOP_VL and TOP_VH framework backbone fragments onto the PDB structure with the highest COMBI-score as identified in step 21. The latter method is formally the same as the method of Sivasubramanian et al. 2009.
6. For a recent benchmark comparison of different methods, see Lu et al. 2008.
7. Various methods exist for de novo loop modeling (Liwo et al. 2008; Olson et al. 2008), as well as specific methods for CDR grafting (Sivasubramanian et al. 2009).
8. It may happen that no substitution can be found for one epitope. In these cases, depending on the expertise available, risky substitutions may be tried.
9. Calculate the energies of the static structures, the original, and the substituted one. The van der Waals energy must be comparable, otherwise short contacts will be detected and the substitution(s) have to be rejected. Note that this energy measurement doesn't take into account solvation (e.g., substitution of a hydrophobic residue by a hydrophilic in a hydrophobic environment will have a prohibitive energetic cost). A scoring function will do the job if available, otherwise use common sense.

References

Baker MP, Jones TD (2007) Identification and removal of immunogenicity in therapeutic proteins. Curr Opin Drug Discov Devel 10:219–227

Boulianne GL, Hozumi N, Shulman MJ (1984) Production of functional chimaeric mouse human-antibody. Nature 312:643–646

Bruggemann M, Williams GT, Bindon CI, Clark MR, Walker MR, Jefferis R, Waldmann H, Neuberger MS (1987) Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. J Exp Med 166:1351–1361

Chamberlain P (2002) Immunogenicity of therapeutic proteins. Regul Rev 5:4–10

Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, Air G, Sheriff S, Padlan EA, Davies D, Tulip WR (1989) Conformations of immunoglobulin hypervariable regions. Nature 342:877–883

Foote J, Winter G (1992) Antibody framework residues affecting the conformation of the hypervariable loops. J Mol Biol 224:487–499

Jones PT, Dear PH, Foote J, Neuberger MS, Winter G (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321:522–525

Khazaeli MB, Saleh MN, Liu TP, Meredith RF, Wheeler RH, Baker TS, King D, Secher D, Allen L, Rogers K (1991) Pharmacokinetics and immune response of 131I-chimeric mouse/human B72.3 (human gamma 4) monoclonal antibody in humans. Cancer Res 51:5461–5466

Kotturi MF, Scott I, Wolfe T, Peters B, Sidney J, Cheroutre H, von Herrath MG, Buchmeier MJ, Grey H, Sette A (2008) Naive Precursor Frequencies and MHC Binding Rather Than the Degree of Epitope Diversity Shape $CD8^+$ T Cell Immunodominance. J Immunol 181:2124–2133

Liwo A, Czaplewski C, Oldziej S, Scheraga HA (2008) Computational techniques for efficient conformational sampling of proteins. Curr Opin Struct Biol 18:134–139

LoBuglio AF, Wheeler RH, Trang J, Haynes A, Rogers K, Harvey EB, Sun L, Ghrayeb J, Khazaeli MB (1989) Mouse/human chimeric monoclonal antibody in man: kinetics and immune response. Proc Natl Acad Sci USA 86:4220–4224

Lu M, Dousis AD, Ma J (2008) OPUS-Rota: a fast and accurate method for side-chain modeling. Protein Sci 17:1576–1585

Maloney DG (1999) Preclinical and phase I and II trials of rituximab. Seminars Oncol 26:74–78

Mian IS, Bradwell AR, Olson AJ (1991) Structure, function and properties of antibody binding sites. J Mol Biol 217:133–151

Morrison SL, Johnson MJ, Herzenberg LA, Oi VT (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci USA 81:6851–6855

Olson MA, Feig M, Brooks CL 3rd (2008) Prediction of protein loop conformations using multi-scale modeling methods with physical energy scoring functions. J Comput Chem 15:820–831

Sivasubramanian A, Sircar A, Chaudhury S, Gray JJ (2009) Toward high-resolution homology modeling of antibody F_v regions and application to antibody-antigen docking. Proteins 74:497–514

Stas P, Pletinckx J, Gansemans Y, Lasters I (2009) Immunogenicity assessment of antibody therapeutics. In: Melvyn L (ed) Recombinant antibodies for immunotherapy. Cambridge University Press, Cambridge

Van Walle I, Gansemans Y, Parren P, Stas P, Lasters I (2007) Immunogenicity screening in protein drug development. Expert Opin Biol Ther 7:405–418

Verhoeyen M, Milstein C, Winter G (1988) Reshaping human antibodies: grafting an antilysozyme activity. Science 239:1534–1536

Wagner CL, Schantz A, Barnathan E, Olson A, Mascelli MA, Ford J, Damaraju L, Schaible T, Maini RN, Tcheng JE (2003) Consequences of immunogenicity to the therapeutic monoclonal antibodies ReoPro and Remicade. Dev Biol (Basel) 112:37–53

Chapter 25
Affinity Maturation by Chain Shuffling and Site Directed Mutagenesis

Jianlong Lou and James D. Marks

25.1 Introduction

In the human immune system, antibodies with high affinities for antigen are created in two stages. A diverse primary repertoire of antibody structures is produced by the combinatorial rearrangement of germline V gene segments, and antibodies are selected from this repertoire by binding to the antigen (Alt et al. 1987). Antibody affinities are then further improved by somatic hypermutation and further rounds of selection (Allen et al. 1987).

In vitro, phage display has been successfully used to increase antibody affinity more than 1,000-fold (Yang et al. 1995; Schier et al. 1996c). The major decision for in vitro antibody affinity maturation is where and how to introduce mutations into the antibody V-genes. The easiest approach has been to introduce mutations randomly into the V-genes, thus mimicking the in vivo process of somatic hypermutation. Mutations have been introduced by chain shuffling (Marks et al. 1992; Figini et al. 1994), error prone PCR (Hawkins et al. 1992), DNA shuffling (Crameri et al. 1996), or by propagation of phage in mutator strains of *Escherichia coli* (Low et al. 1996). While these approaches have yielded large increases in affinity for hapten antigens (up to 300 fold) (Marks et al. 1992; Low et al. 1996), results with protein antigens have been more modest (<10 fold) (Hawkins et al. 1992; Schier et al. 1996c). One limitation to random mutagenesis is that little useful information is generated with respect to the location of mutations that modulate affinity. Such information could be used to guide subsequent mutagenesis efforts if the increase in affinity achieved is inadequate for the desired application. In addition, because of the random introduction of mutations into the framework regions, this approach may create problems of immuogenicity when the antibody is intended for therapeutic use.

J. Lou and J.D. Marks (✉)
Departments of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, USA
e-mail: marksj@anesthesia.ucsf.edu

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_25,

This problem could be minimized using DNA shuffling to remove deleterious or unecessary mutations (Stemmer 1994).

Several groups have shown that targeting mutations to the CDRs can be an effective technique for increasing antibody affinity. During in vivo somatic hypermutation, mutations accumulate preferentially in the CDRs compared to framework residues (Loh et al. 1983). The location of these CDR mutations complements the locations where diversity is generated in the primary antibody repertoire (Tomlinson et al. 1996). However, many CDR residues, especially in V_HCDR3 and V_LCDR3, are responsible for high energy interactions with antigen. Mutating residues in this region can in many cases abolish antigen binding. Fortunately, these residues will be recreated, albeit at low frequencies, given an adequate library size for the number of residues randomized. Mutant residues in the CDRs may increase affinity by introducing new contact residues or by replacing low affinity or "repulsive" contact residues with more favorable energetics (Novotny et al. 1989). However, it appears that many mutations introduced either by somatic hypermutation in vivo or by random mutagenisis in vitro instead exert their effect on affinity indirectly by repositioning the CDRs or the side-chains of contact residues for optimal interaction with the antigen (Foote and Winter 1992). Targeting mutations to the CDR regions of an antibody also may be less likely to generate immunogenic antibodies than mutations in the more conserved framework regions. Finally, with site directed mutagensis, sequence analysis of binding clones identifies both conserved structural and functional residues and those residues that modulate affinity (Schier et al. 1996a). These results can help guide subsequent mutagenic efforts to further improve affinity.

Our lab has used both chain shuffling and site directed mutagenesis to increase antibody affinity and has compared these two techniques using an anti-ErbB2 sigle chain Fv (Marks et al. 1992; Schier et al. 1996a, c). Overall, we find that site directed mutagenesis of the CDR3s of an antibody is the most efficient means to improve affinity by phage display. For example, we increased the affinity of the anti-ErbB2 antibody more than 1,200-fold by sequentially targeting mutagenesis to the two CDR3's. Similarly, Yang et al. increased the affinity of an anti-gp120 Fab 420-fold by mutating four CDRs (V_HCDR1, V_LCDR1, V_HCDR3, and V_LCDR3) in five libraries and combining independently selected mutations (Yang et al. 1995). However, they observed the largest affinity increases when optimizing the CDR3 regions.

Optimization of the two CDR3s are done either in parallel or sequentially. In the parallel strategy, the two CDR3 are randomized independently and beneficial mutations in single clones are combined. Although this strategy has been successfully employed for optimization of several antibodies, not all mutations have been additive (Yang et al. 1995; Schier et al. 1996c). This is no surprise since many residues of the CDR3s pack against one another. Thus, we prefer sequential targeting of mutations to the CDR3 regions of the antibody.

In the following paragraphs, we describe methods for phage displayed antibody affintiy maturation using both chain shuffling and site directed mutagenesis. Our lab

works exclisively with single chain Fv antibody fragments, so the protocols use this format. The general principles can also be applied to maturation of Fabs.

25.2 Materials

- Vent DNA Polymerase (New England Biolabs (NEB))
- Geneclean Turbo (MP Biomedicals, LLC, Cat. 1102-600)
- Custom DNA primers (many vendors)
- Deoxynucleotide triphosphates (NEB)
- *Nco* I, *Not* I, *Sfi* I, *Bss*HII, *Xho*I restriction enzymes (NEB)
- T4 DNA ligase (NEB)
- *E coli* strain TG1 (Zymo Research)
- 37°C incubator
- Electroporator (Gene Pulser^{TM} BioRad or comparable)
- PCR equipment
- 16°C water bath
- Plasmid vector pSYN1 as described (Schier et al. 1995)
- Helper phage (VCSM13, Stratagene)
- PEG 8000
- Biotinylation kit (Pierce, NHS-LC-Biotin)
- Non-fat milk powder (Safeway brand)
- Streptavidin-magnetic beads (Dynal)
- Magnetic rack (Dynal MPC-E)
- PD10 colums (GE Healthcare)
- CM5 sensor chip (BIAcore)
- SOC (recipe: To 950 mL deionized H_2O, add 20 g bacto-tryptone, 5 g yeast extract, and 0.5 g NaCl. Add 10 mL of a 250 mM KCl solution. Adjust the pH to 7.0 with 5 N NaOH, and make up the volume of the solution to 1 L with deionized H_2O. Sterilize by autoclaving for 20 min. Just before use, add 1 mL of a sterile solution of 1 M $MgCl_2$, 1 mL 1 M $MgSO_4$, and 20 mM glucose)
- onto 100 mm TYE plates containing 100 μg/mL Ampicillin and 1% glucose (TYE/Amp/Glu)

25.3 Protocols

25.3.1 Introducing Mutations by Chain Shuffling

Chain shuffling relies on the natural mutation of V-genes that occurs in vivo in response to antigens that the host has been exposed to for the generation of sequence diversity. For chain shuffling of single chain Fv (scFv) from phage

libraries, we first amplify V_H and V_L repertoires from either pre-existing naïve scFv libraries or from total RNA prepared from volunteer human donors. Initially, we used splicing by overlap extension to splice either the wild type V_H or V_L to a gene repertoire of the complementary chain (Clackson et al. 1991; Marks et al. 1992). However this can artifactually generate shortened linker sequence (between VH and VL) leading to scFv dimers (diabodies) (Holliger et al. 1993) which may be preferentially selected on the basis of avidity (Schier et al. 1996b). Therefore, we now prefer to clone the wildtype V_H or V_L gene into a phage display vector containing a repertoire of the complementary chain (Schier et al. 1996b). Such repertoires have been described (Schier et al. 1996b) and it may be possible to obtain these from the authors, saving considerable time. Below we describe the generation of V_H and V_L repertoires in the vector pHEN-1, by amplifying from a pre-existing naïve scFv library, and the subsequent cloning of wild type V_H or V_L into the appropriate vector for creation of chain shuffled libraries.

25.3.2 Construction of Heavy Chain Shuffled Libraries

The strategy used to heavy chain shuffle is shown in Fig. 25.1. To facilitate heavy chain shuffling, libraries are constructed in pHEN-1 (Hoogenboom et al. 1991) containing human V_H gene segment repertoires (FR1 to FR3) and a cloning site at the end of V_H FR3 for inserting the V_H CDR3, V_H FR4, linker DNA, and light chain from a binding scFv as a BssHII-NotI fragment.

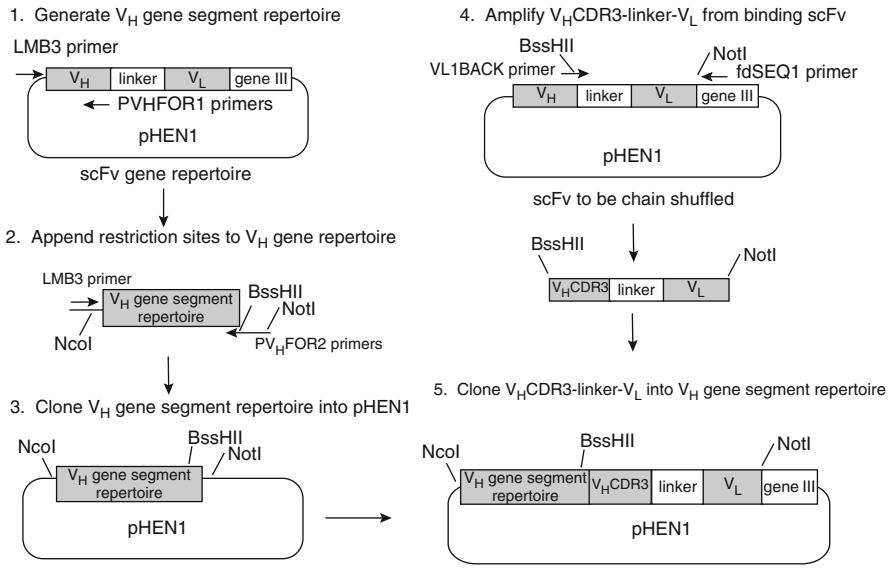


Fig. 25.1 Strategy for heavy chain shuffling

To create the libraries, three V_H gene segment repertoires enriched for human V_H1, V_H3, and V_H5 gene segments are amplified by PCR using as a template single stranded DNA prepared from a 1.8×10^8 member scFv phage antibody library in pHEN-1 (Marks et al. 1992). We used only VH1, VH2 and VH5 as we have not observed any scFv phage antibodies derived from the VH2, four or six families. Primers PVH1FOR1, PVH3FOR1, and PVH5FOR1 are designed to anneal to the consensus V_H1, V_H3, or V_H5 3′ FR3 sequence, respectively (Tomlinson et al. 1992). A second set of primers add the BssHII site for cloning.

25.3.2.1 Construction of Human VH Gene Segment Repertoire Libraries

Primers for heavy chain shuffling:
PVH1FOR1
5′-TCG CGC GCA GTA ATA CAC GGC CGT GTC-3′q
PVH3FOR1
5′-TCG CGC GCA GTA ATA CAC AGC CGT GTC CTC-3′
PVH5FOR1
5′-TCG CGC GCA GTA ATA CAT GGC GGT GTC CGA-3′
PVH1FOR2
5′-GAG TCA TTC TCG ACT TGC GGC CGC TCG CGC GCA GTA ATA CAC GGC CGT GTC-3′
PVH3FOR2
5′-GAG TCA TTC TCG ACT TGC GGC CGC TCG CGC GCA GTA ATA CAC AGC CGT GTC CTC-3′
PVH5FOR2
5′-GAG TCA TTC TCG ACT TGC GGC CGC TCG CGC GCA GTA ATA CAT GGC GGT GTC CGA-3′
LMB3
5′- CAG GAA ACA GCT ATG AC -3′
scFvVL1BACK
5′-AGC GCC GTG TAT TTT TGC GCG CGA CAT GAC GTG GGA TAT TGC-3′

1. Make up three separate 50 μL PCR reaction mixes containing:

water	35.5 μL
20 × dNTPs (5 mM each)	2.5 μL
10 × Vent polymerase buffer (NEB)	5.0 μL
LMB3 primer (10 pm/μL)	2.5 μL
FORWARD primer* (10 pm/μL)	2.5 μL
Single stranded scFv gene template (10 ng)	1.0 μL
Vent DNA polymerase (NEB, 2 units)	1.0 μL

*either PVH1FOR1, PVH3FOR1, or PVH5FOR1

2. Heat to 94°C for 5 min in a PCR thermo-cycler.

3. Cycle 25 times to amplify the V_H genes at 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min.
4. Gel-purify the V_H gene repertoires on a 1.5% agarose gel and extract the DNA using the Geneclean kit. Resuspend the each product in 20 μL of water. Determine DNA concentration by analysis on a 1.5% agarose gel with markers of known size and concentration.

The DNA fragments from the first PCR are then used as templates for a second PCR to introduce a BssHII site at the 3′-end of FR3 followed by a NotI site. The BssHII site corresponds to amino acid residues 93 and 94 (Kabat numbering (Kabat et al. 1991)) and does not change the amino acid sequence (alanine-arginine).

1. Make up 50 μL PCR reaction mixes containing

water	34.5 μL
20 × dNTPs (5 mM each)	2.5 μL
10 × Vent polymerase buffer (NEB)	5.0 μL
LMB3 primer (10 pm/μL)	2.5 μL
FORWARD primer* (10 pM/μL)	2.5 μL
scFv gene template (100 ng)	2.0 μL
Vent DNA polymerase (NEB, 2 units)	1.0 μL

*either PVH1FOR2, PVH3FOR2, or PVH5FOR2

2. Heat to 94°C for 5 min in a PCR thermo-cycler.
3. Cycle 25 times to amplify the V_H genes at 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min.
4. Purify PCR product using the Geneclean purification kit and continue with restriction digest protocol number **25.3.7**, digesting the fragments with *Nco*I and *Not*I. The digested fragments are gel purified, and each V_H gene segment repertoire is ligated separately into gel purified pHEN-1 (Hoogenboom et al. 1991) digested with *Not*I and *Nco*I, and ligated DNA is used to transform *E coli* TG1, as described in Protocol number **25.3.7**.

25.3.2.2 Cloning the VL Gene into VH Gene Segment Repertoires to Generate Heavy Chain Shuffled Libraries

To shuffle the heavy chain gene from a scFv, the light chain gene, linker DNA, and V_H CDR3 and FR4 are amplified using PCR from pHEN-1 containing the target scFv gene using the primers scFvVL1BACK and fdSEQ1. The protocol below uses primers that anneal to gene 3 in pHEN-1 (fdseq) and a VL1back primer designed to anneal to a VH5 framework three and incorportaing a BssHII restriction site. For your specific scFv, it is likely that a different VL1BACK primer will need to be designed to anneal at the 5′ end of the target scFv sequence, and possibly a different 3′ primer will need to be designed as well, depending on the vector backbone that the target scFv is in.

1. Make up 50 µL PCR reaction mixes containing

water	34.5 µL
20× dNTPs (5 mM each)	2.5 µL
10× Vent polymerase buffer (NEB)	5.0 µL
fdSEQ1 primer (10 pm/µL)	2.5 µL
VL1BACK (10 pM/µL)	2.5 µL
scFv light chain gene template (100 ng)	2.0 µL
Vent DNA polymerase (NEB, 2 units)	1.0 µL

2. Heat to 94°C for 5 min in a PCR thermo-cycler.
3. Cycle 25 times to amplify the V_H genes at 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min.
4. Prepare vector DNA from VH gene segment repertoires.
5. Purify PCR product using the Geneclean purification kit. The PCR product and the three repertoires are restriction digested with BssHII and NotI as described in protocol number **25.3.7**. Libraries and insert are gel purified and ligated and transformed, also described in Protocol number **25.3.7**.

25.3.3 Construction of Light Chain Shuffled Libraries

The strategy used to heavy chain shuffle is shown in Fig. 25.2. To facilitate light chain shuffling, a library is constructed in the vector pHEN1-V_λ3 (Hoogenboom

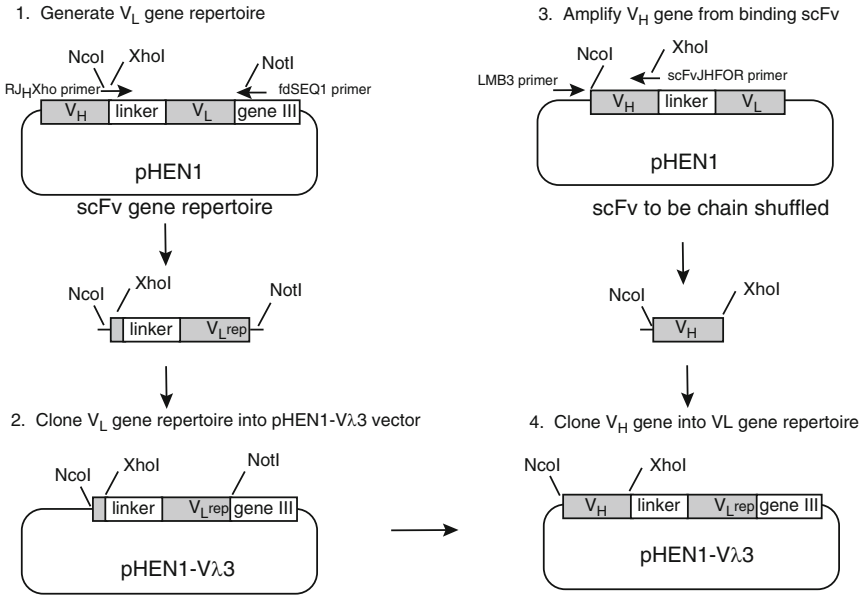


Fig. 25.2 Strategy for light chain shuffling

et al. 1991) containing rearranged human V_κ and V_λ gene repertoires, linker DNA, and cloning sites for inserting a rearranged V_H gene as an *Nco*I-*Xho*I fragment. In this case, an *Xho*I site can be encoded at the end of FR4 without changing the amino acid sequence of residues 102 and 103 (serine-serine) (Kabat et al. 1991).

To create the libraries, V_κ and V_λ gene repertoires and linker DNA are amplified by PCR using as a template single stranded DNA prepared from a 1.8×10^8 member scFv phage antibody library in pHEN-1 (Marks et al. 1992) available from Greg Winter's lab at the MRC Laboratory of Molecular Biology, Cambridge England.

25.3.3.1 Construction of Human VL Gene Repertoire Libraries

Primers for light chain shuffling:
RJH1/2Xho
5′-GGC ACC CTG GTC ACC GTC TCG AGT GGT GGA-3′
RJH3Xho
5′-GGG ACA ATG GTC ACC GTC TCG AGT GGT GGA-3′
RJH4/5Xho
5′-GGA ACC CTG GTC ACC GTC TCG AGT GGT GGA-3′
RJH6Xho
5′-GGG ACC ACG GTC ACC GTC TCG AGT GGT GGA-3′
FdSEQ
5′- GAA TTT TCT GTA TGA GG -3′

1. Make up four separate 50 μL PCR reaction mixes containing

water	35.5 μL
20× dNTPs (5 mM each)	2.5 μL
10× Vent polymerase buffer (NEB)	5.0 μL
fdSEQ1 primer (10 pm/μL)	2.5 μL
BACK primer* (10 pm/μL)	2.5 μL
Single stranded scFv gene template (10 ng)	1.0 μL
Vent DNA polymerase (NEB, 2 units)	1.0 μL

*either RJH1/2Xho, RJH3Xho, RJH4/5Xho, or RJH6Xho

   The BACK primers were designed to anneal to the first 6 nucleotides of the $(G_4S)_3$ linker and either the J_H1, 2,, J_H3, J_H 4,5, or J_H 6 segments respectively and contain the Xho1 cloning site.
2. Heat to 94°C for 5 min in a PCR thermo-cycler.
3. Cycle 25 times to amplify the V_L genes at 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min.
4. Purify PCR product using the Geneclean purification kit. The four PCR products and the pHEN1-V_λ3 vector are digested with XhoI and NotI. Vector and inserts are gel purified and ligated and transformed as described in Protocol number **25.3.7**.

25.3.3.2 Cloning the VH Gene into VL Gene Repertoires to Generate Light Chain Shuffled Libraries

To shuffle the light chain gene from a binding scFv, the rearranged heavy chain gene is amplified using PCR from pHEN-1 containing the target scFv gene using the primers scFvJHXhoFOR and LMB3. The protocol below uses primers that anneal upstream of the pelB leader in pHEN-1 and a scFv specific primer that anneals to the JH gene of the target scFv. One of the JH primers below should be a perfect match for your target scFv VH gene.

scFvJH1-2XhoFOR
5′-GAG TCA TTC TCG TCT CGA GAC GGT GAC CAG GGT GCC-3′
scFvJH3XhoFOR
5′-GAG TCA TTC TCG TCT CGA GAC GGT GAC CAT TGT CCC-3′
scFvJH4-5XhoFOR
5′-GAG TCA TTC TCG TCT CGA GAC GGT GAC CAG GGT TCC-3′
scFvJH6XhoFOR
5′-GAG TCA TTC TCG TCT CGA GAC GGT GAC CGT GGT CCC-3′

To create light chain shuffled libraries, follow protocol number **25.3.2.2** above and substitute in LMB3 for fdseq and one of the JH primers listed above for VL1BACK.

25.3.4 Introducing Mutations by Site Directed Mutagenesis

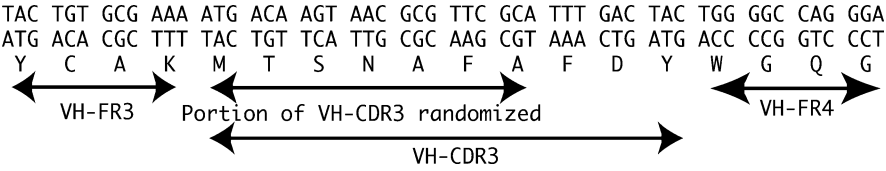
As described above, our preferred approach is to sequentially introduce mutations into V_L and V_H CDR3 using oligonucleotides. As it is difficult to make libraries greater than 10^7–10^8 clones, decisions must be made as to which amino acids to diversify, and to what extent. Conventional oligo-directed mutagenesis uses the nucleotides NNS to randomize each amino acid residue. All parental contacts are discarded and the number of amino acids that can be scanned is limited to five, given typical transformation efficiencies. Rather, we prefer to minimize the number of non-viable structures by using nucleotide mixtures which bias for the wild-type residue, allowing more amino acids to be screened in one library. For V_L CDR3 mutagenesis, we also use molecular modeling using the most homologous V_L in the PDB database to distinguish between residues with solvent accessible side chains from those with buried side chains. Our previous results indicate that randomization of residues with buried side chains is a waste of sequence space, as the wild type sequence returns in most cases (Schier et al. 1996a, c). We also avoid mutating glycines and tryptophans as they invariably return as wild type sequence (Schier et al. 1996a, c). Glycines are frequently critical residues in CDR turns and tryptophans frequently are essential structural or contact residues.

25.3.5 Randomization of the scFv V_LCDR3

We begin randomization with V_L CDR3 as it is typically shorter than V_H CDR3 and more importantly can be modeled based on homologous structures (see above). Randomization of the V_LCDR3 is also technically simpler than V_H CDR3 as it is located at the 3′ end of the scFv gene. The randomization can therefore be carried out in just two PCRs. In the first PCR, a randomized primer (V_LFOR) and the primer LMB3 amplify most of the scFv gene and introduce mutations into the V_L CDR3. The second PCR amplifies the remainder of the scFv and appends a restriction site for cloning of the fragment, Fig. 25.3.

Example of primers used for V_L-radomization:

In this example, seven amino acids of the V_L are randomized (underlined region) with approximately 50% wild type amino acid at each position randomized:



V_L-FR3 randomized V_L-FR4

V_LFOR random primer

5′- CCC TCC GCC GAA CAC CCA ACC 524 513 524 524 542 541 511 CTG GCA GTA ATA ATC AGC CTC -3′

V_L-NotI primer

5′- GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG CAC GGT CAG CTT GGT CCC TCC GCC GAA CAC CCA ACC -3′

Molar compositions:

1: A(70%), C(10%), G(10%), T(10%)
2: A(10%), C(70%), G(10%), T(10%)
3: A(10%), C(10%), G(70%), T(10%)
4: A(10%), C(10%), G(10%), T(70%)
5: G(50%), C(50%)

Underlined portion of V_L-NotI primer anneals to the 5′ end of the V_LFOR random primer.

1. Make up 50 μL PCR reaction mixes containing

water	35.5 μL
20× dNTPs (5 mM each)	2.5 μL
10× Vent polymerase buffer (NEB)	5.0 μL
LMB3 primer (10 pm/μL)	2.5 μL
V_LFOR primer (10 pm/μL)	2.5 μL
scFv gene template (100 ng)	1.0 μL
Vent DNA polymerase (NEB, 2 units)	1.0 μL

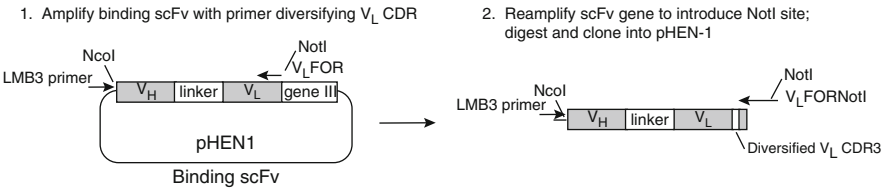


Fig. 25.3 Strategy for diversifying V_L CDR3

2. Heat to 94°C for 5 min in a PCR thermo-cycler.
3. Cycle 30 times to amplify the V_L gene at 94°C for 1 min, 42°C for 1 min, and 72°C for 2 min.
4. Gel-purify the V_L gene repertoires (app. 800 bp) on a 1% agarose gel, and extract the DNA using the Geneclean kit. Resuspend each product in 20 μL of water. Determine DNA concentration by analysis on a 1% agarose gel with markers of known size and concentration.

The scFv V_L gene repertoire is now re-amplified with a primer which appends the restriction site *Not*I.

1. Make up 50 μL PCR reaction mixes containing

water	35.5 μL
20× dNTPs (5 mM each)	2.5 μL
10× Vent polymerase buffer (NEB)	5.0 μL
Lmb3 primer (10 pm/μL)	2.5 μL
V_L-NotI primer (10 pM/μL)	2.5 μL
scFv gene template (100 ng)	1.0 μL
Vent DNA polymerase (NEB, 2 units)	1.0 μL

2. Heat to 94°C for 5 min in a PCR thermo-cycler.
3. Cycle 30 times to amplify the V_L gene at 94°C for 1 min, 42°C for 1 min, and 72°C for 2 min.
4. Purify PCR product using the Wizard PCR purification kit and continue with restriction digest protocol **25.3.7** using Nco1 and Not1.

25.3.6 Randomization of the scFv V_H-CDR3

The PCR for the repertoire is done in two steps. Initially, the V_H is amplified with the randomized primer (V_HFOR) and a primer based in the vector backbone, upstream of the scFv (LMB3). In a separate PCR, the V_L is amplified with a primer based in geneIII of the vector (Fdseq) plus a primer (V_HBACK) which is complementary to parts of the V_HFOR primer. The final V_HCDR3 full-length scFv repertoire is then constructed by PCR assembly of the amplified V_H and V_L fragments (See Fig. 25.4 for strategy).

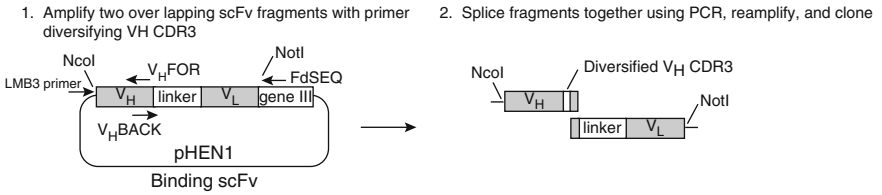
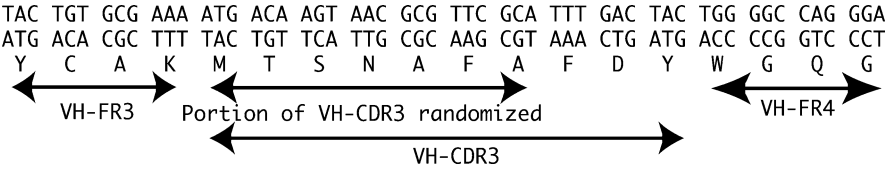


Fig. 25.4 Strategy for diversifying V_H CDR3

Example of primers used for V_H-radomization:
In this example seven amino acids of the V_H are randomized (underlined region):



V_H-FR3 randomized V_H-FR4
The primer to introduce these changes had the following sequence:
V_HFOR primer 5′- CC CTG GCC CCA GTA GTC AAA 532 511 532 544 524 534 514 TTT CGC ACA GTA ATA AAC GGC -3′
Molar compositions:
1: A(70%), C(10%), G(10%), T(10%)
2: A(10%), C(70%), G(10%), T(10%)
3: A(10%), C(10%), G(70%), T(10%)
4: A(10%), C(10%), G(10%), T(70%)
5: G(50%), C(50%)
V_HBACK primer 5′- TTT GAC TAC TGG GGC CAG GG -3′

1. Make up two 50 μL PCR reaction mixes containing

water	35.5 μL
20× dNTPs (5 mM each)	2.5 μL
10× Vent polymerase buffer (NEB)	5.0 μL
reverse primers* (10 pm/μl)	2.5 μL
forward primers# (10 pm/μl)	2.5 μL
scFv gene template (100 ng)	1.0 μL
Vent DNA polymerase (NEB, 2 units)	1.0 μL

*reverse primer for V_H is LMB3; for the V_L the reverse primer is V_HBACK.
#forward primer for V_H is V_HBACK; for the V_L the forward primer is FdSEQ.

2. Heat to 94°C for 5 min in a PCR thermo-cycler.
3. Cycle 30 times to amplify the Vh gene at 94°C for 1 min, 42°C for 1 min, and 72°C for 2 min.
4. Gel-purify the V_H gene repertoires (app. 350 bp) and the V_L gene (app. 350 bp) on a 1.5% agarose gel and extract the DNA using the Geneclean kit. Resuspend

each product in 30 μL of water. Determine DNA concentration by analysis on a 1.5% agarose gel with markers of known size and concentration.

The final V_HCDR3 full-length scFv repertoire is then constructed by PCR assembly of the amplified V_H and V_L fragments.

1. Make up 25 μL PCR reaction mixes containing

water	6.5 μL
10× Vent buffer	2.5 μL
20× dNTP's (5 mM each)	1.0 μL
V_H repertoire (200 ng)	5.0 μL
V_L gene (200 ng)	5.0 μL
Vent DNA polymerase (NEB, 2 units)	1.0 μL

2. Cycle 7 times without amplification at 94°C for 1.5 min, 65°C for 1.5 min, and 72°C for 1.5 min to join the fragments.
3. After 7 cycles, hold at 94°C while adding the 25 μL mix containing the flanking primers:

water	15 μL
10× Vent buffer	2.5 μL
20× dNTP's (5 mM each)	1.3 μL
FdSEQ primer (10 pm/μL)	2.5 μL
Lmb3 primer (10 pm/μL)	2.5 μL
Vent DNA polymerase (NEB, 2 units)	1.0 μL

4. Cycle 30 times to amplify the assembled fragments at 94°C for 1 min, 42°C for 1 min, and 72°C for 2 min.
5. Purify PCR product using the Geneclean purification kit.
6. Proceed to section **25.3.7** for restriction digest with *Nco*I/*Not*I and ligation of fragment.

25.3.7 Restriction Digest, Ligation, and Transformation of scFv Gene Repertoires

We recommend overdigestion of the PCR products because of the poor efficiency with which PCR fragments are digested. For cloning of scFv fragments into pHEN-1, use the enzyme indicated in the specific protocol.

1. Make up 100 μL reaction mix to digest scFv repertoires:

scFv DNA (1–4 μg)	50 μL
water	34 μL
10× NEB Buffer	10 μL
restriction enzyme A (10 u/μL)	3.0 μL
restriction enzyme B (10u/μL)	3.0 μL

2. Incubate at 37°C overnight.

3. Gel-purify the gene repertoires on a 1% agarose gel and extract the DNA using the Geneclean kit. Resuspend the each product in 30 μL of water. Determine DNA concentration by analysis on a 1% agarose gel with markers of known size and concentration.
4. Approximately 4 μg of cesium chloride purified pHEN 1 is digested with the appropiate enzyme. The digested vector DNA is purified on a 0.8% agarose gel, and extracted from the gel , also using the geneclean kit. For optimal digestion, the restriction digest is carried out overnight and vector DNA is gel purified prior to ligation. Efficient digestion is important as a small amount of undigested vector leads to a very large background of non-recombinant clones. Use of vector DNA prepared by techniques other than cesium chloride will give lower transformation effciencies!
   In ligation experiments, the molar ratio of insert to vector should be 2:1. Given that the ratio of sizes of assembled scFv (800 bp) to vector (4,500 bp) is approximately 6:1, this translates into a ratio of insert to vector of 1:3 in weight terms.
5. Make up 50 μL ligation mixture:

10× ligation buffer	5 μL
water	16 μL
digested pHEN 1 (100 ng/μL)	20 μL
scFv gene repertoire (100 ng/μL)	7 μL
T4 DNA ligase (400 u/μL)	2 μL

6. Ligate overnight at 16°C.
7. Ethanol precipitate and wash pellet twice by thoroughly resuspending in 70% ethanol.
8. Resuspend DNA in 10 μL of water and use 2.5 μL/transformation (4 electroporations total) into 50 μL electrocompetent *E. coli* TG1. Set up a "no DNA" control for the electroporation to ensure that the TG1 cells are not contaminated. Typical transformation efficiencies for *E. coli* TG1 cells are at least 5×10^9/μg of supercoiled pUC18 plasmid DNA.
9. Set the electroporator at 200 ohms (resistance), 25 μFD (capacitance) and 2.5 kilo volts. After electroporation, the time constant should be 4.5 s. If time constant is less than 4.3 s, repeat the DNA precipitation.
10. Grow bacteria from each electroporation in 1 mL SOC and incubate at 37°C for 1 h shaking (250 rpm) and plate serial dilutions onto small TYE/amp/glu agar plates for determining the size of the library.
11. Centrifuge remaining bacteria solution at 1,700× *g* for 10 min at 4°C. Resuspend pellet in 250 μl and plate onto four or more large (16 cm) TYE/100 μg/mL ampicillin/2% glucose plates. Incubate overnight at 37°C.
12. Scrape bacteria from large plates by washing each plate with 3 mL 2 × TY/100 μg/mL ampicillin/2%glu. Make glyercol stocks by adding 1.4 mL of bacteria and 0.6 mL 50% glycerol (sterilized by filtration through 0.2 μm filter). Save library stock at −70°C.
    Note: We routinely obtain libraries with complexity $>10^7$ cfu. If library is smaller than 10^6, the cloning should be repeated. It should be possible to

routinely achieve a transformation efficiency for *E. coli* TG1 cells of greater than 5×10^9/μg of supercoiled plasmid DNA. This requires strict attention to the preparation of the electrocompetent cells.

25.3.8 Rescuing Phagemid Libraries for Selection

1. Calculate the number of bacteria per ml from your library glycerol stock (OD_{600} of 1.00 corresponds to approximately 1×10^8 cells). Usually the optical density of your bacterial stock is around 100, or 10^{10} cells/ml. The input of cells for rescuing is dependent on the density of the original bacteria glycerol stock and should be in 10-fold excess of the number of different clones in the library, but should not exceed OD_{600} of 0.05. This step ensures the diversity of the library for subsequent rounds of selections. For example, a library of 10^7 unique members requires at least 10^8 cells for starting the initial culture. A typical inoculum is 20 μL of glycerol stock into 50 mL of 2 × TY/100 μg/mL ampicillin/2% glucose. Use of a starting OD greater than 0.05 will not result in an adequate number of doublings to ensure that the bacteria are healthy and can be easily infected by helper phage.
2. Grow with shaking (250 rpm) at 37°C to an OD_{600} ~ 0.5. If this inoculum has not grown to OD_{600} ~ 0.5 after 1.5–2 h, increase inoculum size accordingly. It is also important that the culture is allowed to pass through several generations; thus, if culture grows to OD_{600} ~ 0.5 in less than 1 h, decrease the inoculum size.
3. Transfer 10 mL (about 5×10^8 bacteria total) to a 50 mL Falcon tube containing the appropriate number of helper phages. To ensure rescue of all clones in the library the ratio of helper phage : bacteria should be 10:1. Therefore, add 5×10^9 plaque forming units (pfu) of helper phage to the bacterial solution. Incubate at 37°C without shaking for 30 min.
4. Plate 1 μL onto TYE/100 μg/mL kanamycin plate to check for infectivity. Incubate at 37°C overnight. The plate should be nearly confluent next day, indicating successful co-infection of the helper phage.
5. Centrifuge cells at 3,000× *g* to remove glucose and resuspend in 50 ml TYE/amp/kan.
6. Grow with shaking (250 rpm) overnight at 30°C.
7. Remove bacteria by centrifugation at 4,000× *g* 20 min, 4°C. Decant the clear supernatant containing phage particles (if cloudy, repeat centrifugation) into a 500 mL centrifuge bottle and add 10 mL 20% PEG8000/2.5 M NaCl per bottle. Mix and incubate on ice for 30 min.
8. Pellet phage by spinning for 15 min, 4,000× *g* at 4°C. Discard the supernatant.
9. Resuspend the white phage pellet in 10 mL PBS. To remove remaining bacteria debris, we recommend spinning down in the centrifuge for 20 min, 4,000× *g* at 4°C.
10. Transfer the supernatant to a 15 mL Falcon tube and repeat PEG precipitation with 1 mL 20% PEG 8000/2.5 M NaCl. Incubate on ice for 15 min.

11. Centrifuge 10 min, 4,000× *g* at 4°C and resuspend the white pellet in 1.5 mL PBS.
12. Filter supernatant through 0.45 μm syringe filter. The phage stock should be used at once for the next round of selection, but can be stored at 4°C approximately 1 week without much loss in binding activity.

25.3.9 Selection of Higher Affinity Clones from Diversified Libraries

Two approaches have been used to select rare higher affintiy scFv from a background of lower affinity scFv or non-binding scFv: selections based on binding kinetics and selections based on equillibrium constant. In either case, it is important to use labeled antigen in solution rather than antigen adsorbed to a solid matrix. This biases towards selections based on binding affinity or binding kinetics, rather than avidity. This is especially important when selecting scFv libraries, where it is known that the scFv can spontaneously dimerize in a sequenc dependent manner (Schier et al. 1996b). Failure to use soluble antigen is likely to result in the selection of dimeric scFv whose monovalent binding constant is no higher than wild type. Even with the Fab format, soluble antigen should also be used to avoid selecting for phage displaying muliple copies of Fab which will have a higher functional affinity (avidity).

In selections based on binding kinetics, also termed off-rate selections, the phage population is allowed to saturate with the labeled antigen before a large molar excess of unlabelled antigen is added to the mix for a given amount of time. The duration of the competition with unlabelled antigen is chosen to allow the majority of the bound wild-type clones to dissociate while the improved mutants remain bound (Hawkins et al. 1992). This approach effectively selects for slower off-rates. As a reduction in koff is typically the major kinetic mechanism resluting in higher affinity when V genes are mutated, both in vivo and in vitro (Marks et al. 1992), this approach should generally result in the selection of scFvs with improved Kds.

We generally prefer equilibrium selections, in which phages are incubated with an antigen concentration below the equilibrium binding constant. This approach effectively selects for improved equilibrium constants. Reduction of the antigen concentration also helps ensure that the selection for higher affinity scFv occurs, rather than selection for scFv that express well on phage or are less toxic to *E coli*. The ability to use soluble antigen for selections affords the control over antigen concentration needed for the equilibrium screen. Phages are allowed to bind to biotinylated antigen and then recovered with streptavidin magnetic beads. Estimates of the optimal antigen concentration used for the selection can be estimated a priori (Boder and Wittrup 1998), but we prefer to use several different concentrations of antigen for the first round of selection. The antigen concentration for the following rounds of selction are guided by determining the fraction of binding

phage present in the polyclonal phage preparation. This is best done by surface plasmon resonance where the binding concentration is determined under mass transport limited conditions (Schier et al. 1996c). Alternatively, the percentage of binding clones can be determined by ELISA. From this data, the optimal antigen concentration can be determined prior to the next round of selection. We typically lower the antigen concentration 10–100 fold after each round of selection. ScFv antibody clones with improved affinity are usually identified after 3–5 rounds of selection.

25.3.9.1 Equilibrium Selection Protocol

1. Prior to selection, block 50 µL streptavidin-magnetic beads with 1 mL 2% MPBS (2% milk powder in PBS) for 1 h at RT in 1.5 mL microcentrifuge tube. Pull beads to one side with magnet. Discard buffer.
2. Block 1.5 mL microcentrifuge tube with 2% MPBS for 1 h at RT and discard the blocking buffer. Incubate biotinylated antigen (for first round of selection, concentrations should be: Kd/1, Kd/10, and Kd/100), prepared according to the manufacturer's instruction (Pierce), with 1 mL of phage preparation (approximately 10^{12} TU) in 2% MPBS (final conc.) by rocking at room temperature for 1 h.
3. Add the streptavidin-magnetic beads to the phage-antigen mix and incubate on rotator at room temperature for 15 min. Place tube in magnetic rack for 30 s. Beads will migrate towards the magnet.
4. Aspirate tubes, leaving the beads on the side of the microcentrifuge tube. This is best done with a 200 µL pipette tip on a Pasteur pipette attached to a vacuum source. Wash beads (1 mL per wash) with PBS-Tween (0.1%) 7 times, followed by MPBS 2 times, then once with PBS. Transfer the beads after every second wash to a fresh Eppendorf tube to facilitate efficient washing.
5. Elute phage with 100 µL 100 mM HCl for 10 min at room temperature. Place tube in a magnetic rack for 30 s. and beads migrate towards the magnet. Remove the supernatant containing eluted phage and neutralize with 1 mL of 1 M Tris, pH 7.4. Save on ice.
6. Add 0.75 mL of the phage stock to 10 mL of exponentially growing TG1 (OD_{600} ~ 0.5). (Store the remaining phage mix at 4°C).
7. Incubate at 37°C for 30 min without shaking.
8. Titer TG1 infection by plating 1 µL and 10 µL onto small TYE/100 µg/mL ampicillin/2% glucose plates (this is a 10^4 and 10^3 dilution, respectively).
9. Centrifuge the remaining bacteria solution at 1,700× *g* for 10 min. Resuspend pellet in 250 µL media and plate onto two large TYE/100 µg/mL ampicillin/2% glucose plates and incubate overnight at 37°C.
10. Add 3 mL of 2 × TY/100 µg/mL ampicillin/2% glucose media to each plate, and then scrape the bacteria from the plate with a bent glass rod. Make glycerol stocks by mixing 1.4 ml of bacteria and 0.6 ml 50% glycerol (filtered). Save stock at −70°C.

Note: For large, randomized libraries (complexity $> 10^7$), the selection titer from the first round of selection should be between 10^4–10^6 cfu. If the titers for all antigen concentrations are larger than 10^7, it is likely that the antigen concentration was too high or that the washing steps have been inadequate. If the titer is below 5×10^4, you have either washed too many times, or too little antigen was used. In this case, reduce the number of washes.

25.3.10 BIAcore Screening for scFv Clones with Improved Off-Rates

Even after stringent selections as described above, only a fraction of the ELISA positive clones will have a higher affinity than wild-type. The strength of the ELISA signal is a poor indicator of which clones have a lower K_d, and more typically correlates with expression level. Therefore, a technique is required to screen ELISA positive clones to identify those with a lower K_d. One such technique is a competition or inhibition ELISA (Friguet et al. 1985). Alternatively, one can take advantage of the fact that a reduction in k_{off} is typically the major kinetic mechanism resulting in higher affinity and thus antibodies with improved affinity can be identified by measuring the off-rate. Since k_{off} is concentration independent (unlike k_{on}), it is possible to measure k_{off}, for example using surface plasmon resonance (SPR) in instruments such as the BIAcore, without purifying the antibody fragment. We have found this a very useful technique for identifying higher affinity scFv and use it to rank affinity maturated clones.

First, we identify binding clones by ELISA as described elsewhere in this book. We then take approximately 20–50 randomly selected ELISA positive clones and rank them by k_{off} as described below. Those clones with the slowest k_{off} are then purified and the K_d is measured. Note that in the case of scFv, the shape of the dissociation curve can indicate whether there is a single k_{off} or multiple k_{off}s present. ScFvs that have multiple k_{off}s (a curve vs a straight line when plotting ln R1/R0 vs T) are a mixture of monomers and dimers and are best avoided for subsequent characterization.

1. Grow a 1 mL overnight culture in 2 × TY/100 μg/mL ampicillin/2%glucose at 30°C (250 rpm).
2. Add 0.5 mL of the overnight culture to 50 mL 2 × TY/amp/0.1% glu in an Erlenmeyer flask and grow at 37°C, shaking at 250 rpm for approximately 2 h to $O.D._{600}$ of 0.9.
3. Induce with 25 μL 1 M IPTG (final concentration of 500 μM) and grow at 30°C at 250 rpm for 4 h. Collect cells by centrifuging in a 50 mL tubes at 4,000× *g* for 15 min.
4. For the periplasmic preparation, resuspend the bacterial pellet in 1 mL PPB buffer (20% sucrose, 1 mM EDTA, 20 mM Tris-HCl, pH = 8), transfer to 1.5 mL Eppendorf tubes, and keep on ice for 20 min. Spin down cells in microcentrifuge

at 6,000 rpm for 5 min. Discard the supernatant. Proceed with the bacterial pellet for the osmotic shock preparation.

5. For the osmotic shock preparation, resuspend the pellet in 1 mL 5 mM $MgSO_4$ (use 1/50 of total growth volume) and incubate on ice for 20 min. Spin down cells in microcentrifuge at full speed (14,000 rpm) for 5 min.
   At this step, the buffer of the osmotic shock fraction is exchanged to the BIAcore running buffer in order to avoid excessive refractive index change during the SPR analysis.
6. The buffer is most effectively exchanged using small columns such as PD10 (Pharmacia) or spin colums containing G-25 M (BioRad).
7. In a BIAcore flow cell, immobilize antigen corresponding to 100–300 RU total scFv binding to a CM5 sensor chip using EDC/NHS chemistry as described by the manufacturer. The immobilization of different antigens varies tremendously. However, 10 μg/mL in 10 mM NaAc, pH 4.5, is a good starting point for most protein antigens.
8. Dissociation of undiluted scFv in the periplasmic fraction is measured under a constant flow of 15 μL/min and HBS as running buffer. An apparent k_{off} can be determined from the dissociation part of the sensorgram for each scFv analyzed. The regeneration of the sensor chip between samples has to be determined for each antigen. However, 4 M $MgCl_2$ will regenerate most antigen surfaces without significant change in the sensorgram baseline after analysis of more than 100 samples.

References

Allen D, Cumano A, Dildrop R, Kocks C, Rajewsky K, Rajewsky N, Sablitzky F, Siekevitz M (1987) Timing, genetic requirements and functional consequences of somatic hypermutation. Immunol Rev 96:5–22

Alt FW, Blackwell TK, Yancopoulos GD (1987) Development of the primary antibody repertoire. Science 238(4830):1079–1087

Boder E, Wittrup K (1998) Optimal screening of surface-displayed polypeptide libraries. Biotechnol Prog 14(1):55–62

Clackson T, Hoogenboom HR, Griffiths AD, Winter G (1991) Making antibody fragments using phage display libraries. Nature 352:624–628

Crameri A, Cwirla S, Stemmer WP (1996) Construction and evolution of antibody-phage libraries by DNA shuffling. Nat Med 2(1):100–102

Figini M, Marks JD, Winter G, Griffiths AD (1994) In vitro assembly of repertoires of antibody chains on the surface of phage by renaturation. J Mol Biol 239(1):68–78

Foote J, Winter G (1992) Antibody framework residues affecting the conformation of the hypervariable loops. J Mol Biol 224:487–499

Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME (1985) Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J Immunol Meth 77:305–319

Hawkins RE, Russell SJ, Winter G (1992) Selection of phage antibodies by binding affinity. Mimicking affinity maturation. J Mol Biol 226(3):889–896

Holliger P, Prospero T, Winter G (1993) 'Diabodies': small bivalent and bispecific antibody fragments. Proc Natl Acad Sci 90:6444–6448

Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, Winter G (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucl Acids Res 19(15):4133–4137

Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C (1991) Sequences of proteins of immunological interest., US Department of Health and Human Services, US Government Printing Office

Loh DY, Bothwell ALM, White-Scharf ME, Imanishi-Kari T, Baltimore D (1983) Molecular basis of a mouse strain specific anti-hapten response. Cell 33:85–93

Low NM, Holliger PH, Winter G (1996) Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. J Mol Biol 260(3): 359–368

Marks JD, Griffiths AD, Malmqvist M, Clackson TP, Bye JM, Winter G (1992) By-passing immunization: building high affinity human antibodies by chain shuffling. Biotechnology (N Y) 10(7):779–783

Novotny J, Bruccoleri RE, Saul FA (1989) On the attribution of binding energy in antigen-antibody complexes McPC 603, D1.3, and HyHEL-5. Biochemistry 28:4735–4749

Schier R, Balint RF, McCall A, Apell G, Larrick JW, Marks JD (1996a) Identification of functional and structural amino-acid residues by parsimonious mutagenesis. Gene 169(2):147–155

Schier R, Bye J, Apell G, McCall A, Adams GP, Malmqvist M, Weiner LM, Marks JD (1996a) Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. J Mol Biol 255(1):28–43

Schier R, Marks JD, Wolf EJ, Apell G, Wong C, McCartney JE, Bookman MA, Huston JS, Houston LL, Weiner LM, Adams GP (1995) In vitro and in vivo characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. Immuno-technology 1(1):73–81

Schier R, McCall A, Adams GP, Marshall KW, Merritt H, Yim M, Crawford RS, Weiner LM, Marks C, Marks JD (1996c) Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. J Mol Biol 263(4):551–567

Stemmer WP (1994) Rapid evolution of a protein in vitro by DNA shuffling [see comments]. Nature 370(6488):389–391

Tomlinson IM, Walter G, Jones PT, Dear PH, Sonnhammer EL, Winter G (1996) The imprint of somatic hypermutation on the repertoire of human germline V genes. J Mol Biol 256(5):813–817

Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G (1992) The repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops. J Mol Biol 227(3):776–798

Yang WP, Green K, Pinz-Sweeney S, Briones AT, Burton DR, Barbas CFr (1995) CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. J Mol Biol 254(3):392–403

Chapter 26
Affinity Maturation by Random Mutagenesis and Phage Display

Holger Thie

26.1 Introduction

In the past two decades, antibodies have been the fastest growing class of pharmaceutical proteins with a predicted marked volume of US$ 30 billion in 2010 (Evans and Das 2005). In vitro antibody selection technologies, most of the antibody phage display offer advantages when compared to the animal based generation of antibodies: enhanced throughput by parallelisation and miniaturisation, the stringent control of selection conditions and the possibility to use toxic proteins or allosteric conformation variants (Hust and Dübel 2004).

Phage display allows the production and screening of large antibody libraries with theoretical complexities of up to 10^{11} different clones (Sblattero and Bradbury 2000). However, even complex naïve (non-immune) antibody libraries sometimes result in the selection and amplification of binders with equilibrium dissociation constant (K_D) ranges >100 nM (Coia et al. 2001). For many applications in research and therapy, antibodies with affinities in the lower nanomolar or subnanomolar range are preferred. Consequently, methods for affinity maturation have been developed for the improvement of these low affinity antibodies. Enhanced K_D values up to two orders of magnitudes are achievable compared to the parental clone (Low et al. 1996).

In general, mutations are introduced into the antibody gene, to create a mutated antibody gene library. Several different mutation strategies have been developed. Site specific mutation of complementarity determining regions (CDRs) by using polymerase chain reaction (PCR) and special randomised primers is one strategy (Yau et al. 2005). Another strategy is random mutagenesis of the complete phagemid carrying the antibody gene. Here, the use of *Escherichia coli* mutator strains

H. Thie
Technische Universität Braunschweig, Institut für Biochemie und Biotechnologie, Abteilung Biotechnologie, Spielmannstr. 7, Braunschweig 38106, Germany
e-mail: h.thie@tu-bs.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_26,

(Irving et al. 1996) or the use of error-prone templi-phi DNA amplification (Fujii et al. 2004) are possible strategies. In both strategies, mutations can also occur in the vector backbone, making subsequent recloning of the antibody ORF necessary.

The most common technique to introduce random mutations is the error-prone PCR of the antibody gene (Martineu 2002). It utilises the natural error-rate of Taq-polymerase (Tindall and Kunkel 1988), which can be increased by suboptimal buffer conditions during DNA amplification. Here, only the gene of interest is affected by mutations and will be subcloned into the phagemid backbone to obtain a "mini library".

Subsequent selection of high affinity mutants is achieved by phage display panning approaches (Hust et al. 2007a) using many harsh and long washing steps.

A disadvantage is that surface-selection often enriches binders with increased tendency for dimerisation, especially when using the scFv format. This problem can be by-passed by panning the antigen in solution (Schier et al. 1996) e.g., by using biotinylated antigen in combination with streptavidin beads. Moreover, it is possible to add unbiotinylated antigen or soluble antibody fragments after antibody phage binding has occurred, to provide competition to improve selection for better off-rates.

Screening of high affinity variants requires the production of monoclonal soluble antibody fragments and can be performed in microtiter plates (96 well scale) (Konthur et al. 2005, Hust et al. 2009). Monoclonal binders can be tested and affinity ranked by using crude cell supernatants in common ELISA techniques or by competitive ELISA (Friguet et al. 1985). Today, affinities are usually be determined by kinetic analysis using surface plasmon resonance (SPR) (Wassaf et al. 2006). Specificity can be analysed e.g., by flow cytometry.

26.2 Outline

These methods describe the affinity maturation of antibody fragments by random mutagenesis. In a first step, genetic diversity will be generated by sequential error-prone PCRs. Subsequently, antibody gene libraries will be generated by cloning the mutated antibody fragment genes into a phagemid and subsequently M13 packaging.

Pannings under stringent conditions will be performed to enrich binders with improved binding properties. Finally, single antibody clones will be produced and analysed in a first ELISA screening.

26.3 Materials

26.3.1 Error-Prone PCR

- Template DNA, phagemid containing antibody fragment gene
- Up to three site specific DNA-oligo primersets

- PCR Thermocycler (Biorad PTC 250)
- GeneMorphII Random Mutagenesis Kit (Stratagene, Amsterdam, Netherlands)
- PCR clean up kit (NucleoSpin Extract II, Macherey-Nagel, Düren, Germany)
- Agarose gel

26.3.2 Affinity Library Creation

- Phage display compatible phagemid (e.g. pHAL14, pSEX81) (Hust et al. 2007b, Pelat et al. 2007, Kirsch et al. 2008, Welschof et al. 1997)
- Restriction enzymes NcoI, NotI (New England Biolabs, Frankfurt/Main, Germany)
- Shrimp alkaline phosphatase, SAP (MBI Fermentas, St. Leon Rot, Germany)
- PCR clean up kit (NucleoSpin Extract II, Macherey-Nagel, Düren, Germany)
- T4 DNA-Ligase (Promega, Mannheim Germany)
- Electrocompetent *E. coli* XL1 Blue MRF' (Stratagene, Amsterdam, Netherlands); Genotype: Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *pro*AB *lac*$I^qZ\Delta M15$ Tn10 (Tet^r)]
- SOC media (0.5% (w/v) yeast extract, 2.0% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose)
- Eppendorf Thermomixer compact
- 2 M Mg solution (1 M MgCl + 1 M $MgSO_4$)
- SOB media pH 7.0 (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, after autoclavation add sterile 1% (v/v) 2 M Mg solution)
- SOB-GA (SOB, containing 100 µg/mL ampicillin, 100 mM glucose, 1.5% (w/v) agar agar) agar plates
- 25 × 25 cm sterile plastic dishes
- 2 × YT medium pH 7.0 (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl)
- 2 × YT-GA medium (2 × YT, containing 100 µg/mL ampicillin, 100 mM glucose)
- Gylcerol solution 80% (v/v)
- 1.8 mL Cryotubes (Nunc, Mannheim, Germany)

26.3.3 Libary Validation

- 9 cm sterile petri dishes
- Phagemid specific DNA-Oligo Primerset
- RedTaq DNA Polymerase (Sigma, Munich, Germany)
- PCR clean up kit (NucleoSpin Extract II, Macherey-Nagel, Düren, Germany)

26.3.4 Library Packaging

- 2 × YT-GA (2 × YT, containing 100 μg/mL ampicillin, 100 mM glucose)
- 100 mL shake flask
- Helperphage M13K07 (Stratagene)
- 2 × YT-AK (2 × YT, containing 100 μg/mL ampicillin, 50 μg/mL kanamycin)
- Polyethylenglycol (PEG) solution (20% (w/v) PEG 6000, 2.5 M NaCl)
- Phage Dilution Buffer (10 mM TrisHCl pH 7.5, 20 mM NaCl, 2 mM EDTA)

26.3.5 Titration

- 2 × YT-T (2 × YT, containing 50 μg/mL tetracyclin)
- 2 × YT-GA (2 × YT, containing 100 μg/mL ampicillin, 100 mM glucose, 1.5% (w/v) agar agar) agar plates
- *E. coli* XL1 Blue MRF' (Stratagene); Genotype: Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *pro*AB *lac*I^qZΔM15 Tn10 (Tet^r)]

26.3.6 Selection by Panning

- Carbonate buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3), pH 9.7
- PBS pH 7.4 (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4*$2H_2O$, 0.24 g KH_2PO_4 in 1 L)
- PBS-T (PBS + 0.1% Tween 20)
- MPBS-T (2% skim milk in PBST, prepare fresh)
- Panning block solution (2% (w/v) skim milk + 2% (w/v) BSA in PBST, prepare fresh)
- Trypsin (10 μg/mL) in PBS
- 2 × YT-T (2 × YT, containing 50 μg/mL tetracyclin)
- 15 cm petri dishes
- Glycerol solution 80% (v/v)

26.3.7 Selection in Solution with Competition

- Soluble antigen
- Biotinylated soluble antigen
- Streptavidin beads (Dynabeads M-280, Invitrogen, Karlsruhe, Germany)
- Overhead shaker (Multi Shaker Rotator RS24, G Kisker)

- Magnet particle concentrator (Dynal, Invitrogen, Karlsruhe, Germany)
- Trypsin (10 μg/mL) in PBS

26.3.8 Production of Soluble Monoclonal AB-Fragments

- 96 well U-bottom polypropylene (PP) microtitre plates (Greiner BioOne, Frickenhausen, Germany)
- AeraSeal breathable sealing film (Excel Scientific, USA)
- 2 × YT-A containing 50 μM isopropyl-beta-D-thiogalactopyranoside (IPTG)
- Thermo Shaker PST-60HL-4, (Lab4You, Berlin, Germany)

26.3.9 Monoclonal ELISA

- Maxisorp Plates (Nunc)
- Mouse α-myc-tag monoclonal antibody (9E10, Sigma-Aldrich, Munich, Germany)
- Mouse α-His-tag monoclonal antibody (α-Penta His, Qiagen, Hilden, Germany)
- Goat α-Mouse IgG serum (Fab specific), HRP conjugated (Sigma-Aldrich, Munich, Germany)
- TMB solution A pH 4.1 (10 g citric acid solved in 100 mL water, add 9.73 g potassium citrate, add H_2O to 1 L)
- TMB solution B (240 mg tetramethylbenzidine, 10 mL acetone, 90 mL ethanol, 907 μL 30% H_2O_2)
- 0.5 M H_2SO_4

26.4 Methods

All methods which are not explained in detail are performed according to Sambrook and Russell (2001).

26.4.1 Error-Prone PCR

1. Design specific primer sets for your phagemid.
   Note: To increase the number of error-prone PCR rounds, design up to three primer sets and perform the PCR in a nested approach.
2. Use <1 ng purified plasmid DNA as template for error-prone PCR.

3. Perform PCR by using GeneMorphII random mutagenesis kit (Stratagene) according to manufacturer's instructions, 35 cycles.
   Note: The advantage of the GeneMorphII kit is the mutational spectra of the MutazymeII DNA polymerase. A,T → N 50.7%; G,C → N 43.8% and the high mutation frequency (up to 16 mutations/kb and PCR) (Stratagene, kit manual)
4. Clean up amplified DNA by using PCR clean up kit and determine DNA concentration photometrically after clean up
5. Use <1 ng of this DNA for the next error-prone PCR.
   Note: 3–4 rounds of error-prone PCR can be performed by using the same primer set. After more rounds, the PCR-product yield will decrease rapidly. We suggest max. 2–3 error-prone PCR rounds with one primer set, then switch to the next inner primer set. After 6 rounds of error-prone PCRs (35 cycles each), around 30 point mutations can be found in a common scFv gene (approx. 800 bp)
6. Repeat error-prone PCR up to 6–8 times with following clean up.
   Note: To get more DNA for the following cloning step, scale up the last PCR
7. Cheque PCR product on a 1% agarose gel. Use only clean PCR fragment (distinct band, no smear) for library creation

26.4.2 Affinity Library Creation

1. Digest your PCR product and phage display vector DNA with suitable restriction enzymes (e.g., NcoI + NotI in case of pHAL14, pSEX81, pIT). Incubate over night at 37°C. Add 1 μL shrimp alkaline phosphatase (SAP) to the vector preparation, incubate 1 h at 37°C. Inactivate all enzymes by heating at 65°C, 15 min
2. If necessary separate your digested vector DNA by agarose gel electrophoresis or directly use a DNA clean up kit for purifying the digested DNA.
   Note: Vectors with discarded inserts < 100 bp do not need agarose gel electrophoresis. A PCR clean up kit gives sufficient DNA purity and a higher recovery yield
3. Determine DNA concentration of digested vector DNA and digested PCR product (photometrically or agarose gel)
4. Use approximately 1 μg vector DNA for ligation, molar vector : insert ratio should be 1:3. Ligate over night at 16°C. Inactivate enzymes at 65°C, 10 min
5. Perform ethanol-precipitation of the DNA, resuspend the pellet in 100 μL H_2O. Split to four aliqots (25 μL each)
6. Mix every ligation aliquot with 25 μL electrocompetent *E. coli* XL1 Blue MRF' (Stratagene) and perform electroporation (BioRad, electroporation unit). Parameter: 1.7 kV, 4–5 ms pulse
7. Resuspend transformed cells in 950 μL prewarmed SOC medium, incubate 1 h at 37°C and 600 rpm (Eppendorf thermomixer)
8. Take 10 μL of the cell suspension and make a dilution series (e.g., 1:100 per step) in SOB medium, up to 10^8 clones can be expected. Plate an aliquot of each dilution and calculate the transformation efficiency.

Note: The transformation efficiency is a benchmark for the theoretical size of the library. The sum of the four transformation yields is the maximum complexity of the new library

9. Plate the rest of the cell suspension to 25×25 cm SOB-GA agar-plates. Incubate o/n at 37°C
10. Add 40 mL SOB medium to each plate. Incubate at room temperature on a rocker for 30 min. Carefully scrape the transformed cells from the plate using a spatula
11. Collect the cell-suspension and centrifuge in a 50 mL Falcon tube at $3{,}200\times\ g$ for 10 min. Discard supernatant, resuspend the pellet in 5 mL $2 \times$ YT medium
12. Pool all resuspended pellets in one tube. Prepare glycerol-stocks of your library by mixing 250 µL 80% glycerol and 750 µL cell suspension in 1.8 mL cryotubes. Shock-freeze this tube in liquid nitrogen and store at −80°C

26.4.3 Library Validation

1. Pick up to 20 clones from the plates used for determination of transformation efficiency. Plate on fresh $2 \times$ YT-GA agar-plates
2. Perform colony-PCR (50 µL scale) using a primer set which will amplify the cloned scFv gene
3. Analyse an aliquot (5 µL) by agarose gel electrophoresis. A band at a defined size (depending on the used primer set, usually 800–1,000 bp for scFv DNA) will be obtained from clones with a "correct" insert. The percentage of these "positive" clones show the quality of the created library
4. Clean up the PCR from these clones, by using a PCR clean up kit. Use this purified DNA for sequencing with appropriate primers.
   Note: Alternatively, perform plasmid-DNA preparation from the plated clones and use the isolated plasmid-DNA for sequencing
5. The average mutation rate can be determined by alignment of the DNA-sequence from the sequenced clones with the parental original sequence of the source clone. Up to 30 point mutations can be expected

26.4.4 Library Packaging

1. Gently thaw a library glycerol stock on ice
2. Inoculate 50 mL $2 \times$ YT-GA medium with 10–100 µL cell suspension directly from the glycerol stock to an initial OD_{600} of about 0.1
3. Grow the cells at 37°C and 250 rpm in a 100 mL Erlenmeyer shake flask to an $OD_{600} \sim 0.5$
4. Transfer 20 mL of the culture into a sterile 50 mL polypropylene tube. Add 5×10^{12} M13K07 pfu for infection, mix gently. Incubate 30 min at 37°C, then incubate another 30 min at 37°C with shaking at 250 rpm

5. Centrifuge the suspension 10 min at 3,200× *g* to pellet the cells. Discard the supernatant to remove the glucose and suspend the pellet in 1 mL fresh 2 × YT-AK medium.
   Note: Due to the selection with kanamycin, only M13K07 (Kan^r) infected cells will survive and produce antibody-phage.
   Use this suspension for inoculation of 50 mL 2 × YT-AK in a 100 mL Erlenmeyer shake flask
6. Incubate cells o/n at 30°C and 250 rpm
7. Precipitate the phage in the supernatant by adding 1/5 volume PEG solution in 50 mL polypropylene tubes. Incubate for 1 h at 4°C with gentle shaking
8. Pellet the phage by centrifugation for 1 h at 3,200× *g* and 4°C. Put the open tubes upside down on tissue paper and let the viscous PEG solution move out completely. Resuspend the phage pellet in 500 µL phage dilution buffer. Centrifuge again with 16,000× *g* to remove debris and residual cells. Transfer the supernatant into a new tube. Titre the phage preparation and use it for the next panning round. Store the remaining phage at 4°C

26.4.5 Titration

1. Inoculate 5 mL 2 × YT-T medium in a 100 mL Erlenmeyer flask with *E. coli* XL1-Blue MRF' and grow over night at 37°C and 250 rpm
2. Inoculate 50 mL 2 × YT-T medium with 500 µL o/n culture and grow at 250 rpm at 37°C up to OD_{600} ~ 0.5.
   Note: If the bacteria have reached OD_{600} ~ 0.5 before they are needed, store the culture on ice to maintain the F pili on the E. coli cells. Store for max. 60 min
3. Make serial dilutions of the phage solution in PBS. The number of eluted phage during panning depends on several parameters (e.g., antigen, library, panning round, washing stringency etc). The phage titre can vary between 10^2–10^7 cfu. The phage preparation after re-amplification of the eluted phage has a titre of about 10^{11}–10^{13} cfu/mL. Infect 50 µL bacteria with 10 µL phage dilution and incubate 30 min at 37°C
4. Plate the 60 µL infected bacteria on 2 × YT-GA agar plates (9 cm petri dishes)
5. Incubate the plates over night at 37°C
6. Count the colonies and calculate the colony forming units (cfu) titre according to the dilution

26.4.6 Selection by Panning

1. Immobilise antigen in different amounts (1–50 ng/well) in a Maxisorp stripe (Nunc), use carbonate buffer (pH 9.7) or PBS (pH 7.4), coat overnight at 4°C
2. Block microtitre plate stripe with 350 µL MPBS-T for 1–3 h at room temperature

3. Wash 3× with PBS-T.
   Note: The washing should be performed with an ELISA washer (e.g., TECAN Columbus Plus) for reproducible washing results. To remove antigen or blocking solutions wash 3× with PBST ("standard washing protocol" for TECAN washer). If no ELISA washer is available, wash manually 3× with PBST
4. Incubate 10^{11}–10^{12} antibody phage from the library in 150 µL panning block solution and add this solution to the antigen coated wells. Incubate 3 h at room temperature
5. After binding of antibody phage, wash 3× with PBS-T.
   Note: After binding of antibody phage, wash 3× with PBST ("stringent bottom washing protocol" in case of TECAN washer). If no ELISA washer is available, wash manually 10× with PBS-T and 10× with PBS
6. Put the complete stripe in 2 L PBS, incubate under soft stirring for 1 week at 4°C
7. Repeat steps 5 + 6 one time weekly (washing 3–4 weeks recommended)
8. Elute with 200 µL trypsin solution for 60 min at 37°C.
   Note: Phagemids like pSEX81 (Welschof et al. 1997) or pHAL14 (Hust et al. 2007b, Pelat et al. 2007, Kirsch et al. 2008) have coding sequences for a trypsin specific cleavage site between the antibody fragment gene and the gIII. Trypsin also cleaves within antibody fragments whereas the phage is very resistant to this procedure. As a result, vectors without a trypsin cleavage site also may be used successfully
9. Use 10 µL of the eluted phage for titration (see section "Titration")
10. Inoculate 50 mL 2 × YT-T with an overnight culture of *E. coli* XL1-Blue MRF' in 100 mL Erlenmeyer flasks and grow at 250 rpm and 37°C
11. Infect exponential growing 20 mL *E. coli* XL1-Blue MRF' culture (OD_{600} ~ 0.5, after 2–3 h) with the remaining 190 µL of the eluted phage. Incubate 30 min at 37°C without shaking and the following 30 min with 250 rpm
12. Harvest the infected bacteria by centrifugation for 10 min at 3,200× *g* in 50 mL polypropylene tubes. Resolve the pellet in 250 µL SOB-GA and plate the bacteria suspension on SOB-GA agar plates (15 cm petri dish). Grow over night at 37°
13. Pick clone colonies from these plates and perform "Production of soluble monoclonal antibody-fragments"

26.4.7 Selection in Solution

1. For all selection processes "in solution", it is obligatory that the antigen is available unlabelled and biotin labelled.
   Note: The antigen can be manually labelled with biotin using the NHS S–S Biotin system (Pierce)
2. The selection process can be performed by systematically reducing the antigen concentration (A) or by competition with free antigen or soluble antibody-fragments (B)

3. Block 50–100 µL streptavidin beads M-280 (3.25–6.5 10^8 beads) with 1.5 mL panning block for 1 h at room temperature. Use magnet particle concentrator (Dynal) for bead separation
4. Use 50 µL blocked streptavidin-beads for library preincubation (remove unwanted binders) for 30 min at room temperature. Discard beads afterwards
5. Use the preincubated library for the selection process
6. (A) Mix 1×10^{11} phage from your preincubated library with 50 nM biotinylated antigen in panning block solution. Incubate 2 h at room temperature and gently mixing using an overhead shaker.
   Note: The initial antigen concentration depends on the equilibrium dissociation constant (K_D) of your parent clone and the K_D you try to achieve by these methods. Variation in the concentration should lead to an optimal result
7. (A) Add 100 µL blocked streptavidin-beads. Perform capturing by incubating 15 min at room temperature and gently mixing in an overhead shaker
8. (A) Separate the streptavidin-beads carrying captured antibody phage from the solution in the magnetic rack
9. (A) Wash the streptavidin-beads by discarding the supernatant and adding fresh washing buffer. Each step 1–2 min at room temperature, using an overhead shaker, bead separation with magnetic rack. Perform washing steps: 3× PBS-T, 2× MPBS-T, 2× PBS, 1× MPBS, 2× PBS
10. (A) Elute captured phage by adding 500 µL trypsin, incubate for 30 min, 37°C. Separate and discard the SA-beads, use supernatant for titration (see section "Titration") and the production of new phage (start at section "Library packaging": step 4, by adding the whole eluted phage to the cells)
11. (A) Start the next round by repeating the complete procedure with the newly produced phage particles. Use reduced amount of biotinylated antigen (e.g., 5 nM). The amount of SA-beads can also be reduced to 50 µL (3.25×10^8 beads)
12. (A) Perform up to 4 selection rounds (if desired). Reduce antigen amount round by round.
    Note: The final antigen concentration should be max. 10 times less than the desired K_D for improved binders (e.g., Choose 0.5 nM antigen, when binders with K_D around 5 nM are expected)
13. (A) Pick colonies from the plates you use for titration and perform "Production of soluble monoclonal AB-Fragments"
14. (B) Perform library and bead blocking as mentioned in points (3–5)
15. (B) Mix 1×10^{12} phage particles from the library with 1 nM biotinylated antigen (Note 15) in 1.5 mL panning block
16. (B) Incubate 2 h at room temperature and gently mix in an overhead shaker
17. Add up to 1 mM (max. factor 1,000) of the unbiotinylated antigen OR the parental antibody fragment
18. Incubate 1 week at 4°C and gently mixing in an overhead shaker to equilibrate the system
19. Wash the beads with 3× panning block solution, 4× PBS-T (the washing steps can be increased, if necessary)
20. Elute the phage as mentioned in point 10 (A)

21. Perfom titration and pick monoclonals for "Production of soluble monoclonal antibody fragments"

26.4.8 Production of Soluble Monoclonal Antibody Fragments

1. Fill each well of a 96 well U-bottom polypropylene microtitre plate with 150 µL 2 × YT-GA medium
2. Pick 96 clones with sterile tips from the desired panning round and inoculate each well. Seal the plate with a breathable sealing film.
   Note: We recommend to pick only 92 clones. Use the wells H3, H6, H9 and H12 for controls. H3 and H6 are negative controls – these wells will not be inoculated. Inoculate the wells H9 and H12 with the "parental" clone containing the phagemid encoding for the antibody fragment. Therefore, the wells H9 and H12 are coated with the corresponding antigen
3. Incubate over night in a microtiter plate shaker (e.g., Thermo Shaker PST-60HL-4, Lab4You, Germany) at 37°C and 1,000 rpm
4. (A) Fill a new 96 well polypropylene microtiter plate with 150 µL 2 × YT-GA and add 10 µL of the over night cultures. Incubate for 2 h at 37°C and 1,000 rpm. (B) Add 30 µL glycerol solution to the remaining 140 µL over night cultures. Mix by pipetting and store this master plate at −80°C
5. Pellet the bacteria in the microtiter plates by centrifugation for 10 min at 3,200× *g* and 4°C. Remove 180 µL glucose containing media by carefully pipetting (do not disturb the pellet)
6. Add 180 µL 2 × YT-A with 50 µM IPTG and incubate over night at 30°C and 1,000 rpm. Seal the plate with a breathable sealing film.
   Note: The appropriate IPTG concentration for induction depends on the vector design. A concentration of 50 µM was well suited for vectors with an Lac promoter like pSEX81 (Welschoff et al. 1997), pIT2 (Goletz et al. 2002), pHENIX (Finnern et al. 1997) and pHAL14 (Hust et al. 2007b, Pelat et al. 2007, Kirsch et al. 2008). The method for the production of soluble antibodies works with vectors with (e.g., pHAL14) and without (e.g., pSEX81) an amber stop codon between antibody fragment and gIII. If the vector has no amber stop codon the antibody::pIII fusion protein will be produced and may be used instead successfully (Mersmann et al. 1998)
7. Pellet the bacteria by centrifugation for 10 min at 3,200× *g* in the microtiter plates. Transfer the antibody fragment containing supernatant to a new polypropylene microtiter plate and store at 4°C

26.4.9 ELISA of Soluble Monoclonal Antibody Fragments

1. To analyse the antigen specificity of the soluble monoclonal antibody fragments, coat 100 ng antigen per well in carbonate buffer (pH 9.7) or PBS (pH 7.4) over night at 4°C in a microtiter plate

2. Wash the coated microtiter plate wells 3× with PBST
3. Block the antigen coated wells with MPBS-T for 1 h at room temperature. The wells must be completely filled
4. Fill 90 µL MPBS-T in each well and add 10 µL of antibody solution. Incubate for 1 h at room temperature
5. Wash the microtiter plate wells 3× with PBS-T
6. Incubate 100 µL α-tag antibody solution for 1 h (appropriate dilution in MPBS-T) at room temperature
7. Wash the microtiter plate wells 3× with PBS-T
8. Incubate 100 µL goat α-mouse HRP conjugate (1:10,000 in MPBS-T) 1 h at room temperature
9. Wash the microtiter plate wells 3× with PBS-T
10. Shortly before use, mix 20 parts TMB solution A and 1 part TMB solution B. Add 100 µL of the prepared TMB solution into each well and incubate for 1–15 min
11. Stop the colour reaction by adding 100 µL 0.5 M sulphuric acid. The colour turns from blue to yellow
12. Measure the extinction at 450 nm in an ELISA reader
13. Identify positive candidates
14. DNA sequencing of antibody clones allows to get rid of duplicate clones

References

Coia G, Hudson PJ, Irving RA (2001) Protein affinity maturation in vivo using E. coli mutator cells. J Immunol Methods 251:187–193

Evans D, Das R (2005) Monoclonal Antibodies: Evolving into a $30 Billion Market. DataMonitor, London, UK

Finnern R, Pedrollo E, Fisch I, Wieslander J, Marks JD, Lockwood CM, Ouwehand WH (1997) Human autoimmune anti-proteinase 3 scFv from a phage display library. Clin Exp Immunol 107:269–281

Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME (1985) Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J Immunol Methods 77:305–319

Fujii R, Kitaoka M, Hayashi K (2004) One-step random mutagenesis by error-prone rolling circle amplification. Nucleic Acids Res 32:e145

Goletz A, Cristensen PA, Kristensen P, Blohm D, Tomlinson I, Winter G, Karsten U (2002) Selection of large diversities of anti idiotypic antibody fragments by phage display. J Mol Biol 315:1087–1097

Hust M, Dübel S (2004) Mating antibody phage display with proteomics. Trends Biotechnol 22:8–14

Hust M, Dübel S, Schirrmann T (2007a) Selection of recombinant antibodies from antibody gene libraries. Gene Function Analysis, Ed: Ochs, M.F. Meth Mol Biol 408:241–254

Hust M, Toleikis L, Dübel S (2007b) Antibody phage display. In: Dübel S (ed) Handbook of therapeutic antibodies. Willey-VCH, Weinheim

Hust M, Steinwand M, Al-Halabil L, Helmsing S, Schirrmann T, Dübel S (2009) Improved microtiter plate production of single chain Fv fragments in *Escherichia coli*. N Biotechnol 25(6):424–428

Irving RA, Kortt AA, Hudson PJ (1996) Affinity maturation of recombinant antibodies using E. coli mutator cells. Immunotechnology 2:127–143

Kirsch MI, Hülseweh B, Nacke C, Rülker T, Schirrmann T, Marschall H-J, Hust M, Dübel S (2008) Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV). BMC-Biotechnology 8:66

Konthur Z, Hust M, Dübel S (2005) Perspectives for systematic in vitro antibody generation. Gene 364:19–29

Low NM, Holliger PH, Winter G (1996) Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. J Mol Biol 260:359–368

Martineau P (2002) Error-prone polymerase chain reaction for modification of scFvs. Methods Mol Biol 178:287–294

Mersmann M, Schmidt A, Tesar M, Schoneberg A, Welschof M, Kipriyanov S, Terness P, Little M, Pfizenmaier K, Moosmayer D (1998) Monitoring of scFv selected by phage display using detection of scFv-pIII fusion proteins in a microtiter scale assay. J Immunol Meth 220:51–58

Pelat T, Hust M, Laffly E, Condemine F, Bottex C, Vidal D, Lefranc M, Dübel S, Thullier P (2007) High-affinity, human antibody-like antibody fragment (single-chain variable fragment) neutralizing the lethal factor (LF) of Bacillus anthracis by inhibiting protective antigen-LF complex formation. Antimicrob Agents Chemother 51:2758–2764

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Sblattero D, Bradbury A (2000) Exploiting recombination in single bacteria to make large phage antibody libraries. Nat Biotech 18:75–80

Schier R, Bye J, Apell G, McCall A, Adams GP, Malmqvist M, Weiner LM, Marks JD (1996) Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. J Mol Biol 255:28–43

Tindall KR, Kunkel TA (1988) Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. Biochemistry 27:6008–6013

Wassaf D, Kuang G, Kopacz K, Wu Q, Nguyen Q, Toews M, Cosic J, Jacques J, Wiltshire S, Lambert J, Pazmany CC, Hogan S, Ladner RC, Nixon AE, Sexton DJ (2006) High-throughput affinity ranking of antibodies using surface plasmon resonance microarrays. Anal Biochem 351:241–253

Welschof M, Terness P, Kipriyanov S, Stanescu D, Breitling F, Dörsam H, Dübel S, Little M, Opelz G (1997) The antigen binding domain of a human IgG-anti-F(ab')2 autoantibody. Proc Natl Acad Sci USA 94:1902–1907

Yau KYF, Dubuc G, Li S, Hirama T, Mackenzie CR, Jermutus L, Hall JC, Tanha J (2005) Affinity maturation of a V(H)H by mutational hotspot randomization. J Immunol Methods 297:213–224

Chapter 27
Engineering of the Fc Region for Improved PK (FcRn Interaction)

Vania E. Kenanova, Tove Olafsen, Jan T. Andersen, Inger Sandlie, and Anna M. Wu

27.1 Introduction

Interest in developing antibody drugs continues to grow, proven by the number of antibody products (over 20 antibodies, antibody fragments, and antibody-conjugate proteins) approved for clinical use by the U.S. Food and Drug Administration. The pharmacokinetic (PK) and pharmacodynamic (PD) properties of antibodies are crucial for achieving their effect, in both imaging and therapeutic applications. It is important to note that this class of macromolecules is characterized by PK and PD that are often more complex than those exhibited by small-molecule drugs. Specifically, antibodies possess non-linear distribution and metabolism (Fig. 27.1). Because of their large molecular mass and high polarity, immunoglobulins extravasate mostly through convective transport (movement of antibody with fluid flow from blood to tissue (Covell et al. 1986; Baxter et al. 1994; Flessner et al. 1997), as well as receptor-mediated or fluid-phase endocytosis. Receptor-mediated endocytosis occurs through binding of immunoglobulins to Fcγ receptors (FcγRI, FcγRII, and FcγRIII), expressed by a variety of cells (B-lymphocytes, macrophages, monocytes), or by internalization of antibody bound to a membrane antigen (e.g., Her2/neu). Nevertheless, research suggests that the majority of antibody extravasation occurs through fluid-phase pinocytosis of antibody into vascular endothelial cells (Lobo et al. 2004) (Fig. 27.2a). Specifically, when antibodies enter the acidic environment of endosomes (pH ~ 6.5), the affinity of

V.E. Kenanova, T. Olafsen, and A.M. Wu (✉)
Crump Institute for Molecular Imaging, Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at University of California Los Angeles, 570 Westwood Plaza, Los Angeles, CA 90095, USA
e-mail: vkenanova@mednet.ucla.edu; tolafsen@mednet.ucla.edu; awu@mednet.ucla.edu
J.T. Andersen and I. Sandlie
Department of Molecular Biosciences and Centre for Immune Regulation, University of Oslo, Olso, Norway
e-mail: j.t.andersen@imbv.uio.no; inger.sandlie@imbv.uio.no

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_27,

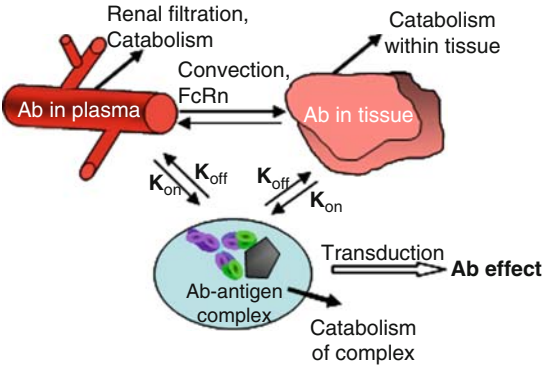


Fig. 27.1 Diagram of antibody pharmacokinetics and pharmacodynamics. For many antibodies used as drugs, the primary parameters affecting their bioavailability and distribution include (**a**) the rates of antibody movement from blood into tissues and back from tissues into blood (through the lymphatic circulation) – convection, (**b**) FcRn-mediated transcytosis or recycling, (**c**) interaction with antigen targets, and (**d**) catabolism. Antigen binding is often characterized with a second order rate constant of association (k_{on}) and a first order rate constant of antibody−antigen complex dissociation (k_{off})

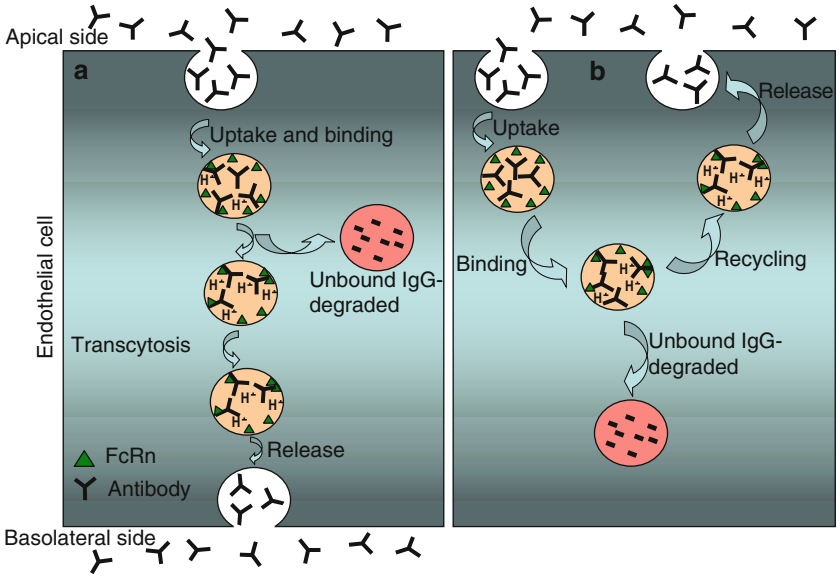


Fig. 27.2 Schematic representation of IgG trafficking. (**a**) IgGs in the circulation are non-specifically endocytosed by endothelial cells. Endocytosed IgG molecules bind unoccupied FcRn receptors in the acidified endosomes (pH ~ 6.5). Unbound IgG is degraded in lysosomes. FcRn bound IgGs transcytose and are released in the interstitial space (pH ~ 7.4). The same process occurs in the opposite direction (bidirectional transcytosis) to return IgGs from tissues/interstitial space back to the general circulation. (**b**) Pinocytosed IgGs bind free FcRn in the endosome, which recycles back to the circulation, where IgGs are released. IgGs that did not bind the FcRn are catabolized

IgG for the Brambell receptor, FcRn (the Fc-receptor of the neonate), increases. FcRn interacts with the constant Fc region of IgG in a pH dependent fashion, binding at acidic pH and release or no binding at physiologic pH 7.4 (Burmeister et al. 1994). While in a complex with FcRn, IgGs escape from intracellular degradation. When the endosome transcytoses to the basolateral side of the cell, IgGs are released into the interstitial space.

How are antibodies degraded? Because of their large molecular weight (~150 kDa), only a minute quantity of intact immunoglobulin is filtered by the kidneys, and even less is excreted in the urine. Smaller antibody fragments (e.g., scFv, Fab, diabody) are filtered, but the protein is mostly re-absorbed and/or metabolized by the proximal tubule cells of the kidneys. Catabolism accounts for most antibody clearance (Waldmann and Strober 1969) (Fig. 27.1). The serum half-life of IgGs (~ 23 days) is significantly longer compared with other immunoglobulin isotypes (2.5–6 days) (Lobo et al. 2004), and it is now known that their persistence is directly related to their binding affinity for FcRn (Ghetie et al. 1997; Dall'Acqua et al. 2002). Figure 27.2b illustrates the role of FcRn in IgG catabolism. IgG enters acidified endosomes of catabolic cells by fluid phase pinocytosis, where the low pH triggers binding to FcRn. Subsequently, IgG in complex with FcRn is recycled to the cell surface where exposure to the physiologic pH of the blood initiates release into the circulation. Unbound IgGs proceed to the lysosome where they undergo proteolytic digestion. Recent reports strongly support the notion that vascular endothelium, in addition to hematopoietic cells, is the principle site for FcRn mediated recycling (Akilesh et al. 2007; Qiao et al. 2008; Montoyo et al. 2009). Furthermore, FcRn is expressed in both mouse and human renal epithelial cells, where it clears IgG from the glomerular basement membrane (Haymann et al. 2000; Akilesh et al. 2008). Therefore, FcRn probably plays an important role in the re-absorbtion of filtered immunoglobulins, explaining the minor role that urinary excretion plays in IgG elimination.

As the Fc portion of IgG is the region of the molecule interacting with FcRn (Fig. 27.3), introduction of specific Fc mutations is strategized to increase the half-lives of therapeutic IgGs by engineering antibodies with augmented affinity for FcRn (Dall'Acqua et al. 2006; Hinton et al. 2006; Petkova et al. 2006). In contrast, when antibodies are used to deliver a cytotoxic payload (radionuclide, drug, toxin,

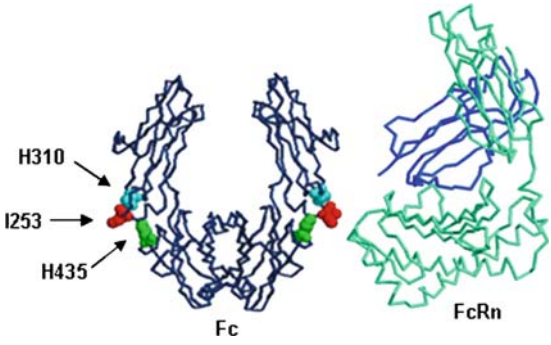


Fig. 27.3 Rasmol generated models of human IgG1 Fc and human FcRn (Fc binding site). The Fc chains are colored in *dark blue*. I253, H310, and H435 amino acid residues are depicted in *red, light blue* and *green*, respectively. The β2m is *blue*, and the FcRn chain is *light green*

etc.), reduction in serum half-life by decreasing the affinity for FcRn is sought in order to minimize systemic toxicity. In imaging, antibodies with faster serum clearance kinetics are also preferred as high-contrast images can be acquired at earlier time points. Therefore, manipulation of the Fc−FcRn interaction gives a rare opportunity for achieving the optimal PK for any desired application.

Several approaches, involving mutated derivatives of human and mouse IgG/Fc fragments in binding studies, have led to localization of the interaction site on IgG for FcRn (Kim et al. 1994; Raghavan et al. 1995; Medesan et al. 1996; Medesan et al. 1997). These studies have identified several conserved amino acids (e.g., I253 and H310; Fig. 27.3) at the C_H2-C_H3 domain interface that play a critical role in the interaction of IgGs with FcRn. We have also confirmed this conclusion in a study where a single chain Fv-Fc (scFv-Fc) antibody fragment with an I253 to A mutation showed longer serum half-life than the H310 to A mutant (Fig. 27.4). Nevertheless, both I253A and H310A mutants persisted in serum shorter than the wild type (WT), non-mutated scFv-Fc (Kenanova et al. 2005). In different functional studies in mice, mutation of H435 to A resulted in a loss of FcRn binding, while the simultaneous mutation of H433 and N434 to A had a moderate effect (Kim et al. 1994), explained by perturbing the critical H435 residue conformation. Furthermore, there is a sequence deviation of human IgG1 and human IgG3 (G3m,s^-,t^- allotype) at position 435 (H in IgG1; R in G3m,s^-,t^- allotype of IgG3). We have shown that a

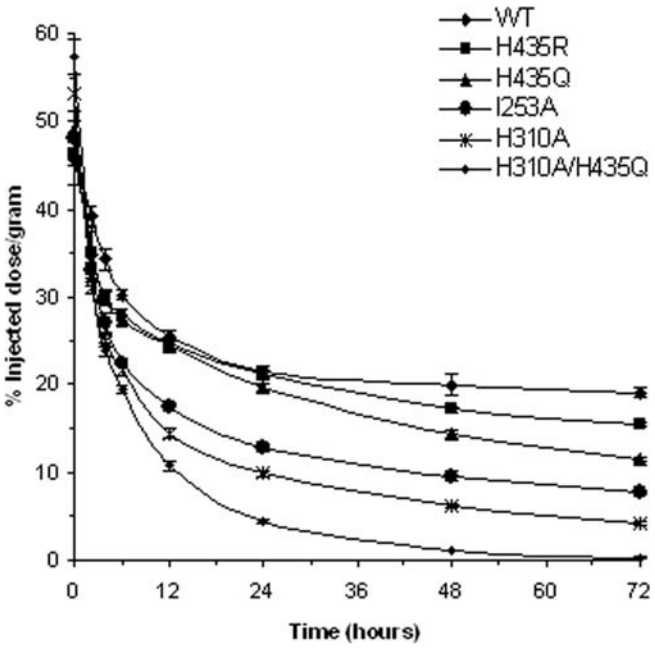


Fig. 27.4 Activity curves of the serum persistence of radioiodinated scFv-Fc WT and five variants (with Fc mutations) as a function of time in Balb/c mice. These data have been used to generate the serum half-life of each protein

scFv-Fc fragment with H435 mutated to R persisted in the mouse circulation shorter than the WT scFv-Fc but longer than the H435 to Q variant (Fig. 27.4) (Kenanova et al. 2005). The most dramatic effect was achieved when both H435 and H310 were mutated to generate the scFv-Fc H435Q/H310A double mutant. In Balb/c mice, the serum half-life of the double mutant was 8 h, compared to 289 h (12 days) for the non-mutated protein. Taken together, we and others have shown that careful manipulation of the Fc−FcRn interaction can be used as a fine control of antibody PK.

This chapter focuses on the steps required to produce antibodies, antibody fragments, or fusion proteins (containing an Fc region) with perturbed affinity for the FcRn receptor, resulting in altered *in vivo* PK.

27.2 Materials

27.2.1 Mutagenesis of the Fc Gene

27.2.1.1 Mutagenic Oligonucleotide Design Instructions

The instructions for designing mutation primers given in this chapter follow closely the mutagenic primer design section of Stratagene's QuickChange XL site-directed mutagenesis kit instruction manual, which can be accessed at http://www.stratagene.com/manuals/200516.pdf. Stratagene also offers web-based primer design software, specifically developed for optimizing the design of mutagenic primers for use with their QuickChange site-directed mutagenesis kit. The program offers additional primer design parameters, such as free energy, mismatch, and codon replacement strategies. To use this program, go to http://labtools.stratagene.com/QC.

- Both of the mutagenic oligonucleotide primers (forward and reverse) should contain the desired mutation and should anneal to the same sequence on opposite strands of the plasmid template.
- The length of primers must be between 25 and 45 bases, with a melting temperature (T_m) $\geq$ 78°C. The formula below can be used for estimating the T_m of the primers:

$$T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$$

N is the number of bases composing the primer.
Percentage GC is calculated by taking the total number of G and C nucleotides and dividing it by the total number of bases. The product is multiplied by 100 and the result is rounded to a whole number.
Percentage mismatch is calculated by taking the number of bases that are being changed from the original template sequence and dividing it by the total number of bases. The product is multiplied by 100, and the result is rounded to a whole number.

- The desired mutation (point mutation or insertion) should be in the middle of the primer, flanked by 10–15 bases of non-mutated sequence on both ends.
- The GC content of the primers should be minimum 40%. The ends should also be made of one or more G or C bases.
- Primer concentration should always be kept in excess, even when the template DNA concentration is varied.
- Primers do not need to be 5′-phophorylated but should be purified either by polyacrylamide gel electrophoresis (PAGE) or by high performance liquid chromatography (HPLC). Inadequate purification of the primers will lead to significant decrease in mutation efficiency.
- Using a smaller vector, such as pUC19 plasmid for the mutagenesis reaction is recommended to avoid problems with amplification and fidelity.

For example, the mutagenesis primers introducing I253 to A, H310 to A, H435 to Q, and H435 to R mutations in the scFv-Fc antibody fragment were designed as follows, with their corresponding T_m:

Forward	I253A:	5′ acccaaggacaccctcat**ggc**ctcccggacccctgag 3′	$T_m = 85.6°C$
Reverse	I253A:	5′ ctcaggggtccgggag**gcc**atgagggtgtccttgggt 3′	
F	H310A:	5′ gtcctcaccgtcctg**gc**ccaggactggttgaatg 3′	$T_m = 81.2°C$
R	H310A:	5′ cattcaaccagtcctgg**gc**caggacggtgaggac 3′	
F	H435Q:	5′ gaggctctgcacaacca**g**tacacgcagaag 3′	$T_m = 85.7°C$
R	H435Q:	5′ cttctgcgtgta**c**tggttgtgcagagcctc 3′	
F	H435R:	5′ gaggctctgcacaac**agg**tacacgcagaag 3′	$T_m = 91.5°C$
R	H435R:	5′ cttctgcgtgta**cct**gttgtgcagagcctc 3′	

27.2.1.2 Reagents and Equipment

- Circular plasmid containing the antibody Fc gene
- QuickChange site-directed mutagenesis kit (Stratagene), containing high fidelity *PfuTurbo* DNA polymerase with proof reading activity, 10× reaction buffer, dNTP mixture, *Dpn*I restriction enzyme, XL-1-Blue supercompetent Escherichia coli (*E. coli*) cells, and reagents for the control reactions
- DNA primers for mutagenesis (125 ng/μL)
- Chemically competent *E. coli* cells (e.g., DH5α (Invitrogen))
- DNA plasmid isolation and purification kit (e.g, Qiaprep mini- and midiprep kits (Qiagen))
- SOC medium (Invitrogen)
- SeaKem LE agarose (Cambrex BioSciences, Rockland)
- Ampicillin (Sigma)
- LB Broth Miller (USBiological)
- Agar (USBiological)
- 6 × DNA loading buffer (Promega)
- Ethidium bromide (10 mg/mL) solution (Sigma) ! Caution – review MSDS before use
- Hand-held pipettes

- 0.2 mL thin-walled PCR tubes (Roche Diagnostics)
- Petri dishes (USA Scientific)
- Waterbaths or heatblocks preset to 37 and 42°C
- Shaker with temperature and rpm controls (New Brunswick Scientific)
- Bench top centrifuge for 1.6 and 0.5 mL Eppendorf tubes (Thermo Electron).
- Thermal cycler, preferably one with a heated top (PTC-100^{TM} Peltier Thermal Cycler, MJ Research)
- Agarose gel-running apparatus (Embi Tec)
- Ultraviolet (UV) transilluminator ! Caution – Wear a protective shield to prevent damage to eyes and skin
- CCD camera for taking gel pictures

27.2.1.3 Solutions/Media to Prepare

50 × TAE running buffer (500 mL) for agarose gels:

Tris	121 g
Na_2EDTA	18.6 g

Dissolve in about 400 mL diH_2O

Glacial acetic acid	28.6 mL

Bring final volume to 500 mL with diH_2O

Dilute 50 × TAE to 1 × working solution with diH_2O

Luria-Bertani (LB) – ampicillin medium:

Dissolve 25 g of LB Broth Miller in 1,000 mL of diH_2O. Autoclave at 121°C for 15 min, cool to 50°C, and add ampicillin to 100 μg/mL final concentration.

LB-ampicillin agar plates:

Add 25 g of LB Broth Miller and 15 g of agar to 1,000 mL of diH_2O. Autoclave at 121°C for 15 min, cool down to 50°C, and add ampicillin to 100 μg/mL final concentration. Aliquot about 20 mL of solution into each 10 cm Petri dish. Once the agar solidifies, store the plates at 4°C for up to 3 weeks.

Note: For detailed protocols on stable transfection by electroporation, screening and expansion of expressing clones, and purification and biochemical characterization, refer to Sect. 6.3.4–6.3.6.

27.2.2 Binding Studies Using Surface Plasmon Resonance (SPR)

27.2.2.1 Reagents and Equipment

- Recombinant soluble human FcRn (shFcRn) protein (Berntzen et al. 2005; Andersen et al. 2008a)
- Purified antibody Fc protein for binding studies (0.5 μM), suspended in phosphate buffer at pH 6.0 or pH 7.4
- Buffer pH 7.4 (HBS/EP buffer, 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, pH 7.4) (GE Healthcare)
- Buffer pH 6.0 (67 mM phosphate buffer, 0.15 M NaCl, 0.05% Tween20, pH 6.0).
- 10 mM Sodium acetate, pH 5.0 (GE Healthcare)
- CM5 dextran based sensor chips (GE Healthcare)

- N-Hydroxysuccinimide (NHS) coupling solution (GE Healthcare)
- 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) solution (GE Healthcare)
- 1.0 M Ethanolamine-HCl, pH 8.5 solution (GE Healthcare)
- Biacore 3000 instrument, including BIAevaluation software 4.1 (GE Healthcare)
  Note: If you only need to determine the serum half-life of the antibody Fc mutant(s), it is recommended to use Balb/c mice (no tumors), as they are immunecompetent and the absence of T cells (athymic nude mice) alters the serum persistence of injected antibodies. For detailed protocol regarding establishment of tumor xenografts in athymic nude mice and tumor targeting *in vivo*, refer to Sect. 37.3.1.

27.2.3 Radioiodination and Biodistribution

27.2.3.1 Reagents and Equipment

- ^{125}I- or ^{131}I-labeled Sodium Iodide (ICN Biomedicals, PerkinElmer Life Sciences) ! Caution – review safety protocols regarding handling and disposal of radioactivity and radioiodine in particular. A specialized iodination chamber and appropriate shielding should be used.
- Iodogen (1,3,4,6-tetrachloro-3α-6α-diphenylglycouril) (Pierce)
- Acetone (Fisher)
- Hamilton gas-tight syringes (Hamilton)
- Certified iodination hood
- Kimble conical glass vial, 3 mL, 20 mm top (Fisher, Cat. No. 06-100-2E)
- Kimble PTFE/butyl stopper with Al-seal, 20 mm (Fisher, Cat. No. 03-340-71H)
- Wheaton crimping tool for 20 mm Al-seals (Chiron Scientific)
- ITLC Tec-Control kit (Biodex Medical System)
- Dose calibrator (Biodex Medial Systems)
- Gamma counter, test tubes, and caps (PerkinElmer)
- Disposable insulin syringes, 1.0 mL (Becton Dickinson)
- Inhalation anesthetic (e.g., isoflurane (Abbott))
- Mouse restrainer (Stoelting)
- Mice (Balb/c (Charles River))
- Dissection instruments
- Wheaton vials for tissue counting

27.2.3.2 Setup to Prepare

Iodogen (1,3,4,6-tetrachloro-3α-6α-diphenylglycouril)-coated tubes:

Dissolve 0.5 mg Iodogen in 1 mL acetone to prepare 0.5 mg/mL solution by vortexing. Aliquot 40 μL (20 μg) into each Eppendorf tube. Slowly evaporate the acetone to complete dryness by using N_2 gas and a pipette tip while continuously

rotating the tube. This will result in the formation of a thin film of Iodogen covering the bottom portion of the tube. Alternatively, iodination beads can be used, following Thermo Scientific protocol, found at http://www.piercenet.com/files/0331dh5.pdf. Store iodogen-coated tubes in a desiccator at −20°C in sealed plastic bags. Open only when tubes are needed for radioiodination.

27.3 Protocols

27.3.1 Mutagenesis Reaction

Figure 27.5 is a schematic illustration of the oligonucleotide-directed mutagenesis reaction.

1. Prepare a bacterial culture by picking a single colony from a plate with bacteria transformed with the plasmid DNA that contains the antibody Fc gene. Grow bacteria in 15 mL LB/ampicillin media. Incubate the culture overnight in a shaking incubator at 37°C and 200 rpm.
2. The following morning, when bacteria have grown, extract plasmid DNA using a plasmid miniprep kit, according to the manufacturer's protocol. Measure the absorbance (A) of the purified DNA at 260 and 280 nm and calculate both the yield (A_{260}) and purity (A_{260}/A_{280}). Run the plasmid DNA on a 0.8% agarose gel to check for DNA integrity/degradation.
3. The mutagenesis reaction steps follow the Stratagene procedure available at http://www.stratagene.com/manuals/200516.pdf. Keep all reagents on ice. Use 0.2 mL PCR tubes for setting up the PCR reaction.
4. Follow the steps in the order listed below for preparing the PCR reaction: Prepare two negative controls. 1) Follow the steps, outlined below, but do not add the primers. Make up the 50 µL volume by adding extra H_2O; 2) Follow the steps, outlined below, but do not add a DNA template. Again, complete the 50 µL volume by adding extra H_2O. A positive control is provided in the kit.

5 μL 10 $\times$ reaction buffer
1 μL dNTP mix
1 μL (125 ng) primer 1 (forward)
1 μL (125 ng) primer 2 (reverse) $\times$ μL (20 or 50 ng) of double stranded DNA (dsDNA) template (from DNA isolation above)
Complete a final volume of 50 μL by adding DNase free H_2O.
As a last step, add 1 μL(2.5 U/μL)of *PfuTurbo* DNApolymerase
Use a heated top thermalcycler. If such is not available, add 30 μL of mineral oil to the surface of the reaction mixture to prevent evaporation.

5. Program the thermal cycler following the program below (parameters are used specifically for the scFv-Fc antibody fragment, inserted in a pUC19 vector).

Denaturation	98°C for 30 s	
Denaturation	98°C for 30 s	} × 12
Annealing	55°C for 1 min	
Polymerization	68°C for 9 min	
	4°C thereafter	

Note: Point mutations require 12 cycles, single amino acid substitutions – 16 cycles, multiple amino acid insertions or deletions – 18 cycles. Plasmid length determines the time for polymerization (e.g., 5 kb requires 5 min at 68°C).

6. Run 5 μL of a PCR product on a 0.8% agarose gel to determine if the amplification reaction was successful and which template amount (20 ng or 50 ng) yielded more amplified plasmid DNA.
7. Add 1 μL (10 U) of *Dpn*I enzyme to the PCR reactions and incubate in a water bath at 37°C for 1 h.
8. Aliquot 50 μL of competent cells that have been slowly thawed on ice into pre-chilled 1.6 mL Eppendorf tubes.
9. To each aliquot of competent cells, add 1 μL of digested DNA from each reaction (including controls). If the gel electrophoresis analysis from step 6 above has shown low DNA yield, then more digested DNA should be added to the competent cells. *Warning:* If you used mineral oil in your PCR reaction, avoid transferring it to the transformation reaction.
10. The transformation reaction (competent cells and DNA) is incubated on ice for 30 min, heat shocked at 42°C for 45 s in a water bath and transferred back on ice for 3 min.
11. Add 500 μL of room temperature (RT) SOC medium directly to the transformation reaction tubes and incubate for 1 h at 37°C in a water bath.
12. Allow two LB-ampicillin agar plates per reaction to warm up to RT (while waiting for the incubation from step 11 above).
13. Spread 50 μL of the transformation reaction on a plate. Centrifuge the remaining in the tube volume for 2 min using a bench top centrifuge. Aspirate the supernatant, leaving about 50 μL. Resuspend and spread the cells on the second plate. This step is needed in case the transformation efficiency of the reaction is low.
14. Invert plates, with the agar on top, and incubate at 37°C overnight or until colonies are big enough to be picked (> 16 h).
15. The following day, pick three colonies for DNA isolation (miniprep) and sequencing.
16. Digest the antibody Fc gene (incorporating the desired mutations) and the expression vector with the appropriate restriction enzymes and ligate the gene in the expression vector. For example, the anti-CEA scFv-Fc was subcloned into pEE12 mammalian expression vector, with *Xba*I and *EcoR*I at the 5′ and 3′ ends, respectively.

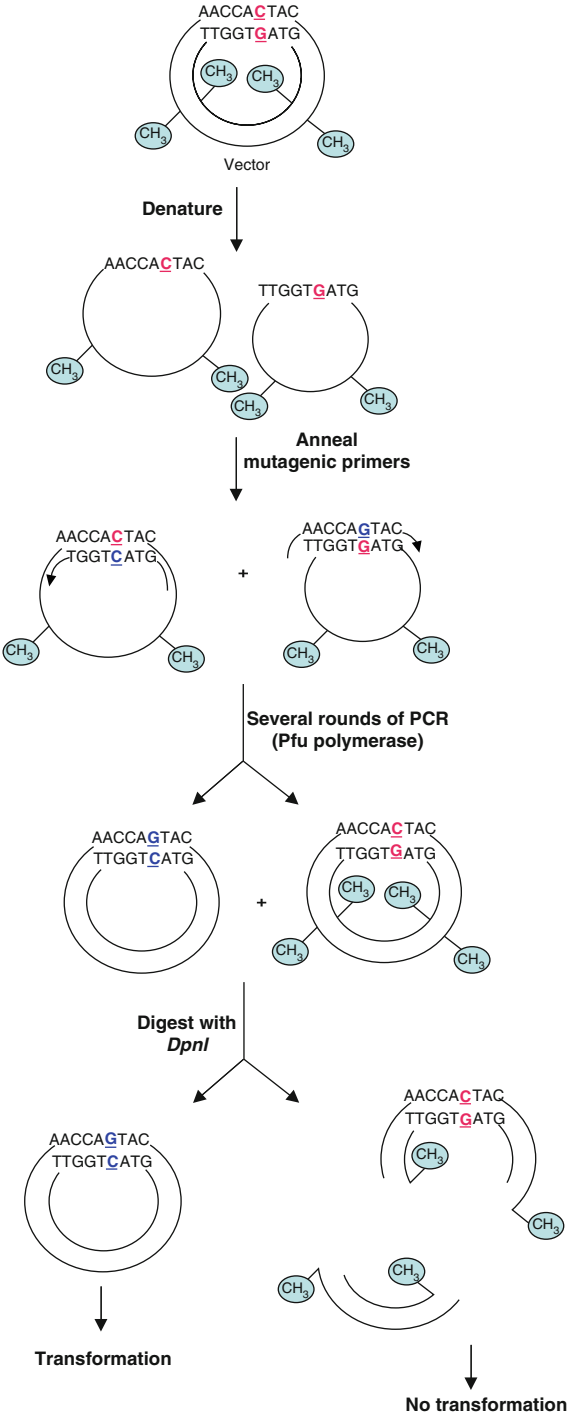


Fig. 27.5 Schematic representation of the oligonucleotide-directed mutagenesis reaction, using plasmid double stranded DNA (dsDNA). The antibody Fc gene is cloned into the plasmid vector. The dsDNA is denatured to serve as a template strand available for hybridization with the mutagenic oligonucleotides (primers). The mutant strand is synthesized by primer extension, using high fidelity *Pfu* Polymerase, from the hybridized oligonucleotides. The restriction enzyme *Dpn*I is capable of cleaving only fully and hemi-adenomethylated dam^{+} sites within DNA sequences (target sequence - 5′-Gm^{6}ATC-3′). The template DNA, isolated from dam^{+} *E. coli* strain culture is methylated. In contrast, the *in vitro*-synthesized vector/insert DNA produced in the mutagenesis reaction is not methylated. Therefore, *Dpn*I can be used to efficiently degrade the methylated, non-mutated template DNA at the end of the mutagenesis reaction, leaving only the mutated DNA for transformation into competent cells

27.3.2 Binding Studies with shFcRn Using SPR

1. Couple shFcRn protein to a CM5 sensor chip, using amine coupling chemistry and a semiautomated Surface Preparation Wizard supplied with the Biacore 3000 control software:
   (a) Prime the instrument several times with filtered and degassed HBS/EP buffer (pH 7.4).
   (b) Open the "Run Application Wizard," select "Surface Preparation" and click on Start. In the "Surface Preparation dialog box," select "immobilization." In the "Type of Sensor Chip" box, select "CM5 chip," followed by "Amine Coupling" and "Aim for Immobilization Level." Use flow cells 1 and 2 (or 3 and 4). Flow cell 2 (FC-2) is the experimental cell and flow cell 1 (FC-1) is the reference cell.
   (c) Plot in requested immobilization level (~300–1,000 RU, depending on the sensitivity needed).
   (d) Prepare the following: 120 μL NHS, 120 μL EDC, and 120 μL 1.0 M ethanolamine-HCl, pH 8.5 for each immobilization.
   (e) 200 μL of shFcRn protein (10 μg/mL) in 10 mM sodium acetate (pH 5.0) for FC2, 220 μL of HBS/EP buffer for FC1.
   (f) Place the vials with diluted solutions, in addition to empty vials, in the rack positions given by the "Surface Preparation Wizard."
   (g) Start the immobilization wizard.
2. If the immobilization level is reached, prime the system several times with the appropriate running buffer (Buffer pH 7.4 or Buffer pH 6.0).
3. Use the "Run Application Wizard" and select "Kinetic Analyzes." Inject 0.5 M antibody Fc protein in the appropriate buffer over immobilized shFcRn at a flow rate of 30–50 μL/min for 2–5 min at 25°C following the Wizard guidelines.
4. Regenerate the flow surfaces right after completion of each round of injection using Buffer pH 7.4 until baseline is reached.
5. Evaluate the binding data using the BIAevaluation 4.1 software. Zero-adjust data and subtract the reference cell (FL1) value.
6. Select "Overlay plot" to compare the relative binding of the different injected antibody Fc variants. For kinetic analyzes, serial dilutions of the antibody Fc variant must be injected at high flow rate. Analyze the binding data using the heterogeneous ligand-binding model supplemented with the BIAevaluation Wizard.
7. The immobilized CM5 chip may be stored in Buffer pH 6.0/Buffer pH 7.4 at 4°C. *Note: A*. To compare the relative binding differences of mutated antibody Fc variants with the wild type counterpart, the same shFcRn immobilized CM5 surface should be used. Furthermore, the activity of immobilized shFcRn may decrease over time, thus multiple rounds of injection of the antibody Fc variants should be performed simultaneously. *B*. Several different Biacore instruments exist on the market. The principle step-by-step immobilization

procedure described here may vary depending on the Biacore instrument that is being used.

27.3.3 Radioiodination

Work in an iodination chamber with a vent and activated charcoal filter. Have the appropriate shielding. For additional information on radioiodination of antibody fragments, please refer to Sect. 37.3.2

1. Aliquot 100–200 μg of pure protein (1–4 mg/mL) in 1× PBS into an iodogen-coated tube.
2. Cut the lid off the tube and place inside a glass vial. Close the glass vial with an Al-seal, using the crimping tool.
3. Puncture the seal few times with an 18-G syringe needle, in order to make openings for the Hamilton syringe needle.
4. Prepare potassium iodide (KI; Sigma) at a working concentration of 10 μg/mL in 0.5 M phosphate buffer (pH 8) at a ratio of 0.5 iodides per protein molecule.
5. Determine the volume of sodium iodide needed for a labeling ration with a specific activity of 1–2 μCi/μg. Withdraw the volume in a designated iodination hood using a gas-tight Hamilton syringe.
6. Pierce the Al-seal and dispense the radioactive sodium iodide into the protein solution. Incubate at RT for 5–7 min.
7. To terminate the reaction, transfer the reaction mixture into a new tube using the Hamilton syringe. Rinse the syringe in PBS and then in water.
8. Separate the radiolabeled protein from the free label by centrifuging in a multispin column containing Sephadex G-25. Prepare the column by washing it three times with 1× PBS. Apply 50 μL of labeled protein mixture to a bed volume of 0.5–0.7 mL and centrifuge for 2 min at 750× *g* at RT.
9. Measure the activity of the total recovered radiolabeled protein (void volume) using a dose calibrator.
10. Determine the purity by instant thin-layer chromatography (ITLC).
    (a) Each ITLC strip has an origin line, cut line, and solvent line. Dilute 1 μL of the radioiodinated protein 50–100 fold and spot a drop (5–10 μL) on the origin line (~ 1 cm from the bottom) of the ITLC strip. Allow to air dry for 30 s.
    (b) Place the strip in a vial filled with the solvent (0.9% NaCl solution) to a depth of 3–5 mm. Do not allow the origin to fall below the level of the solvent.
    (c) Watch the solvent front migrate up to the solvent line (~ 1 cm from the top).
    (d) Remove the strip, let it air dry, cut the strip in half at the cut line and place each half in a vial.
    (e) Count the lower half (where the radiolabeled protein should be) and the upper half (free iodine) in a dose calibrator or in a gamma well counter, using the appropriate counting energy window (15–70 keV for ^{125}I and 260–430 keV for ^{131}I).

(f) Determine the amount of incorporated radioiodine or labeling efficiency (LE) by the formula: %LE = [(activity of lower strip half)/(sum of the total activity of both halves)]x100.

27.3.4 Biodistribution

1. Dilute the radioiodinated protein in saline 0.9% NaCl/1% HSA to a concentration of 20–50 μg/mL.
2. Load the radioactive material in a 1.0 mL insulin syringe. Measure each dose using a dose calibrator and note the time of measurement. Transport syringes to the place of injection in a lead container.
3. Place the mouse in a mouse restrainer tube with its tail protruding out. Heat the tail by wrapping it in warm, wet gauze.
4. Insert the needle into the lateral vein of the tail. Slowly inject 2–10 μCi (2–5 μg) of radiolabeled protein in a volume of 200 μL.
5. Withdraw the needle and apply pressure over the wound using an alcohol swab to stop the bleeding. Inject groups of 4–5 mice for each time point of analysis.
6. Euthanize one group of mice at each time point, from 0 to 72 h by exsanguinations via cardiac puncture under terminal anesthesia. Remove as much blood as possible to decrease the blood trapped in organs. In addition, remove organs (liver, spleen, kidneys, and lungs), bone and muscle. Place each plus the mouse carcass in a separate, pre-weighed Wheaton vial.
7. Prepare two counting standards in the following way: Dilute 50 μL of the radiolabeled protein from the injection doses in 950 μL saline/1% HSA solution (1:20 dilution). Take 40 μL diluted dose into 5 mL saline and count (1:2500 dilution of the original dose). Also, count two tubes with 5 mL saline to correct for background activity.
8. Weigh tubes with tissues and measure their radioactive content using a gamma counter set to the appropriate energy window (15–70 keV for ^{125}I and 260–430 keV for ^{131}I). Decay correct and convert to percentage of injected dose per gram (% ID/g) using the standards (subtracting the background activity).
9. Fit a bi-exponential decay estimate for the blood curves and two rate constants k_1 and k_2 (ADEPTII software). $T_{1/2}$ is related to the inverse of k_1 and k_2 by the formula $T_{1/2} = 0.693/k$.
10. Compare the serum half–life of the mutated antibody Fc protein to the wild type.

27.4 Results

The amplification and template digestion should be checked by gel electrophoresis. Figure 27.6 is an example of a typical gel, though it is not necessary that both template concentrations (20 ng and 50 ng) yield a product. An undigested PCR

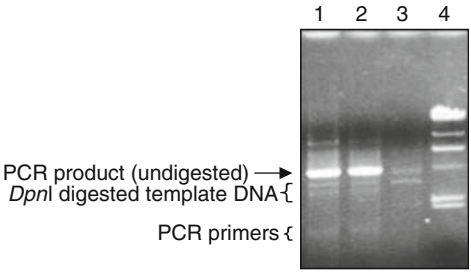


Fig. 27.6 An example gel electrophoresis image of site-directed mutagenesis reaction. Lane 1: mutagenesis reaction using 50 ng template DNA; lane 2: mutagenesis reaction using 20 ng template DNA; lane 3: negative control with template DNA only (no primers); lane 4: molecular weight standard (λDNA-*Hind*III digest; New England Biolabs)

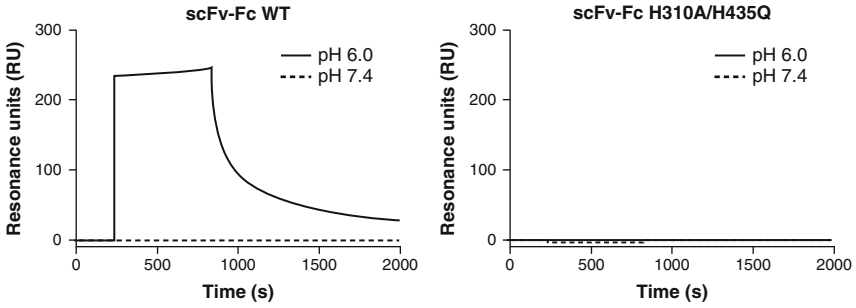


Fig. 27.7 Example of SPR analyzes of scFv-Fc WT and H310A/H435Q variant binding to immobilized shFcRn at pH 6.0 and pH 7.4 (Andersen et al. 2008b)

product should be visible (lanes 1 and 2) and should be the full size of the original template DNA (plasmid). The *Dpn*I-digested template (lanes 1, 2, and 3) may or may not be visible, depending on how much template was used in the reaction. Primer dimers are often noticeable at the bottom of the gel.

SPR is a quantitative method which can be used for assaying the binding of the antibody Fc mutant(s) to the FcRn receptor *in vitro*. The Fc mutant binding affinity for the FcRn is related to the Fc protein serum persistence *in vivo*. The sensorgrams on Fig. 27.7 are examples of different analyte-receptor binding interactions. The higher the RU values, the stronger the binding of the ligand to the immobilized receptor. The data clearly demonstrate that the WT Fc protein exhibits strong interaction with the shFcRn, whereas the H310A/H435Q mutant has lost its affinity for the receptor at acidic pH. The sensorgram of the scFv-Fc WT also demonstrates reversible, pH-dependent binding to the immobilized FcRn at pH 6.0 and no interaction at pH 7.4.

Biodistribution and clearance studies of radiolabeled antibody Fc mutants in mice are necessary to determine the effect of the Fc mutations on the overall serum persistence of the protein. An example of blood clearance data of six scFv-Fc antibody fragments (WT and five Fc mutants) is depicted on Fig. 27.4. The curves show the order of clearance and indicate how specific mutations in the Fc region of

Table 27.1 Troubleshooting of possible problems with the mutagenesis (also check Stratagene troubleshooting section at http://www.stratagene.com/manuals/200516.pdf), binding, radiolabeling and biodistribution steps

Problem	Action
If no PCR product is seen on the gel after the mutagenesis reaction or no colonies grow after transformation of the PCR product	Use different concentration of template DNA (0.7–100 ng), while keeping the primer concentration in excess (600 ng). Increase the number of cycles (e.g., from 18 to 25). Increase the time for polymerization (extension) (e.g., 5–10 min). Decrease the annealing temperature. Use a different batch of dNTPs, as frequent freezing/thawing may have damaged them. Prepare aliquots to prevent dNTP inactivation. Run a sample from the PCR reaction before *Dpn*I digestion. If you do not see anything, then the template DNA is degraded. Repeat the protocol including 5% DMSO in the reaction mix. DMSO helps in strand separation of GC rich portions of DNA. If the plasmid-insert DNA is very long (e.g., 12 kb), include "non-mutated sequence" primers (with 5' phosphate) that anneal to the plasmid every 1.5–2 kb. If there are partial repeats in the primers (due to primer sequence), introduce 1 or 2 silent mutations. Disadvantage – must check more clones to get the correct sequence. Ensure that the positive control, provided with the kit, is working. If no colonies grow after transformation of the control, then check if the primer design is correct.
Lack of binding activity upon injection of the antibody Fc (SPR measurement)	Try immobilizing a higher amount of shFcRn. Avoid acidic solutions (especially during regeneration steps), as this will affect the activity of immobilized shFcRn. The purity of immobilized shFcRn must be >95%. Immobilization of impurities will decrease the observed binding activity of the surface.
Abnormal sensorgram profiles	The purity and composition of injected antibody Fc variants must be >95% and aggregates must be removed.
Non-specific binding	Always include a reference surface as a negative control to correct for bulk refractive index changes due to difference between the analyte and running buffer composition.
The reference curve during injection appears to be increasing, or the response does not return to baseline at the end of the injection	The reference sensorgram should have a rectangular shape. If this is not the case, then non-specific binding is occurring. Use a

(*continued*)

Table 27.1 (continued)

Problem	Action
	different sensor chip (e.g., CM4 with less carboxymethyl groups and overall less negative charge).
	Increasing the salt concentration of the analyte and running buffers may be beneficial.
	Do not use acidic solution to regenerate the surface with immobilized shFcRn, as this will destroy the functionality of the receptor.
Binding of the antibody Fc variant to the dextran	Include soluble dextran (~1 mg/mL) in the analyte and running buffers.
	Use a different chip (e.g., CM3 with a dextran length that is 1/3 the length of sensor chip CM5; CM1 does not contain any dextran).
	Increase the amount of detergent, such as surfactant P20 or Tween20 in the running buffer and use salts other than NaCl.
Other technical problems	Use the troubleshooting guidelines supplied with the Biacore instrument and software Wizard.
The radioactivity in the blood decreases rapidly after injection	Check the activity of the protein after radioiodination. If the radioiodinated WT Fc protein does not bind shFcRn at pH 6.0, a different radiolabeling method should be used (e.g., DOTA-radiometal).
	Check the labeling efficiency of the protein. If most injected radiolabel is free (not bound to the protein), it will quickly be excreted in the urine.

antibodies affect their serum half–life. The scFv-Fc order of serum clearance from the slowest to the fastest clearing fragment in immunecompetent mice is as follows: WT > H435R > H435Q > I253A > H310A > H310A/H435Q.

Table 27.1 is a troubleshooting guide, which may be helpful in providing possible solutions of common problems with the mutagenesis reaction, as well as the SPR binding studies, radioiodination of the purified protein, and biodistribution.

27.5 Conclusion

Potential limitations of using mice as preclinical models for the analysis of engineered antibodies exist. Although human and mouse FcRn share substantial sequence homology, mouse FcRn exhibits a much broader binding specificity relative to human FcRn (Ober et al. 2001; Roopenian and Akilesh 2007). Therefore, additional experiments are required to confirm the validity of the data as well as the degree of variability between the mouse and human systems. Generation of human FcRn for binding studies (e.g., SPR) has been simplified (Berntzen et al. 2005;

Andersen et al. 2008a) and will provide preliminary data on the binding affinity of the antibody Fc mutant for the human FcRn. In addition, flow cytometry or cell-based ELISA using human FcRn expressing cells (Petkova et al. 2006) may be useful to elucidate if the generated Fc mutations comply with the strict pH requirement for FcRn binding (low pH) and release (neutral pH). Finally, a human FcRn transgenic mouse model has already been developed (Petkova et al. 2006) and will be most useful in predicting the PK of the antibody Fc mutant in a human system. To generate blood curves and determine serum half-life, small numbers of transgenic mice injected with a radiolabeled antibody (Fc mutant) can be bled serially (not sacrificed), in order to reduce the number of mice and cost. A complete biodistribution, aiming to investigate targeting, cross reactivity, or other normal organ involvement in the protein clearance can still be completed in Balb/c, athymic nude, or SCID mice.

IgG elimination may be dominated by its affinity for FcRn; however, there are other factors that must be considered. Antibodies are often selected to display very high affinity binding to specific cellular antigens. It is important to note that the interaction of the antibody with its antigen may significantly alter the overall antibody bioavailability and distribution. There is scientific evidence suggesting that this antigen−antibody interaction may play an important role in defining the overall rate of antibody elimination (Baselga et al. 1999; Bauer et al. 1999; Meijer et al. 2002; Mager et al. 2003). Therefore, antibodies with different specificities, sharing the same affinity for FcRn, may exhibit different PK and PD. In addition, factors such as the immunogenicity of the antibody (Kuus-Reichel et al. 1994), its glycosylation pattern (Wawrzynczak et al. 1992; Meier et al. 1995; Newkirk et al. 1996), and its susceptibility to proteolysis (Gillies et al. 2002) may also contribute to the rate of antibody elimination. Because of the complexity of antibody interactions and mechanisms of action, the investigation and manipulation of antibody PK/PD continue to be a challenge. Improvements have been made and there is a basis for optimism that the learned lessons about antibody PK/PD will lead to advances in the development of antibody-based drugs.

References

Akilesh S, Christianson GJ, Roopenian DC, Shaw AS (2007) Neonatal FcR expression in bone marrow-derived cells functions to protect serum IgG from catabolism. J Immunol 179:4580–4588

Akilesh S, Huber TB, Wu H, Wang G, Hartleben B, Kopp JB, Miner JH, Roopenian DC, Unanue ER, Shaw AS (2008) Podocytes use FcRn to clear IgG from the glomerular basement membrane. Proc Natl Acad Sci USA 105:967–972

Andersen JT, Justesen S, Berntzen G, Michaelsen TE, Lauvrak V, Fleckenstein B, Buus S, Sandlie I (2008a) A strategy for bacterial production of a soluble functional human neonatal Fc receptor. J Immunol Methods 331:39–49

Andersen JT, Justesen S, Fleckenstein B, Michaelsen TE, Berntzen G, Kenanova VE, Daba MB, Lauvrak V, Buus S, Sandlie I (2008b) Ligand binding and antigenic properties of a human neonatal Fc receptor with mutation of two unpaired cysteine residues. Febs J 275:4097–4110

Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L (1999) Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. Semin Oncol 26:78–83

Bauer RJ, Dedrick RL, White ML, Murray MJ, Garovoy MR (1999) Population pharmacokinetics and pharmacodynamics of the anti-CD11a antibody hu1124 in human subjects with psoriasis. J Pharmacokinet Biopharm 27:397–420

Baxter LT, Zhu H, Mackensen DG, Jain RK (1994) Physiologically based pharmacokinetic model for specific and nonspecific monoclonal antibodies and fragments in normal tissues and human tumor xenografts in nude mice. Cancer Res 54:1517–1528

Berntzen G, Lunde E, Flobakk M, Andersen JT, Lauvrak V, Sandlie I (2005) Prolonged and increased expression of soluble Fc receptors, IgG and a TCR-Ig fusion protein by transiently transfected adherent 293E cells. J Immunol Methods 298:93–104

Burmeister WP, Huber AH, Bjorkman PJ (1994) Crystal structure of the complex of rat neonatal Fc receptor with Fc. Nature 372:379–383

Covell DG, Barbet J, Holton OD, Black CD, Parker RJ, Weinstein JN (1986) Pharmacokinetics of monoclonal immunoglobulin G1, F(ab')2, and Fab' in mice. Cancer Res 46:3969–3978

Dall'Acqua WF, Woods RM, Ward ES, Palaszynski SR, Patel NK, Brewah YA, Wu H, Kiener PA, Langermann S (2002) Increasing the affinity of a human IgG1 for the neonatal Fc receptor: biological consequences. J Immunol 169:5171–5180

Dall'Acqua WF, Kiener PA, Wu H (2006) Properties of human IgG1s engineered for enhanced binding to the neonatal Fc receptor (FcRn). J Biol Chem 281:23514–23524

Flessner MF, Lofthouse J, Zakaria el-R (1997) In vivo diffusion of immunoglobulin G in muscle: effects of binding, solute exclusion, and lymphatic removal. Am J Physiol 273:H2783–H2793

Ghetie V, Popov S, Borvak J, Radu C, Matesoi D, Medesan C, Ober RJ, Ward ES (1997) Increasing the serum persistence of an IgG fragment by random mutagenesis. Nat Biotechnol 15:637–640

Gillies SD, Lo KM, Burger C, Lan Y, Dahl T, Wong WK (2002) Improved circulating half-life and efficacy of an antibody-interleukin 2 immunocytokine based on reduced intracellular proteolysis. Clin Cancer Res 8:210–216

Haymann JP, Levraud JP, Bouet S, Kappes V, Hagege J, Nguyen G, Xu Y, Rondeau E, Sraer JD (2000) Characterization and localization of the neonatal Fc receptor in adult human kidney. J Am Soc Nephrol 11:632–639

Hinton PR, Xiong JM, Johlfs MG, Tang MT, Keller S, Tsurushita N (2006) An engineered human IgG1 antibody with longer serum half-life. J Immunol 176:346–356

Kenanova V, Olafsen T, Crow DM, Sundaresan G, Subbarayan M, Carter NH, Ikle DN, Yazaki PJ, Chatziioannou AF, Gambhir SS, Williams LE, Shively JE, Colcher D, Raubitschek AA, Wu AM (2005) Tailoring the pharmacokinetics and positron emission tomography imaging properties of anti-carcinoembryonic antigen single-chain Fv-Fc antibody fragments. Cancer Res 65:622–631

Kim JK, Tsen MF, Ghetie V, Ward ES (1994) Localization of the site of the murine IgG1 molecule that is involved in binding to the murine intestinal Fc receptor. Eur J Immunol 24:2429–2434

Kuus-Reichel K, Grauer LS, Karavodin LM, Knott C, Krusemeier M, Kay NE (1994) Will immunogenicity limit the use, efficacy, and future development of therapeutic monoclonal antibodies? Clin Diagn Lab Immunol 1:365–372

Lobo ED, Hansen RJ, Balthasar JP (2004) Antibody pharmacokinetics and pharmacodynamics. J Pharm Sci 93:2645–2668

Mager DE, Mascelli MA, Kleiman NS, Fitzgerald DJ, Abernethy DR (2003) Simultaneous modeling of abciximab plasma concentrations and ex vivo pharmacodynamics in patients undergoing coronary angioplasty. J Pharmacol Exp Ther 307:969–976

Medesan C, Radu C, Kim JK, Ghetie V, Ward ES (1996) Localization of the site of the IgG molecule that regulates maternofetal transmission in mice. Eur J Immunol 26:2533–2536

Medesan C, Matesoi D, Radu C, Ghetie V, Ward ES (1997) Delineation of the amino acid residues involved in transcytosis and catabolism of mouse IgG1. J Immunol 158:2211–2217

Meier W, Gill A, Rogge M, Dabora R, Majeau GR, Oleson FB, Jones WE, Frazier D, Miatkowski K, Hochman PS (1995) Immunomodulation by LFA3TIP, an LFA-3/IgG1 fusion protein: cell line dependent glycosylation effects on pharmacokinetics and pharmacodynamic markers. Ther Immunol 2:159–171

Meijer RT, Koopmans RP, ten Berge IJ, Schellekens PT (2002) Pharmacokinetics of murine anti-human CD3 antibodies in man are determined by the disappearance of target antigen. J Pharmacol Exp Ther 300:346–353

Montoyo HP, Vaccaro C, Hafner M, Ober RJ, Mueller W, Ward ES (2009) Conditional deletion of the MHC class I-related receptor FcRn reveals the sites of IgG homeostasis in mice. Proc Natl Acad Sci USA 106:2788–2793

Newkirk MM, Novick J, Stevenson MM, Fournier MJ, Apostolakos P (1996) Differential clearance of glycoforms of IgG in normal and autoimmune-prone mice. Clin Exp Immunol 106:259–264

Ober RJ, Radu CG, Ghetie V, Ward ES (2001) Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. Int Immunol 13:1551–1559

Petkova SB, Akilesh S, Sproule TJ, Christianson GJ, Al Khabbaz H, Brown AC, Presta LG, Meng YG, Roopenian DC (2006) Enhanced half-life of genetically engineered human IgG1 antibodies in a humanized FcRn mouse model: potential application in humorally mediated autoimmune disease. Int Immunol 18:1759–1769

Qiao SW, Kobayashi K, Johansen FE, Sollid LM, Andersen JT, Milford E, Roopenian DC, Lencer WI, Blumberg RS (2008) Dependence of antibody-mediated presentation of antigen on FcRn. Proc Natl Acad Sci USA 105:9337–9342

Raghavan M, Bonagura VR, Morrison SL, Bjorkman PJ (1995) Analysis of the pH dependence of the neonatal Fc receptor/immunoglobulin G interaction using antibody and receptor variants. Biochemistry 34:14649–14657

Roopenian DC, Akilesh S (2007) FcRn: the neonatal Fc receptor comes of age. Nat Rev Immunol 7:715–725

Waldmann TA, Strober W (1969) Metabolism of immunoglobulins. Prog Allergy 13:1–110

Wawrzynczak EJ, Cumber AJ, Parnell GD, Jones PT, Winter G (1992) Blood clearance in the rat of a recombinant mouse monoclonal antibody lacking the N-linked oligosaccharide side chains of the C_H2 domains. Mol Immunol 29:213–220

Chapter 28
Antibody-Dependent Enzyme Prodrug Therapy (ADEPT)

Richard Begent, Surinder Sharma, and Kerry Chester

28.1 Introduction

28.1.1 Principles

Antibody-directed enzyme prodrug therapy (ADEPT) generates a cytotoxic drug selectively within deposits of cancer and is designed to give therapy with high levels of tumour selectivity coupled with exceptional potency. This is achieved by giving an enzyme linked to an antibody which targets it to the cancer; prodrug is then given when there are minimal enzyme levels in normal tissues but sufficient in the cancer for prodrug activation. The multi-component nature of ADEPT makes drug development more demanding than it is for developing single agents but is nevertheless being vigorously pursued, and if mastered will have broad application. This review considers how much progress there has been towards practical cancer treatment.

28.1.2 Background

Cancer is characterised by multiple genetic and epigenetic aberrations coupled with genetic instability, which confers capacity to evolve drug resistance during treatment. (Hanahan and Weinberg 2000; Jones and Baylin 2007). For many people with a common metastatic cancer, today's typical therapies are not potent enough to eradicate the diverse and evolving population of malignant cells within the limits of acceptable normal tissue toxicity. By contrast, natural immunity against cancer minimises the problem of toxicity by local tumour activation of humoral and

R. Begent (✉), S. Sharma, and K. Chester
Department of Oncology, UCL Cancer Institute, 72 Huntley Street, London WC1E 6BT, UK
e-mail: r.begent@ucl.ac.uk

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_28,

cellular mechanisms, which are too potent to be tolerable if they are activated systemically (Suntharalingam et al. 2006).

Generating drugs locally within tumours by ADEPT has the potential to deliver high-dose therapy to cancer without corresponding normal tissue damage. There is also the potential for a high proportional cell kill in a short time, diminishing the opportunity for development of drug resistance that is a feature of the more protracted regimens currently in use.

Prodrug activation by enzymes located in or around the cancer cells combines the advantages of tumour selectivity, of potency through generation of many molecules of active drug by each enzyme molecule and of a bystander effect by diffusion of drug away from the immediate site of generation. Compared with the systemic therapies that are the mainstay of current cancer therapy, toxicity can be expected to be reduced for the same level of potency or potency greatly increased for the same level of toxicity.

Although the principles of enzyme prodrug therapy are compelling, the detailed design criteria for prodrugs, enzymes and antibodies present a significant challenge. Emerging technologies have been critical for generation of recombinant antibody-enzyme molecules. Complex model systems and clinical trial designs have been needed to elucidate the function of the multiple individual components and their integration into effective therapeutic systems. We are beginning to emerge from this phase into one where investment in pre-clinical and clinical studies has a real prospect of delivering practical and effective therapeutics.

28.1.3 Designing ADEPT Systems

The first challenge is to use antibody targeting to achieve high level of enzyme in the tumour with minimal activity in the blood and other normal tissues. The second is to develop a prodrug, which is converted to a potent active drug that is retained largely within the tumour, giving high potency. The first functioning systems achieved selective anti-tumour effects in animals (Bagshawe 1989; Senter 1990) (Fig. 28.1).

28.2 Developing ADEPT

The conventional approach of determining efficacy and safety in relation to dose in cellular and animal models is unlikely to succeed because the therapy is too complex. Antibody delivery, enzyme, prodrug and drug have to work in concert and with appropriate timing. The strategy taken has therefore been to model the therapy representing the components and their relationships and to plan pre-clinical and clinical experiments on this basis. The elements that make up an ADEPT system are shown in Table 28.1.

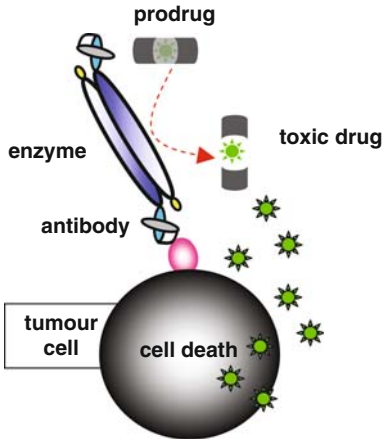


Fig. 28.1 Principles of ADEPT

Table 28.1 The elements of ADEPT

Elements of ADEPT	
Target	
Therapeutic	Targeting moiety
	Enzyme
	Prodrug
	Drug
Function in cells	
Function in animal models	
Function in clinic	

28.2.1 The Target

Requirements of the target for delivery of enzyme are:

- High tumour specificity
- Accessibility to antibody-enzyme molecules
- Abundance
- Presence on the majority of tumour cells.
- No excessive shedding into the circulation.

Characterisation of tumour cell surfaces and identification of targets using antibodies from hybridoma and phage library antibody technologies has, over the last four decades, identified antigens and yielded specific antibodies for several of the most common types of cancer and a number of rare ones.

Targets to date have been cell surface tumour-associated antigens but tumour stroma including blood vessels is a rational target as long as tumour specificity is high. Active drug generated at or near the cell membrane will need to reach its site of action for instance, with internalisation facilitated by lipophilicity. There is

evidence that this is effective for DNA-interactive drugs and that DNA cross-links distinctive of alkylating agent binding can be produced in cytotoxic amounts in tissue culture animal models and tumour biopsies from patients (Webley et al. 2001; Mayer et al. 2006). Any population of tumour cells will have a variation in the abundance of target on individual cells and delivery to cells at the lower end of the range will tend to be better if the overall abundance is high. An excess or antibody-enzyme over antigen in the circulation is required for efficient tumour targeting and levels of antigen shed into the blood need to be measured for this reason (Boxer et al. 1992).

28.2.2 The Therapeutic

28.2.2.1 Antibody

Antibodies have been used to target cancer therapy for more than a century. Problems which limit their use relate to lack of knowledge of molecular pathology and targets, low specificity, and difficulty in obtaining pure and stable products. These have progressively been overcome so that antibodies are now well established as important therapeutics in several disease areas. Hybridoma technology is one of the major advances that is giving access to pure, reproducible and homogeneous products. Another key advance is recombinant DNA technology, which makes it possible to engineer antibodies for specific functions and to select from a great diversity of antibody specificities in antibody libraries. The remaining area of important progress is in structural biology, which gives knowledge of structure/function relationships of antibodies and the surfaces with which they react. The result is that stable antibodies can be created to give high affinity binding and great selectivity, particularly for proteins in which relatively flat surfaces often limit the specificity with which small molecules can bind.

28.2.2.2 Enzymes

Many enzymes have been used to activate prodrugs and release drugs in ADEPT (see Table 28.2). Enzymes should have high catalytic activity and not have any active human counterpart to avoid endogenous activation of prodrug in normal tissues. This has led to the choice of bacterial enzymes, in particular, carboxypeptidase G2 in the clinical studies conducted to date. However, derivatives of human enzymes have also been investigated in pre-clinical models.

28.2.2.3 Antibody-Enzyme

The antibody regions for activation of complement and antibody-dependent cell cytotoxicity and maintenance of antibody in the circulation are not needed for

Table 28.2 ADEPT target and therapeutic systems

Target	Antibody and linkage	Enzyme	Prodrug/drug	Tumour	Authors
HCG	F(ab')2 conjugate	CPG2[a]	Bis-chloro mustard	Choriocarcinoma	Bagshawe
CEA	F(ab')2 conjugate	CPG2	Benzoic acid mustard	Colon (LS)	Blakey
CEA	F(ab')2 conjugate	CPG2	Bis Iodo phenol mustard (BIP)	Colon	Blakey
CEA	scFv fusion protein	CPG2	BIP	Colon	Sharma
c-erbB2	F(ab')2 conjugate	CPG2	Benzoic acid mustard	Breast	Eccles
CEA	F(ab')2 conjugate	CPG2	Benzoic acid mustard	Ovary	Sharma
CEA	F(ab') fusion protein	human βglucuronidase			Bosslet
melanotransferrin p97		bacterial beta lactamase	cephalosporin mustard	melanoma	Siemers NO
CD20		Human β glucuronidase	doxorubicin glucuronyl carbamate	lymphoma	Haisma HJ
L6	Antibody-enzyme conjugate	antibody-penicillin-G amidase conjugate	cephalosporin mitomycin	Lung, melanoma	Vrudhula VM
L6	Antibody-enzyme conjugate	βlactamase	cephalosporin mitomycin	lung, melanoma	Vrudhula VM
		bacterial glucuronidase	glucuronylated nornitrogen mustard	colon (LoVo)	Thomas M
HMFG1	HMFG1	βglucosidase	Amygdalin	Bladder	Syrigos
EPCAM		human βglucuronidase	doxorubicin glucuronyl carbamate		de Graaf M
TNT		hu βglucuronidase	doxorubicin glucuronide	mu lung	Biela BH

[a]*CPG2* carboxypeptidase G2

ADEPT, in which only the target-binding function of the Fv regions is required. This makes it rational to engineer antibody-enzyme molecules containing just the targeting function. This was achieved partially with the first product used clinically in which F(ab')2 fragments were chemically conjugated to carboxypeptidase G2. Pre-clinical studies and clinical trials with this type of molecule enabled ADEPT to become established in experimental therapeutics (Bagshawe 1989; Senter 1990; Bagshawe et al. 1995; Napier et al. 2000; Francis et al. 2002). Genetic fusion proteins, however, have advantages in terms of versatility of design possibilities, stability, homogeneity, reproducibility of the product and potential for production at a moderate cost using modern fermentation technology. Bosslet et al. (1992) produced the first example of a genetic fusion protein for ADEPT by fusing the gene for Fab' anti-carcinoembryonic antigen (CEA) antibody to human βglucuronidase.

More recently, an scFv antibody to CEA was selected from an immunised mouse phage library and shown to give selective localisation clinically. (Chester et al. 1994; Begent et al. 1996; Mayer et al. 2000). The gene for MFE-23 was fused with the gene for the enzyme carboxypeptidase G2 (CPG2) to give 'MFE-CP' fusion proteins (Michael et al. 1996), Initially this was expressed in *Escherichia coli* but yields were too low for a clinical trial and *in vivo* pharmacokinetics were not optimal, although a distinct improvement over the results obtained previously with chemical conjugates (Bhatia et al. 2000). It was then shown that expression in the eukaryote yeast, *Pichia pastoris* gave higher yields. Futhermore, the glycosylation added by the yeast as a post-translational modification gave favourable tumour localisation and pharmacokinetic properties (Medzihradszky et al. 2004; Sharma et al. 2005). A *C*-terminal hexahistidine tag (His-tag) was engineered to MFE-CP to permit purification by immobilised metal affinity chromatography (IMAC) A cartoon of MFECP1, the first MFE-CP to enter clinical trial is shown in Fig. 28.2. MFECP1 when purified, is stable over 2 years and complies with UK regulations for an investigational medicinal product (Tolner et al. 2006a, b).

Examples of targets, antibodies and enzymes used in ADEPT are given in Table 28.2.

28.2.2.4 Immunogenicity

The main disadvantage of non-human enzymes is their potential to elicit an immune response in humans. Clinical trials with a chemical conjugate of a murine F(ab')2 and the bacterial enzyme carboxypeptidase G2 showed that, even with cyclosporine immunosuppression, there was only a window of about 21 days in which enzyme could be given without formation of human anti-carboxypeptidase antibody (HACA) (Napier et al. 2000). Use of a genetic fusion protein of a murine sFv and the same enzyme was less immunogenic with only one third of patients developing HACA without immunosuppressive therapy (Mayer et al. 2006). The improved purity, homogeneity, lack of aggregation and of denatured components of the

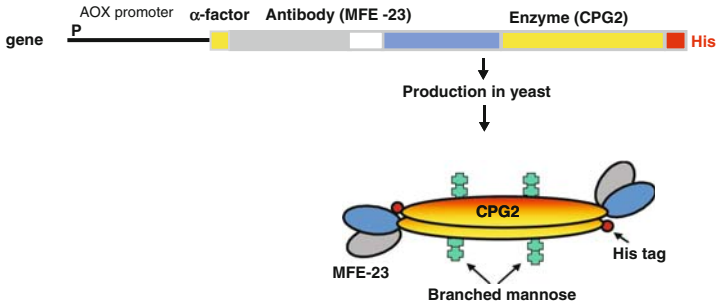


Fig. 28.2 MFECP1 is a recombinant fusion protein composed of MFE-23, an anti-CEA scFv, fused to the amino-terminus of the enzyme CPG2. MFECP1 is a homo-dimer in its native functional state. For expression in the methylotrophic yeast *Pichia pastoris*, MFECP1 is fused to the α-factor leader sequence that allows secretion of the fusion product into the medium. MFECP1 is excreted as a soluble protein during methanol fermentation

recombinant product probably contribute to this as well as the lack of antibody constant regions in the scFv. The immune response to the antibody component can probably be further reduced by use of humanised or human antibodies. For the enzyme, identification and mutation of T cell epitopes to reduce immunogenicity has shown promise with βlactamase (Harding et al. 2005) and probably has applicability to other enzymes. Identification and mutation of surface B cell epitopes of the enzyme has also been shown to be clinically relevant for the bacterial enzyme, carboxypeptidaseG2 (Spencer et al. 2002. Mayer et al. 2004). Part of the epitope concerned was at the *C*-terminus of MFE-CP, where a His-tag had been added for purification by metal affinity chromatography. Compared to HACA positive sera from previous trials (Napier et al. 2000), patients treated with MFEC-P1his enzyme also had a reduced antibody response to the *C*-terminal epitope and their polyclonal HACA response was delayed. However, it appears that multiple epitopes need to be silenced, and as with T cell epitopes, it is not clear whether this can always be attained while retaining enzyme activity.

28.2.2.5 Prodrugs and Drugs

The general structure of a prodrug comprises a trigger that provides specificity and is converted by the enzyme in ADEPT and an effector, which is the active drug or metabolite. This field has been reviewed by Senter and Springer (2001)

Consideration of the multiple genetic and epigenetic defects and genetic instability of common cancers is critical for appropriate selection of drugs to be generated locally in tumours. Drugs targeted at metabolic processes such as BCR/ABL or components of the EGFR pathway make their most effective contribution when added to cytotoxic therapy because of the development of resistance

due to genetic instability. While effective as single agents in chronic myeloid leukaemia and gastrointestinal stromal tumours, these are atypical in having relatively stable genomes and drug resistance only becomes limiting when genetic instability develops (Melo and Barnes 2007; Allander et al. 2001; Rubin et al. 2007). By contrast, cytotoxic chemotherapy can be effective in common cancers in the presence of genetic instability probably because they produce relatively non-specific cellular toxicity.

There are pre-clinical and clinical indications that increasing the concentration and duration of exposure of tumour cells to cytotoxic drugs will enhance effect, particularly with alkylating agents. This is also illustrated by studies linking dose intensity to outcome (Aapro et al. 2006) and by trials of high-dose chemotherapy with bone marrow stem cell support. The latter is established as part of the treatment of relapsed haematopoietic and germ cell malignancy (Einhorn et al. 2007). High response rates are also reported with high-dose chemotherapy in common epithelial cancers but survival gain is limited, probably by deaths caused by the therapy. For these reasons, potent alkylating agents have been chosen for clinical trials to date.

28.3 Preparing for Clinical Trials

28.3.1 Studies in Cells

28.3.1.1 Mechanism and Effect

The mechanism depends on the K_m and k_{cat} of the enzyme and its substrate, the prodrug, giving an active drug with greater potency than the prodrug. A difference of 100-fold is considered desirable but the value required for effectiveness also depends on the drug sensitivity of the tumour treated. Measurements of the IC50 of the drug are valuable in understanding this. Several enzyme prodrug systems and tumour cell types have been investigated (Table 28.2) and evidence of a selective anti-tumour effect not seen with appropriate controls shown in all of them.

28.3.1.2 Repair of Effect

Webley et al. (2001) investigated the effect of ADEPT with mustard prodrugs on production of the DNA cross-links, which are the potentially lethal lesions produced by this class of drug. They found induction of the lesions within minutes but that there was a population of surviving cells in which the cross-links appeared to have been repaired within 24 h both in tissue culture and in human colon carcinoma xenografts in nude mice.

28.3.1.3 Dose Response

Dose response has been shown in the examples above but the complexity of the relationship between enzyme, prodrug, drug and duration of exposure means that there is probably considerable opportunity for tuning of these systems for optimal efficacy. The ideal drug for ADEPT has a progressive and continuous increase in response in relation to dose. If the response has a plateau at too low a level, the advantage of local generation of drug may be lost. Alkylating agents appear particularly favourable from this point of view and examples are described above.

The discrimination between activation in the target and non-target cells depends on the relative toxicity of prodrug and drug. A ratio of 100:1 is considered desirable (Senter 1990) but the ratio of 10:1 reported for a system for treating lymphoma (Haisma et al. 1998) may be sufficient for lymphomas due to the drug sensitivity usually found in this tumour.

28.3.2 Studies in Animal Models

28.3.2.1 Target Molecule

Human tumour xenografts expressing the target that will be used in clinical trials have been widely used. The distribution of the target molecule in different xenografts may vary considerably with consequences for therapeutic effect because of failure to reach tumour cells not expressing the target. The overall concentration of target in different tumours is very variable (Pedley et al. 1990). There is theoretical relationship between the total amount of target molecule and capacity of a given amount of antibody-enzyme and prodrug to reach it in sufficient quantity for effectiveness. This may well be an issue when tumour deposits are of many hundreds of grammes in man and is not reflected in animal models where small tumour deposits are normally studied to minimise suffering to animals.

28.3.2.2 Tissue Pathology

Human tumour xenografts probably continue to exhibit genetic instability when passaged and may deviate in their behaviour from their original state. Nevertheless, they tend to emulate the pathology seen in the host of origin with areas of necrosis and haemorrhage in many carcinomas and diversity in the stroma, which is of course of murine origin. Tumour cells may remain viable within these areas and yet present diverse challenges for access of therapy and drug resistance that are pertinent to the clinical situation. They need to be understood as far as possible in assessing the effectiveness of ADEPT.

28.3.2.3 Tissue Physiology

Tumour deposits of 1 mm diameter commonly have uniform delivery of nutrients and correspondingly good access for therapeutics including gene therapy vectors and antibody-enzyme molecules. As tumour grows beyond this point, hypoxic areas appear leading to areas of necrosis and correspondingly poor access of drugs. Tumour cells in these areas are resistant to cytotoxic drugs and radiation.

28.3.2.4 Pharmaco-Kinetics and Pharmaco-Dynamics

In Bagshawe's original experiments with a choriocarcinoma xenograft, there was a major anti-tumour effect with glutamated mustard prodrug. This probably relates to the rapid blood clearance of the anti-HCG antibody-carboxypeptidase G2 conjugate giving a high tumour to blood ratio and to the high chemo-sensitivity of this tumour type. Similar results were not found in a colon carcinoma xenograft with the same enzyme and prodrug but an anti-CEA antibody and an antibody-enzyme conjugate, which cleared slowly from the blood. A low tumour to blood ratio of enzyme and the relative chemo-resistance of the colon carcinoma xenograft were probably important here.

If prodrug was given early, there was lethal toxicity attributed to enzyme-activating prodrug in the circulation and if it was given late when enzyme had cleared from the circulation. By contrast, Senter 1990 reported anti-tumour effects in a different colon carcinoma xenograft by giving a different antibody (L6) linked to alkaline phosphatise to activate etoposide phosphate when the prodrug was given only 18–24 h after conjugate. The degree of differentiation between tumour and blood enzyme was not given but there was 10 times the level of radiolabelled conjugate present in tumour compared with a non-specific antibody.

Eccles treating breast carcinoma xenografts with antibody to cErbB2 linked to carboxypeptidase G2 and a nitrogen mustard prodrug showed marked anti-tumour effects, but it was necessary to wait 12–14 days before giving the prodrug in order to obtain satisfactory tumour to blood ratios of enzyme, which were 3:1 by the time that prodrug was administered.

In a different and drug-sensitive colon carcinoma xenograft, Blakey et al. 1996 reported anti-tumour effects with the same antiCEA-carboxypeptidase conjugate used by Bagshawe but with a bis-iodophenol mustard prodrug, generating a drug of great potency and with tumour to blood enzyme ratios of 10:1 at 72 h produced effective anti-tumour therapy. This system produced DNA cross-links in tumour cells in human tumour xenografts (the lesions through which alkylating agents are cytotoxic) and in cells surviving the initial treatment, there was evidence of DNA repair within 24 h of treatment (Webley et al. 2001).

It is evident that there are substantial differences between different tumour xenografts and pharmacodynamics of antibody-enzyme molecules used in them. There is a clear need to have the highest possible tumour:normal tissue ratio of enzyme to avoid normal tissue toxicity.

The issue of tumour to normal tissue ratio of enzyme delivery was addressed by Bagshawe's group using a second antibody directed against the enzyme to accelerate clearance from the circulation without a corresponding reduction in tumour enzyme levels. This principle had already been shown to have clinical relevance in radioimmunotherapy of colorectal cancer (Begent et al. 1989). It was further developed for ADEPT by using a second antibody, which inactivated the enzyme and added galactosylatioin in order to accelerate clearance via the reticuloendothelial system. Using the system, high tumour to blood ratios of enzyme were obtained in the system that had previously proved resistant to ADEPT, and this formed the pre-clinical basis for the first ADEPT clinical trial.

The complexity of this system and difficulty in making reproducible, stable and pure chemical conjugates of antibody enzyme led to the use of genetic fusion proteins. That generated by Chester and colleagues (Sharma et al. 2005; Tolner et al. 2006a, b) with mannosylated anti-CEA-carboxypeptidase G2 was designed for rapid clearance whose mechanism was demonstrated by Kogelberg et al. 2007) to be achieved via mannose receptors, principally in the sinusoidal endothelial cells of the liver. The enzyme activity is rapidly degraded at this location but is retained in the tumour, giving tumour:blood enzyme ratios of 1000:1 or more. This product has also been taken into clinical trial.

28.3.2.5 Dose Response

Antibody-Enzyme

For each of the systems above, a dose of antibody-enzyme was identified which would produce a therapeutic effect when prodrug was given. In some instances, the minimum tumour level of enzyme was measured along with the enzyme level in blood, which produced toxicity. In all the examples, it was possible to generate a therapeutic effect.

Prodrug/Drug

The dose of prodrug required for effectiveness was also identified in the animal model studies. The critical values for tumour response and avoidance of toxicity from prodrug in normal tissues and tumour have been identified. Much of the work has been conducted with bolus injections of prodrug and it is not clear that this is optimal as the sustained presence of enzyme in the tumour would lend itself best to generation of active drug if a reasonably constant level of prodrug was maintained by infusion.

The window between the six parameters needs to be understood and while this is a challenge for developing a safe clinical protocol with a reasonable prospect of effectiveness, the animal investigations described provide a means of choosing

Table 28.3 Minimum anticipated biological effect level (MABEL) for ADEPT and starting doses derived and used for clinical trial of antiCEA/carboxypeptidase G2 fusion protein with bis-iodophenol mustard prodrug (Mayer et al. 2006)

	MABEL	Starting dose for clinical trial	References
Fusion protein dose	>500 mg/m^2	50 mg/m^2	Sharma et al. (2005) Mayer et al. (2006)
Serum Enzyme when prodrug given	0.05 u/ml	0.05 u/ml	Springer et al. (1995) Blakey et al. (1996) Francis et al. (2002)
Prodrug dose (AUC)	52 mg/kg × 3	12.4 mg/m^2 × 3 (AUC_{INF} 34 mg ml min)	Blakey et al. (1996) Springer et al. (1995) Francis et al. (2002) Mayer et al. (2006)

appropriate starting drug doses for clinical trials. This is done partly by determining the pre-clinical determination of the level at which there is no anticipated adverse effect (NOAEL) but also through the determination of the minimum anticipated biological effect level (MABEL) (Duff report 2006). The MABEL calculations for one clinical trial are shown in Table 28.3.

In summary, the investigations discussed above collectively provide evidence for function of the key elements of ADEPT listed below

- Enzyme
  - Specific antibody targeting to tumour
  - Retention in tumour
  - Normal tissue clearance and inactivation
- Prodrug
  - Delivery to tumour
  - Activation in tumour
  - Normal tissue clearance of prodrug
  - Toxicity when not activated
- Active drug
  - Generation in tumour and normal tissues
  - Clearance from normal tissues
  - Toxicity to tumour
  - Repair of damage to surviving tumour cells
  - Toxicity to normal tissues

Although the relationships between these elements in the different examples are quantitatively different, the same general principles appear to apply throughout these diverse systems and indicate that ADEPT is a robust proposition for clinical application.

28.3.3 Clinical Trials

28.3.3.1 Tumour and Target

Although ADEPT has shown the greatest effects in xenograft animal models of tumours known to be chemosensitive such as choriocarcinoma, breast and ovarian cancer, a decision was taken to use it initially in gastrointestinal cancer, principally colon or rectal adenocarcinomas because of the clinical need for improved treatments, the success of therapy in colon carcinoma xenografts and the availability of anti-CEA antibodies. These trials are summarised in Table 28.4.

28.3.3.2 Administration of Therapy

Dose

The dose of antibody-enzyme ideally needs to be sufficient to provide antibody excess in all of the target antigen-bearing sites where enzyme is to be delivered. In practise, it is difficult to know the total body quantity of the target. However, persistence of antibody-enzyme in the circulation as seen in most of the clinical trials implies that sufficient antibody has been administered. In the clinical trials reported, the administered antibody enzyme dose has been scaled up from doses shown to be effective in animal models usually with corresponding evidence of adequate enzyme concentration in the tumour (Sharma et al. 2005). However, patients with a large tumour burden may require a higher dose in a way that is not paralleled in non-targeted therapeutics.

For prodrug, pharmacokinetic profiles related to efficacy and achievement of IC50 derived from animal models are helpful in planning clinical dose.

Table 28.4 Summary of clinical trials of ADEPT

	Target	Antibody/ enzyme	Clearance system	Tumour/blood enzyme ratio when prodrug given	Evidence of response
Bagshawe et al. (1995)	CEA	F(ab')2/CPG2 conjugate	Glycosylated anti-enzyme antibody	–	yes
Napier et al. (2000)	CEA	”	”	10,000:1	yes
Francis et al. (2002)	CEA	”	None	1.2:1	no
Mayer et al. (2006)	CEA	ScFv/CPG2 fusion protein	Mannosylated fusion protein	>20:1	yes

Route

ADEPT is being developed for systemic use although local administration, for instance, intra-arterially seems perfectly feasible.

Time

All antibody-based products should be given slowly because of the risk of immediate hypersensitivity reactions, cytokine release or tumour lysis syndromes. The timing of prodrug administration after antibody enzyme administration is determined by knowledge of the level of enzyme in the circulation below which significant toxicity from prodrug activation is avoided. The duration for which prodrug activation can be continued is determined by the duration for which tumour levels of enzyme can be expected to be sufficient for prodrug activation.

Pharmacokinetics and Pharmacodynamics

The aim is to achieve sufficient enzyme in tumour for prodrug activation and levels in normal tissues, which do not activate enough prodrug to cause toxicity. Although it was not the first trial, it is useful to consider the simplest system in which anti-CEA F(ab')2 mouse monoclonal antibody was chemically conjugated to carboxypeptidase G2 and given intravenously followed by bis-iodophenol prodrug when serum enzyme levels were below 0.05 u/ml. Enzyme activity cleared from the blood with a half-life of about 24 h, permitting prodrug to be given after about 72 h. Radiolabelled antibody enzyme distribution was monitored by single photon emission tomography and tumour biopsies were obtained from some patients. These studies showed that blood was the prime location of residual enzyme with no excess accumulation in other tissues. Tumour to blood ratios varied from $<1:1$ to a maximum of 10:1 (the value also achieved in the colon carcinoma xenograft model). No tumour responses were seen and myelosuppression was dose limiting. Because of this, it was not possible to escalate the prodrug dose to levels expected to give effective therapy on the basis of animal model studies. It was concluded from this trial that higher tumour to normal tissue ratios were needed.

28.3.3.3 The First Clinical Trial

In the first clinical trial, Bagshawe et al. used the system which had been successful in mice that bore human colon carcinoma xenografts. It comprised a F(ab')2 anti-CEA antibody chemically conjugated to the bacterial enzyme carboxypeptidase G2 administered intravenously. Twenty-four hours later, clearance of antibody-enzyme

from the circulation was achieved by giving a galactosylated mouse monoclonal antibody to carboxypeptidase G2 to inactivate and clear circulating enzyme. Twenty-four hours after this and when enzyme was undetectable in the blood, a monomesyl benzoic acid mustard prodrug was given intravenously. As a control, prodrug was given without antibody enzyme before the full treatment, at each dose level, as prodrug dose was escalated. This produced no measurable effect. The full therapy produced responses in patients with advanced colorectal cancer as the prodrug dose was increased (Bagshawe and Begent 1996). The principle toxicity was bone marrow suppression but this was ameliorated by infusing prodrug rather than giving it as a bolus.

28.3.3.4 The Second Clinical Trial

This followed the same system as the first trial but with a focus on measurement of the parameters which were important for the function of ADEPT to determine as far as possible whether the system was operating as designed (Napier et al. 2000). This trial also showed examples of anti-tumour efficacy. Clearance of enzyme activity from the circulation appeared complete within 24 h of administration of the clearing second antibody. Radiolabelled antibody enzyme conjugate was given with the main dose and distribution mapped serially in tumour and normal tissues by single photon emission computed tomography (SPECT). Antibody enzyme was initially substantially retained in the circulation and major organs with a large blood supply. Following administration of the clearing antibody at 24 h, there was rapid clearance of radioactivity from blood and normal tissues but retention in tumour. Quantitative estimates of enzyme derived from the SPECT data showed tumour to normal ratios of 10,000:1. This was confirmed by enzyme activity measurements in tumour biopsies in four of the patients, which confirmed the high tumour to blood ratios of enzyme. There was evidence that response was related to prodrug dose (Bagshawe and Begent 1996). The principle toxicity was bone marrow suppression, which was attributed to leak-back of active drug into the circulation after generation in the tumour.

28.3.3.5 The Third Clinical Trial

This used the same chemical conjugate as before but with no clearing system (Francis et al. 2002). The prodrug, a bis-iodophenol mustard (Springer et al. 1995; Blakey et al. 1996), was more potent and the active drug had a shorter half life than the benzoic acid mustard used before. It was activated through cleavage of glutamate by carboxypeptidate as before. A clearance system was not used and tumour to normal ratios were low (Table 28.4). There was no clinical evidence of response but valuable information was acquired about clinical use of the prodrug.

28.3.3.6 The Fourth Clinical Trial

This was the first clinical use of a genetic fusion protein of antibody and enzyme. MFECP antibody enzyme fusion protein. The prodrug was the bisiodophenol mustard. The fusion protein was mannosylated as a result of production in yeast and this had resulted in very rapid clearance from the circulation in animal models.

The processes used in the GMP recombinant protein production unit are described in two papers (Tolner et al. 2006a, b).

Initial results relating to pharmacokinetics and pharmacodynamics were published by Mayer et al. (2006). Safety was established, immunogenicity assessed and optimal doses and timing of antibody-enzyme and prodrug established. Toxicity and immune response are reported in this paper.

Pharmacokinetics and Pharmacodynamics

Measurements of CPG2 enzyme activity by high-performance liquid chromatography showed a median blood α half-life of 0.44 h and β half-life of 4.6 and 1.96 h for MFECP1. The level for giving prodrug <0.005 units/ml.

Tumour localization of MFECP1

Gamma camera imaging. MFECP1 localization was determined by gamma camera imaging. The median amount of CPG2 in tumour was calculated to be 0.18 units/g at 4 h, falling to 0.07 units/g after 20 h: this gives tumour to blood ratios of between 14:1 and 36:1 given that blood enzyme levels were <0.005 u/ml, but these are probably underestimates because of the heterogeneity of tumours which had large areas of necrosis and stroma as shown on histology of tumour biopsies.

These tumour biopsies were done at a median, 17.7 h after the end of the infusion of MFECP1 and immunohistochemistry with anti-CPG2 antibody showed MFECP1 localization in tumour in patients up to 19 h.

The mean half-life for the BIP prodrug in plasma was 10.3 min (F3.45 min), the median half-life being 9.96 min. The rate of clearance was reduced as the prodrug dose increased, commensurate with the increased C0 and AUCINF.

Tumour was available for assessment of cross-link formation in one patient. Reduction in tail moment by 58% was seen in this sample when compared with pretreatment peripheral blood lymphocytes, indicating a significant level of DNA interstrand cross-linking consistent with prodrug activation in tumour.

Immune Responses

Immune response HACA were detected in 36% (11 of 30) of patients after a single treatment but subsequent studies with repeated therapy have shown formation of HACA in all the patients.

Safety and Toxicity

MFECP1 was safe and well tolerated with no serous adverse events attributable to the antibody-enzyme fusion proteins. SPECT gamma camera imaging of patients who received radiolabeled MFECP1 showed that it cleared rapidly through the liver with prominent hepatic tracer activity seen by 1 h. There was subsequent reduction in hepatic activity and excretion of tracer as expected through the urinary system and bowel. No unexpected areas of tracer uptake were identified.

Toxicity of BIP prodrug

The dose of prodrug was escalated from 12.4, progressively to 3,226 mg/m^2.

Toxicity attributable to prodrug occurred at 1,613 mg/m^2 and above comprising renal and liver toxicity comparable to that seen with prodrug alone in animal toxicity studies.

The principal toxicity attributable to active drug was myelosuppression, seen particularly with repeated therapy given over 10 days, which reached grade 3 and 4 above cumulative prodrug doses of 1,200 mg/m^2.

Efficacy

Responses were seen in patients receiving a cumulative dose of 900 mg/m^2 or more of prodrug in single doses or with the whole ADEPT treatment given two or three times within 10 days. These evidenced by reductions in FDG-PET tumour uptake, serum CA19/9 levels and CT. Taking these data with those toxicity there is a clear therapeutic window which may be exploited using the current pharmaceuticals and as a basis for future development.

28.4 Conclusion

ADEPT is based on a concept that could make a major contribution to cancer therapy. However, the complexity of the system makes it a substantial development challenge. This review seeks to present a systematic approach to the components and their interactions. The evidence now accumulated shows that there do not appear to be fundamental barriers to development of a practical and widely used therapy. Each of the components used can be shown to perform singly and in concert giving the essential element of ADEPT as illustrated in Fig. 28.3.

The clinical trials have shown that the treatment is practical and manageable in existing clinical facilities. Collectively, this means that there are opportunities to

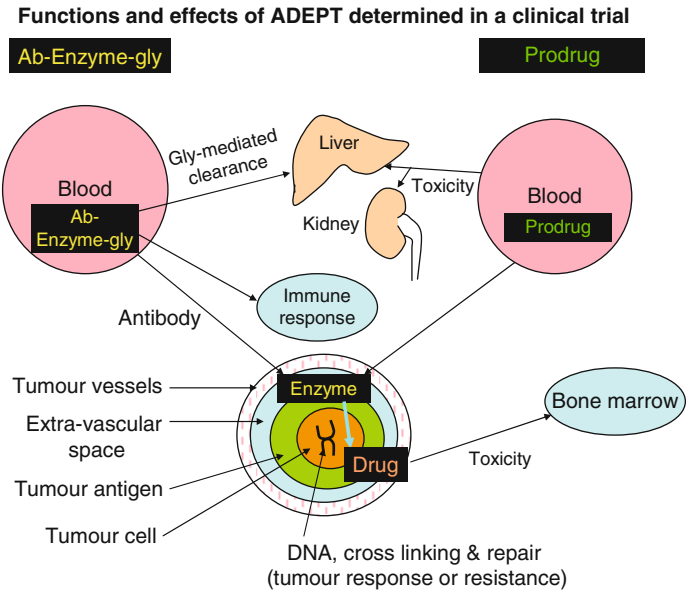


Fig. 28.3 Components of ADEPT and their interactions. All of the elements shown have been investigated as described in this chapter

develop ADEPT for diverse tumours and targets and antibodies, using derivatives of human enzymes or de-immunised enzymes to avoid immunogenicity and novel prodrug/drug systems.

Acknowledgements The work described was supported by Cancer Research UK, the UCL Experimental Cancer Medicine Centre and the UCL Cancer Institute Research Trust. The author is partly supported by the UCL/UCLH Comprehensive Biomedical Research Centre.

References

Aapro MS, Cameron DA, Pettengell R, Bohlius J, Crawford J, Ellis M, Kearney N, Lyman GH, Tjan-Heijnen VC, Walewski J, Weber DC, Zielinski C (2006) European. Organisation for Research and Treatment of Cancer (EORTC) Granulocyte Colony-Stimulating Factor (G-CSF) Guidelines Working Party EORTC guidelines for the use of granulocyte-colony stimulating factor to reduce the incidence of chemotherapy-induced febrile neutropenia in adult patients with lymphomas and solid tumours. Eur J Cancer 42(15):2433–2453

Allander SV, Nupponen NN, Ringner M, Hostetter G, Maher GW, Goldberger N, Chen Y, Carpten J, Elkahloun AG, Meltzer PS (2001) Gastrointestinal stromal tumours with *kit* mutations exhibit a remarkably homogeneous gene expression profile. Cancer Res 61:8624–8628

Bagshawe KD (1989) Toward generating cytotoxic agents at cancer sites. Br J Cancer 60:275–281

Bagshawe KD, Begent RHJ (1996) First clinical experience with ADEPT. Adv Drug Deliv Rev 22(3):365–367

Bagshawe KD, Sharma SK, Springer CJ, Antoniw P (1995) Antibody directed enzyme prodrug therapy: a pilot scale clinical trial. Tumour Target 1:17–30

Begent RHJ, Ledermann JA, Green AJ, Bagshawe KD, Riggs SJ, Searle F, Keep PA, Adam T, Dale RG, Glaser MG (1989) Antibody distribution and dosimetry in patients receiving radiolabelled antibody therapy for colorectal cancer. Br J Cancer 60:406–412

Begent RHJ, Verhaar MJ, Chester KA, Casey JL, Green AJ, Napier MP, Hope-Stone LD, Cushen N, Keep PA, Johnson CJ, Hawkins RE, Hilson AJW, Robson L (1996) Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. Nat Med 2:979–984

Bhatia J, Sharma SK, Chester KA, Pedley RB, Boden RW, Read DA, Boxer GM, Michael NP, Begent RHJ (2000) Catalytic activity of an in vivo tumor targeted anti-CEA scFv:: carboxypeptidase G2 fusion protein. Int J Cancer 85:571–577

Biela BH, Khawli LA, Hu P, Epstein AL (2003) Chimeric TNT-3/human beta-glucuronidase fusion proteins for antibody-directed enzyme prodrug therapy (ADEPT). Cancer Biother Radiopharm 18(3):339–353

Blakey DC, Burke PJ, Davies DH, Dowell RI, East SJ, Eckersley KP, Fitton JE, McDaid J, Melton RG, Niculescu-Duvaz IA, Pinder PE, Sharma SK, Wright AF, Springer CJ (1996) ZD2767, an improved system for antibody-directed enzyme prodrug therapy that results in tumor regressions in colorectal tumor xenografts. Cancer Res 56(14):3287–3292

Bosslet K, Czech J, Lorenz P, Sedlacek HH, Schuermann M, Seemann G (1992) Molecular and functional characterisation of a fusion protein suited for tumour specific prodrug activation. Br J Cancer 65(2):234–238

Boxer GM, Begent RH, Kelly AM, Southall PJ, Blair SB, Theodorou NA, Dawson PM, Ledermann JA (1992) Factors influencing variability of localisation of antibodies to carcinoembryonic antigen (CEA) in patients with colorectal carcinoma–implications for radioimmunotherapy. Br J Cancer 65:825–831

Chester KA, Begent RHJ, Robson L, Keep P, Pedley RB, Boden JA, Boxer G, Green A, Winter G, Cochet O, Hawkins RE (1994) Phage libraries for generation of clinically useful antibodies. Lancet 343:455–456

Duff 2006 Expert scientific group on phase one clinical trials. 2006. The Stationery Office, ISBN-13 978 0 11 703722 9

Eccles SA, Court WJ, Box GA, Dean CJ, Melton RG, Springer CJ (1994) Regression of established breast carcinoma xenografts with antibody-directed enzyme prodrug therapy against c-erbB2 p185. Cancer Res 54(19):5171–5177

Einhorn LH, Williams SD, Chamness A, Brames MJ, Perkins SM, Abonour R (2007) High-dose chemotherapy and stem-cell rescue for metastatic germ-cell tumours. N Engl J Med 357 (4):340–348

Francis RJ, Sharma SK, Springer C, Green AJ, Hope-Stone LD, Sena L, Martin J, Adamson KL, Robbins A, Gumbrell L, O'Malley D, Tsiompanou E, Shahbakhti H, Webley S, Hochhauser D, Hilson AJ, Blakey D, Begent RH (2002) A phase I trial of antibody directed enzyme prodrug therapy (ADEPT) in patients with advanced colorectal carcinoma or other CEA producing tumours. Br J Cancer 87(6):600–607

Haisma HJ, Brakenhoff RH, vd Meulen-Muileman I, Pinedo HM, Boven E (1998) Construction and characterization of a fusion protein of single-chain anti-carcinoma antibody 323/A3 and human beta-glucuronidase. Cancer Immunol Immunother 45(5):266–272

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70

Harding FA, Liu AD, Stickler M, Razo OJ, Chin R, Faravashi N, Viola W, Graycar T, Yeung VP, Aehle W, Meijer D, Wong S, Rashid MH, Valdes AM, Schellenberger V (2005) B-lactamase with reduced immunogenicity for the targeted delivery of chemotherapeutics using antibody-directed enzyme prodrug therapy. Mol Cancer Ther 4(11):1791–1800

Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128:683–692

Kogelberg H, Tolner B, Sharma SK, Lowdell MW, Qureshi U, Robson M, Hillyer T, Pedley RB, Vervecken W, Contreras R, Begent RH, Chester KA (2007) Clearance mechanism of a mannosylated antibody-enzyme fusion protein used in experimental cancer therapy. Glycobiology 17(1):36–45

Mayer A, Tsiompanou E, O'Malley D, Boxer GM, Bhatia J, Flynn AA, Chester KA, Davidson BR, Lewis AA, Winslet MC, Dhillon AP, Hilson AJ, Begent RH (2000) Radioimmunoguided surgery in colorectal cancer using a genetically engineeredanti-CEA single-chain Fv antibody. Clin. Cancer Res 6:1711–1719

Mayer A, Sharma SK, Tolner B, Minton NP, Purdy D, Amlot P, Tharakan G, Begent RH, Chester KA (2004) Modifying an immunogenic epitope on a therapeutic protein: a step towards an improved system for antibody-directed enzyme prodrug therapy (ADEPT). Br J Cancer 90 (12):2404–2410

Mayer A, Francis RJ, Sharma SK, Tolner B, Springer CJ, Martin J, Boxer GM, Bell J, Green AJ, Hartley JA, Cruickshank C, Wren J, Chester KA, Begent RHJ (2006) A phase I study of single administration of antibody-directed enzyme prodrug therapy with the recombinant anti-carcinoembryonic antigen antibody-enzyme fusion protein mfecp1 and a bis-iodo phenol mustard prodrug. Clin Cancer Res 12:6509–6516

Medzihradszky KF, Spencer DI, Sharma SK, Bhatia J, Pedley RB, Read DA, Begent RH, Chester KA (2004) Glycoforms obtained by expression in Pichia pastoris improve cancer targeting potential of a recombinant antibody-enzyme fusion protein. Glycobiology 14(1):27–37 Epub 2003 Sep 26

Melo JV, Barnes DJ (2007) Chronic myeloid leukaemia as a model of disease evolution in human cancer. Nat Rev Cancer 7:441–453

Michael NP, Chester KA, Melton RG, Robson L, Nicholas W, Boden JA, Pedley RB, Begent RHJ, Sherwood RF, Minton NP (1996) *In vitro* and *in vivo* characterisation of a recombinant carboxypeptidase G_2::anti-CEA scFv fusion protein. Immunotechnology 2:47–57

Napier MP, Sharma SK, Springer CJ, Bagshawe KD, Green AJ, Martin J, Stribbling SM, Cushen N, O'Malley D, Begent RHJ (2000) Antibody-directed enzyme prodrug therapy: efficacy and mechanism of action in colorectal carcinoma. Clin Cancer Res 6:765–772

Pedley RB, Boxer GM, Boden JA, Southall PJ, Begent RH, Bagshawe KD, Humm J, Searle F (1990) Preliminary observations on the microdistribution of labelled antibodies in human colonic adenocarcinoma xenografts: relevance to microdosimetry. Br J Cancer 61:218–220

Rubin CP, Heinrich MC, Corless CL (2007) Gastrointestinal stromal tumour. Lancet 369: 1731–1741

Senter PD (1990) Activation of prodrugs by antibody-enzyme conjugates: a new approach to cancer therapy. FASEB J 4:188–193

Senter PD, Springer CJ (2001) Selective activation of anticancer prodrugs by monoclonal antibody-enzyme conjugates. Adv Drug Deliv Rev 53(3):247–264

Sharma SK, Pedley RB, Bhatia J, Boxer GM, El-Emir E, Qureshi U, Tolner B, Lowe H, Michael NP, Minton N, Begent RH, Chester KA (2005) Sustained tumour regression of human colorectal cancer xenografts using a multifunctional mannosylated fusion protein in antibody-directed enzyme prodrug therapy. Clin Cancer Res 11:814–825

Spencer DI, Robson L, Purdy D, Whitelegg NR, Michael NP, Bhatia J, Sharma SK, Rees AR, Minton NP, Begent RH, Chester KA (2002) A strategy for mapping and neutralizing conformational immunogenic sites on protein therapeutics. Proteomics 2(3):271–279

Springer CJ, Dowell R, Burke PJ, Hadley E, Davis DH, Blakey DC, Melton RG, Niculescu-Duvaz I (1995) Optimization of alkylating agent prodrugs derived from phenol and aniline mustards: a new clinical candidate prodrug (ZD2767) for antibody-directed enzyme prodrug therapy (ADEPT). J Med Chem 38(26):5051–5065

Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, Panoskaltsis N (2006) Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med 355:1018–1028

Tolner B, Smith L, Begent RHJ, Chester KA (2006a) Production of recombinant protein in *Pichia pastoris* by fermentation. Nat Protoc 1:1006–1021

Tolner B, Smith L, Begent RHJ (2006b) Chester KA Expanded-bed adsorption immobilized-metal affinity chromatography. Nat Protocart 1:1213–1222

Webley SD, Francis RJ, Pedley RB, Sharma SK, Begent RH, Hartley JA, Hochhauser D (2001) Measurement of the critical DNA lesions produced by antibody-directed enzyme prodrug therapy (ADEPT) in vitro, in vivo and in clinical material. Br J Cancer 84(12):1671–1676

Chapter 29
Production of Recombinant Human IgG Antibodies in the Baculovirus Expression System

Mifang Liang and Stefan Dübel

29.1 Introduction

As a well-established method, phage display has been widely used to select scFv or Fab antibody fragments specific for many different antigens (see Part II (Generation of Antibody Repertoires) of this book). For some purposes, it is required to reassemble the variable regions of the selected antibodies with constant regions to generate complete recombinant human antibodies.The expression of entire IgG molecules, however, is not generally achievable for every antibody in *Escherichia coli*, thus, an eukaryotic IgG expression vector system is needed.

The baculovirus expression system has been used for the expression of a wide variety of heterologous genes. Since its first description for Ig expression (Hasemann and Capra 1990), it has been continuously improved to be one of the most powerful and convenient eukaryotic expression systems available for the production of recombinant chimeric, humanized or human antibodies (Nesbit et al. 1992; Poul et al. 1995; Liang et al. 1997). Using baculovirus systems for antibody production has several advantages over either prokaryotic or transfection-based eukaryotic expression systems. First, the baculovirus expression system provides appropriate signal peptide cleavage, folding, and disulfide bond formation. The glycosylation is different from mammalian cells, but although the addition of high mannose oligosaccharides has been reported, it does not appear to affect the immunoglobulin expression. Second, compared to other eukaryotic expression systems, baculovirus expression allows the production of IgGs with yields significantly larger than those

M. Liang (✉)
State Key Laboratory for Infectious Diseases Control and Prevention, National Institute for Viral Disease Control and Prevention, China CDC, 100 Ying Xin Jie, Xuan Wu Qu, Beijing 100052, China
e-mail: mifangl@vip.sina.com
S. Dübel
Department of Biotechnology, Technische Universität Braunschweig, Spielmannstr. 7, Braunschweig 38106, Germany

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_29,

typically obtained from transfected eukaryotic cells. About 10-mg secreted human IgG antibodies per liter can be obtained from the supernatant of infected SF9 cells cultured in flasks (Liang et al. 1997). Third, it is not necessary to store transfected productive cells in liquid nitrogen or repeatedly passage them, since the recombinant baculovirus stock can be easily stored for weeks at 4°C or for years in normal deep freezers and used to infect fresh cells for the next production batch.

The most common baculovirus strain used in the baculovirus expression system is *Autographa californica* nuclear polyhedrosis virus (AcNPV), which has a double stranded circular DNA genome of 131 kb (Miller 1996). Infectious AcNPV particles enter susceptible insect cells by an endocytosis or fusion mechanism, and the viral DNA is uncoated in the nucleus where the viral DNA is transcribed, replicated and packaged into nucleocapsid protein. During the early phase of viral infection, the infectious viral particles are released extracellularly by budding from infected cell membranes. Late in infection (>48 h), viral particles are found in occlusion bodies, crystal-like structures mainly composed of*** a 29-kDa protein named polyhedrin, and accumulated within the nucleus of the infected cells. The later phase is marked by a dramatic increase in the transcription of the polyhedrin and p10 genes (Weyer et al. 1990). The temporarily regulated hyperexpression of the late genes, as polyhedrin and p10, which is nonesssential for baculovirus production, made baculoviruses perfect vectors for the expression of foreign genes. Current commercial baculovirus transfection kits provide linearized modified AcNPV baculovirus DNA, which contain a lethal deletion with Bsu361, resulting in the removal of an essential part of the open reading frame ORF1629. The antibody gene is supplied on a transfer vector plasmid under the control of polyhedrin and/or p10 promoters and flanked by regions homologous to AcNPV. When the linearized modified AcNPV baculovirus DNA is cotransfected with the shuttle vector plasmid into insect cells, recombination takes place between the homologous regions (ORF603 and ORF1629) of viral DNA and plasmid, resulting in recombinant Baculoviruses carrying and expressing the foreign genes. If the expressed protein is preceded by a signal sequence, the insect cells secrete it into the medium in large amounts. In addition, the recombinant Baculoviruses will be released into the supernatant, from where they can be recovered and stored until they will be used to infect fresh insect cells for a new batch of antibody production.

To achieve expression of complete human immunoglobulins, we have constructed a set of "cassette" baculovirus expression vectors, which were designed for the convenient insertion of heavy and light chain genes of Fab or scFv antibodies selected from phage display libraries. The IgG expression vector system (Fig. 29.1) is based on the backbone vector pAcUW51 (Pharmingen Cat# 21205P), which contains a SV40 transcription terminatior, the f1 origin of phage DNA replication, an Amplicillin resistance gene for selection in *E. coli,* and the two very late baculovirus expression promoters of polyhedrin and p10, located in back-to-back orientation. The IgG expression elements were cloned separately into pAcUW51 via the BglII and BamHI sites. The heavy chain gene is under the control of the polyhedrin promoter, is preceded by the authentic IgG signal sequence from IgG1 subgloup VHIII, and contains the mutant in-frame cloning sites XhoI and NheI for

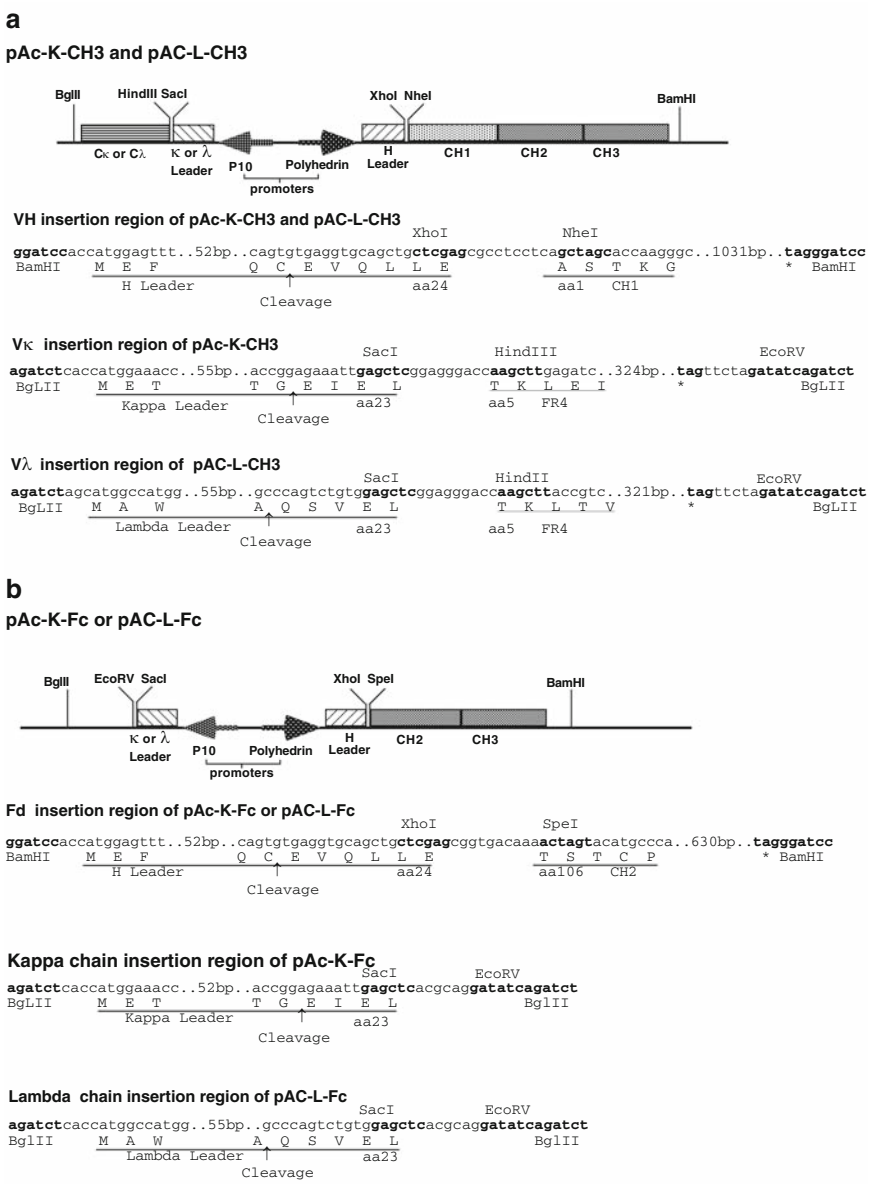


Fig. 29.1 The antibody coding regions of the baculovirus vectors for human IgG expression used in this protocol. (**a**) vectors for the cloning of V region DNA obtained from scFv phage display systems; (**b**) vectors for the cloning of Fab region DNA obtained from Fab phage display systems. The vector backbone in all cases (outside Bgl II/BamHI) is pAcUW51. Abbreviations: *aa* amino acid number; *C*(*x*) constant human immunoglobulin regions; *H* κ or λ leader, sequence coding for the respective human immunoglobuline signal peptides

inserting the VH gene of the scFv (Fig. 29.1a) or XhoI and SpeI for inserting the Fab Fd gene (Fig. 29.1b). The entire constant region genes of human IgG1 or the Fc region gene of human IgG1, respectively, are located downstream of signal sequence and cloning sites. In opposite orientation to the heavy chain operon, the light chain elements start with the authentic signal sequence of human lambda chain or kappa chain, in the scFv cassette vectors followed by the constant region of lambda or kappa chains (Fig. 29.1a). The cloning sites for the insertion of lambda or kappa VL genes are SacI and HindIII; or SacI and EcoRV for the insertion of lambda or kappa VL Fab genes. The original HindII site in the vector pACUW51 was removed. Thus, either of VL or Fab-L and VH or Fab-Fd genes can be cloned into one of the above vectors. This design of the vectors results in the secretion of correctly processed and assembled immunoglobulins from recombinant baculovirus-infected insect cells, without apparent differences between the expression level of heavy and light chains.

29.2 Outline

The key steps to the successful expression of recombinant IgG antibodies are as follows: (1) The phage library-derived Fab or scFv genes must be cloned in frame into the baculovirus expression vectors the cloning sites of your Fab or scFv gene fragments must match the correct amino acid positions; which are calculated from the first start codon of IgG heavy or light chain genes mRNA. (2) The Fab genes derived from the pComb3 phagemid vector system (Barbas and Lerner 1991) can be directly cloned into our expression vectors pAc-L(K)-Fc. To clone scFv single chain genes derived from panning using the pSEX phagemid vector system (Welschof et al. 1997) or others, the primers may need to be designed according to the gene sequences. (3) The sequences of the inserts have to be checked to confirm that no mutation happened in the ORF of complete IgG heavy or light chain genes. The consecutive steps for the baculovirus expression of IgG antibodies can be summarized as follows: (4) The right amino acids at position H5, H6, and H9 of VH region, which are mostly close to your antibody sequence, will be more 100 important for your antibody secession (Jung et al. 2001).

Your strategy for production shall therefore include the following steps:

- Choose on appropriate expression vector (depending on the antibody format).
- Clone Fab or scFv genes into one of expression vectors pAc-l(k)-CH3 or pAc-l(k)-Fc.
- Propagate and purify the vector DNA containing Fab or scFv genes from *E. coli*.
- Cotransfer the purified plasmid DNA with linearized AcNPV baculovirus DNA into SF9 insect cells.
- Verify the production of functional human IgG antibodies in SF9 cells by immunofluorescent assay (IFA) and ELISA.
- Amplify and plaque purify the recombinant virus, and prepare the recombinant virus stock for storage and reinfection.

- Use recombinant virus to reinfect insect cells for the production of recombinant IgG immunoglobulin molecules.
- Affinity purify the expressed IgG from the supernatants of infected cells, and characterize recombinant antibodies by SDS-PAGE, Western-Blot, IFA, ELISA, etc.

29.3 Materials

29.3.1 Equipment

– 27°C cell culture incubator
– Sterile workbench and basic cell culture equipment
– Immunofluorescence, gel electrophoresis, and ELISA equipment

29.3.2 Reagents

– Cloning and expression vectors: pAc-H(k)-CH3, and pAc-H(k)-Fc, pAc-H(l)-CH3, pAc-H(l)-Fc
– Insect cell lines: SF9 cells and H5 cells (Pharmingen or Invitrogen)
– *E. coli* DH5<alpha> cells (GIBCO-BRL), or XLI- Blue cells (Stratagene)
– Restriction endonucleases: Xho1, SpeI, NheI, HindIII, SacI, and EcoRV (New England BioLabs or Life Technology)
– PCR reagents: ELONGase Enzyme Mix and buffers (Life Technology) and, 10 mM dNTP
– Insect cells culture media: TNF-FH (BD, Cat# 554760) and Sf900-II SFM (Serum-Free Medium) (Invitrogen, Cat# 10902-088)
– QIAGEN Spin Miniprep kit (Cat# 27104), QIAGEN-tip 100 Midiprep kit (Cat# 12143 or 12144), and QIAquick Gel-Extraction Kit (cat # 28704)
– BaculoGold DNA (PharMingen Cat #21227M), and Transfection buffer A and B set (PharMingen cat# 21483A)
– Low temperature melting agarose (Agarplaque Agarose, PharMingen Cat# 21403A)
– Protein-free insect medium (PharMingen Cat# 21228M)
– T25, T75 cell culture flasks, spinner flasks, 60-mm tissue culture plates, 6-wells tissue culture plates, and 96-wells tissue culture plates (Greiner)
– 96-well polyvinylchloride ELISA plates (Nunc, Wiesbaden, Germany)
– Skimmed milk powder (Oxoid, Basingstoke, UK)
– TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution (Sigma Aldrich, Steinheim, Germany)
– 10 or 12-wells glass slides for microscopy

- PBS (140-mM NaCl, 20-mM phosphate, pH7.4)
- FITC or HRP or nonconjugated anti-human IgG, Fc specific, and Fab-specific antibodies (Sigma, Deisenhofen, Germany)
- Protein G-sepharose affinity column (Pharmacia Biotech) and suitable pump

29.4 Procedure

29.4.1 Selecting an Appropriate Expression Vector for Your IgG Expression

As we described in the instruction, the vector system was specially designed for the cloning of Fab or scFv genes obtained from a phage display library.

- If your antibody genes were derived from scFV single-chain antibody expression system, use vectors pAc-K(L)-CH3. If your light chain gene is kappa, the vector pAc-K-CH3 is recommended. If your light gene is lambda, the vector pAc-L-CH3 is recommended.
- If your antibody genes were derived from Fab fragments antibody expression system, use vectors pAc-K(L)-Fc. If your light chain gene is kappa, the vector pAc-K-Fc is recommended. If your light gene is lambda, the vector pAc-L-Fc is recommended.

29.4.2 PCR Amplification of the Variable Region of Light and Heavy Chain Genes

Primer design for the PCR amplification of VH and VL genes:

The vectors pAC-K(L)-Fc were specially designed for cloning from the pComb3 phage display system. If the cloning sites of your antibody genes do not match our baculo vector system, you have to design primers according to the sequences of your antibody genes. The key issue for your cloning strategies is that the insert gene must be in frame of the vector that you are going to use. As Fig. 1 indicated, the heavy chain 5′ cloning site XhoI must start at 72 bp, at amino acid (aa) position 24 since ATG of the IgG1 gene amplified from mRNA, with CTC=Leu; the 3′ cloning site NheI must start at first amino acid of constant region, with GCT=Ala; SpeI must start at 318 bp, aa106 of constant region, with ACT=Thr; the kappa or lambda 5′ cloning site SacI must start at 69bp, aa23 since ATG of kappa or lambda gene from mRNA; the 3′cloning site HindIII must start at 18bp, aa6 since first bp of FR4, with AAG=Lys. Table 1 below outlines the reference primers for cloning VH and VL genes into our vector system.

A List of Primers for cloning human antibody genes into baculovirus IgG expression vectors

Human IgG variable chain 5′ primers
VH3a 5′-GAG GTG CAG CTC GAG GAG TCT GGG-3′*
VH1f 5′-CAG GTG CAG CTG CTC GAG TCT GGG-3′*
VH3f 5′-GAG GTG CAG CTG CTC GAG TCT GGG-3′*
VH4f 5′-CAG GTG CAG CTG CTC GAG TCG GG-3′*
VH6a 5′-CAG GTA CAG CTG CTC GAG TCA GG-3′
Human kappa variable chain 5′ primers*
VK1a 5′-GAC ATC GAG CTC ACC CAG TCT CCA-3′
VK3a 5′-GAA ATT GAG CTC ACG CAG TCT CCA-3′
VK2a 5′-GAT ATT GAG CTC ACT CAG TCT CCA-3′
Human Lambda variable chain 5′ primers
VL1 5′-AAT TTT GAG GAG CTC CAG CCC CAC-3′
VL2 5′-TCT GCC GAG CTC CAG CCT GCC TCC GTG-3′*
VL3 5′-TCT GTG GAG CTC CAG CCG CCC TCA GTG-3′*
VL4 5′-TCT GAA GAG CTC CAG GAC CCT GTT GTG TCT GTG-3′*
VL5 5′-CAG TCT GTG GAG CTC CAG CCG CCC-3′
VL6 5′-5′-CAG ACT GAG GAG CTC CAG GAG CCC-3′*
Human IgG (Fd) heavy chain 3′ primers (SpeI)[a]
CG1z 5′-GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG-3′
Human IgG variable chain 3′ primer
NheI-HR 5′- TGG GCC CTT GGT GCT AGC TGA GGA GAC GGT GACC-3′
Human lambda variable chain 3′ primer
HindII-LR 5′- GAC GGT AAG CTT GGT CCC TCC-3′
Human Kappa variable chain 3′ primer
HindIII-KR1 5′-CAG TTC GTT TGA TTT CAA GCT TGG TCCC-3′
HindIII-KR2 5′-CAG TTC GTC TGA TCT CAA GCT TGG TCCC-3′
Human Lambda chain 3′ primer
EcoR-CL 5′-CCG GAT ATC TAG AAC TAT GAA CAT TCT GTA GG-3′
Human Kappa chain 3′ primer
EcoRV-CK 5′-CCG GAT ATC TAG AAC TAA CAC TCT CCC CTG TTGA-3′

[a]Primers identical to the pComb 3 system primers (Kang et al. 1991)

Protocol

1. PCR amplification of ScFV or Fab antibody genes:

DNA(plasmid)	1 μl (0.1 μg)
Forward Primer	1 μl (60pm)
Reverse Primer	1 μl (60pm)
E-longase(GIBCO-BRL)	2 μl
BufferA	10 μl
BufferB	10 μl
H2O to	100 μl

   PCR reaction: 94°C 2 min, followed by 25 cycles with 94°C 50", 54°C 50", 72°C 1 min, followed by a final incubation at 72°C for 10 min, cool down to 4°C
2. Purify the PCR products via agarose gel electrophoresis using a QIAquick Gel Extraction Kit.

29.4.3 Cloning into a Baculovirus IgG Expression Vector

1. To clone the light chain gene into the IgG expression vector, double-digest the gel-purified PCR products of your light gene (or your phagemid DNA) and the vector pAc-k-CH3 or pAc-L-CH3 with SacI and HindIII (For scFv genes) or the vector pAc-k-Fc or pAc-L-Fc with SacI and EcoRV (for Fab genes). Ligate the insert DNA with the vector DNA at a concentration molar ratio of 1:4. Ligate with 1U ligase (Life Technology) at 16°C overnight. Following the ligation reaction, transform the ligated plasmid DNA into competent cells of *E.coli* XLI-Blue or DH5<alpha>, plate the bacteria on LB plates containing 100 ug/ml ampicillin, incubate at 37°C overnight. Pick colonies for DNA miniprep and restriction endonuclease analysis to select the positive clones with insert.
2. To clone the heavy chain gene into the IgG expression vector that contains the whole light chain gene, double–digest the gel-purified VH or Fd PCR products (or your phagemid DNA) and the vector DNA containing your light chain insert with XhoI and NheI (for the VH gene) or XhoI and SpeI (for Fd gene). Ligate the insert DNA with the vector DNA at a concentration molar ratio of 1:4. The subsequent steps are identical to those of Step 1.
3. Prepare the plasmid DNA by using the QIAgen miniprep kit, and sequence the inserts of the new constructs obtained from Step 2 or 3. Use the following primers for sequencing:

   (a) For VH and Fd:

   Forward Primer: 5′-TCCACCATGGAGTTTGGGCTGAGC-3′
   Reverse Primer: 5′-TCCCATGTGACCTCAGGGGTCCGGGAGAT-3′

   (b) For VL or VL-CL:

   Forward Primer: 5′-CCGGGACCTTTAATTCAACCCAACAC-3
   Reverse Primer: 5′-GGCAGACATGGCCTGCCCGGTTAT-3′

4. Propagate and purify the plasmid DNA for co-transfection. The quality of the plasmid DNA is critical for successful co-transfections. We recommend the of use the QIAGEN-tip100 Midiprep Kit. The IgG expression vectors are high copy number vectors, usually, 40 ml of a 10–12 h culture of bacteria should yield a total of 100 μg DNA per column (tip-100) with a concentration of at least 0.5 μg/ul.
   Notes:
   i. Generally, we suggest that you clone the light chain first, followed by the heavy chain. However When you start to clone your Fab or scFv antibody genes into our IgG expression vectors, you may need to check the variable region sequences for internal restriction sites and decide the cloning strategy.
   ii. For cloning the light chain gene of Fab antibody, make sure your light chain gene fragment contains a stop codon. If not, you need to re-amplify your gene with the respective primer (see Table 1). If your gene comes from a

pComb3 phage display system, you can digest your gene from plasmid DNA with XbaI first, make it blunt, and then digest it with SacI.

iii. It is very important to sequence your insert genes completely to make sure that the antibody genes (scFv or Fab) are in heavy or light chains open-reading frame and that no significant mutations occur after the insertion of your genes.

iv. When you are preparing the high quality plasmid DNA for co-transfection, the bacteria should not be growing too much into the stationary phase, i.e., the O.D 600 nm value of the bacterial culture should not exceed 1.5.

29.4.4 Preparation of Insect Cells

29.4.4.1 Insect Cell Lines

Several insect cell lines are established to be highly susceptible to AcNPV virus infection. The two more frequently used insect cell lines for virus infection and protein expression are Sf9 and H5 cells. Sf9 cells are most frequently used for co-tranfection, recombinant virus propagation, and protein expression, and H5 cells are generally used for high expression of foreign proteins.

29.4.4.2 Thawing Frozen Insect Cells

1. Remove a vial from liquid nitrogen and thaw it rapidly with gentle agitation in 37°C waterbath.
2. Transfer the 1ml of cell suspension into 7ml of complete TC-100 medium (other insect cell culture media such as Grace's, TNM-FH or, SF900-II are suitable as well) in a 15-ml centrifugation tube, centrifuge at 230×g, 4°C for 5–8 min.
3. Carefully discard the supernatants, re-suspend the cells in 5 ml (for 1×10^6 cells/ml) or 15 ml (for 1×10^7 cells/ml) of complete TC-100 medium, transfer the cell suspension into a T25 (25 cm^2) or T75 (75 cm^2) flask.
4. Put the flask to a 27°C incubator, and allow the cells to attach to the bottom of flask. Change the medium after 12 h or 24 h. The viability of the recovered cells should be more than 70%. Check the cells daily until a confluent monolayer has formed. Once such a monolayer has formed, the cells can be divided (see next step).

29.4.4.3 Culturing and Passaging Insect Cells

1. Culture Sf9 cells either in flasks or in a spinner flask (Suspension culture) at 27°C. To maintain a healthy culture, passage the cells 1:3–4 in a T25 or T75 flask when they reach a confluent monolayer, or reach 1×10^6 cells/ml in suspension culture. A Complete insect cell culture medium with serum can be used to subculture cells in both culture systems; the serum-free medium SF-900-II is usually used to subculture cells in suspension culture; however, the medium can also be used for nonsuspension culture with serum.

2. To passage cells from regular flasks, remove all but 3 or 9 ml of medium from the T25 or T75 flask with confluent monolayer cells, respectively. Dislodge the cells by gently blowing down the medium from a 10-ml pipette while sweeping the tip of the pipette across the monolayer from side to side. Start at the bottom and work your way to the top of the flask.
3. Transfer the 3 ml (or 9 ml) of cells suspension into three T25 (or T75) flasks containing 4 ml (or 12 ml) of fresh complete medium. Rock gently to humidify the entire growth surface, and distribute the cells evenly.
4. Incubate at 27°C until the cells are confluent, then passage as described above.

29.4.4.4 Freezing Insect Cells

1. Freeze the Cells in their logarithmic growth phase at 98% viability.
2. Remove cells from flasks and transfer into 15-ml (or 50-ml) centrifugation tubes, pellet down the cells by centrifugation at 230× *g* for 10 min, carefully remove all of the supernatant.
3. Gently resuspend the cells in a certain volume of cells storage solution containing 10% dimethylsulfoxide (DMSO) and 90% fetal bovine serum, at final cell density of 1×10^7 cells/ml.
4. Aliquot the cells into cryogenic tubes, and place the tubes into a slowly cooling container; store the container at −70°C overnight. Then, transfer the tubes very quickly to liquid nitrogen for long-term storage.
   Notes:
   i. Sf9 cells can be grown as monolayer or suspension cultures and transitioned repeatedly between either type of culture without significant changes in cell viability or growth rate. After 30–35 passages, if the cell viability or growth rate tends to decrease over time, we recommend that you recover new Sf9 cells from your storage.
   ii. Sf9 cells are sensitive to centrifugal forces and any other forces. Therefore do not exceed 250× *g* in centrifugation. When dislodging cells from flasks, avoid creating aerosols that can promote cell damage.
   iii. Always allow your cell culture medium to acquire room temperature before use.

29.4.5 Generation of Recombinant Baculoviruses by Co-transfection

1. Prepare at least 10 μg of highly purified plasmid DNA using the QIAGEN tip-100 midiprep kit. Resuspend the DNA in pure water with a final concentration of at least 0.5 μg/ul.
2. Seed 2×10^6 Sf9 cells into 60-mm tissue culture plates or 1×10^6 Sf9 cells into a well of six wells plate, and incubate the plates at 27°C for 20–30 min. The cells

should be attached and form a flat and even surface in the plate. If cells do not attach well after 30 min, they are not suitable to be used for transfection.

3. To co-transfect your plasmid DNA with linearized baculovirus DNA, combine 0.5-μg (5 μl) BaculoGold DNA (Pharmingen, Cat# 21100D) and 2–5-μg (5–10 μl) plasmid DNA in a microcentrifuge tube, mix well by pipetting up and down, incubate the mixture at room temperature for 5 min, and then add 1 ml of transfection Buffer B (Pharmingeb Cat# 21483A) to the mixture.
4. Remove the medium from the cells attached on the co-transfection plate and replace with 1 ml of transfection Buffer A (Pharmingen, Cat# 21100D). Make sure that all cells are covered to prevent their dehydration.
5. Slowly add 1-ml of the above mixture of Transfection buffer B plus DNA drop by drop to the co-transfection plate. After every three drops, gently rock the plate to mix the drops with medium. After the addition of transfection buffer B plus DNA, the medium in the plate should appear slightly cloudy due to the calcium phosphate/DNA precipitate.
6. Incubate the co-transfection plate at 27°C for 4 h, remove the medium from the plate, wash the plate with 4 ml of fresh TC-100 complete medium (or the insect cell culture complete medium) by gently rocking the plate. Remove the wash medium, add 3.5 ml of fresh medium to the plate, and incubate at 27°C for 4–5 days.
7. After 4–5 days, check the plate for signs of infection (larger cells, enlarged nuclei – for comparison, and keep a nontransfected culture in parallel. Please note that the appearance of cytopathic effects in recombinant virus-infected Sf9 cells is delayed in comparison with wildtype virus-infected cells, and does not contain crystal-like inclusion bodies). Harvest the supernatant medium; it is the original recombinant virus stock, designated P0 (passage zero). This stock can be stored at 4°C for at least half a year. The transfected Sf9 cells can be used to prepare cell slides to check for antibody expression by immunofluorecence staining (see below).
   Notes:
   i. The density of the Sf9 cells in the co-transfection plate should not be above 60–70%, since in 24–48 h after co-transfection, uninfected cells will still divide and can be infected.
   ii. Always keep transfection buffer B on ice before adding to the mixture of Baculovirus DNA and plasmid DNA to maintain high transfection efficiency.

29.4.6 Fast Detection of Recombinant IgG Expression in SF9 Cells After Transfection

29.4.6.1 Immunofluorescent Assay of the Recombinant Intact IgG Expressed in SF9 Cells

1. Remove the medium from recombinant baculovirus-infected SF9 cells attached on 60 mm co-transfection plates or a T25 flask, and gently wash the cells twice with PBS.

2. Add 3–3.5 ml PBS, gently dislodge the cells from the monolayer by pipetting, drop the cell suspension to multiple-well slides. Air dry at room temperature.
3. Put the slides into a slide-fixing glass container, and cover the entire slide with acetone for 10 min at room temperature for fixation. Remove the slides from the container. Air dry.
4. Add 25–30 μl of FITC-conjugated anti-human IgG, Fc or Fab antibodies per well (concentration according to manufacturer), and incubate at 37°C for 30 min.
5. Wash the slide three times with PBS and observe by regular or confocal immunofluorescent microscopy.
   Notes:
   i. Infected insect cells must be washed with PBS before fixing the cells to multiple slides since cells cultured in a medium containing serum are difficult to fix to the slides.
   ii. The observation time can be prolonged by embedding the stained cells in 50% glycerol in PBS (or commercial embedding media, e.g. Moviol).

29.4.6.2 Detection of the Expression by Sandwich ELISA

1. Dilute first antibody (unconjugated anti-human Fab antibody) in 0.1 M $NaHCO_3$, pH 8.6 coating buffer to final concentration about 5–10 μg/ml. Coat the unconjugated antibodies to the wells of a 96-well polyvinylchloride ELISA plate by adding 100 μl of the diluted antibody solution per well. Incubate at 4°C overnight.
2. Wash the wells three times with PBS.
3. Series dilute the supernatant medium from recombinant virus-infected SF9 cells (Could start at a range 1:2–1:100) with 3% skimmed milk powder in PBS. Add 100 μl of the dilutions to each well, and incubate at 37°C for 1 h.
4. Wash the wells three times with PBS.
5. Add 100 μl of diluted HRP-conjugated anti-human IgG Fc antibody. The amount of conjugated second antibody depends on the recommendation of the manufacturer. Incubate the plate at 37°C for 1 h.
6. Wash the wells five times with PBS
7. Develop the color by adding TMB substrate solution and detect the O. D value at 490 mm.

29.4.7 Purification and Titration of Recombinant Baculoviruses by Plaque Assay

The plaque assay can be used to purify the recombinant virus to increase the virus titer and to determine the recombinant virus titer in plaque-forming units per milliliter (pfu/ml)

1. Use a six-well cell culture plate. Remove the medium from a T75 flask with a confluent monolayer of SF9 cells. Add 20 ml of fresh TC-100 complete medium, and dislodge the cells by gently pipetting. Add 3 ml of the SF9 cell suspension to each well (about 2×10^6 cells) of the six-well cell culture plate. Allow the cells to attach to the plate at 27°C for 1 h.
2. Prepare a dilution series (from 10^{-2} to 10^{-7}) of the recombinant virus stock with SF-900 II medium. Carefully remove the medium from the six-well plate, and add 200 µl of the respective virus dilution per well. Make sure that the entire bottom surface of the well is covered with the solution. Incubate at 27°C in a humid atmosphere for 1 h (e.g., by putting the six-well pate into a larger box containing a moistured tissue) to allow infection of the cells by the virus particles.
3. During the above mentioned process of incubation, prepare a 2% agarose solution of cell culture quality low melting point agarose (Agarplaque Agarose, Pharmingen, cat# 21403A or Seakem ME, FMC Corp) in a protein-free medium (Pharmingen Cat#21228M#) and, heat the solution in a microwave oven until the solution begins to boil and the agarose dissolves completely (about 30 s to 1 min). Cool to 40°C by putting into a waterbath 15 min before use. Let it warm up the Sf-900-II SFM medium (Life Technology) to room temperature (takes approx. 20 min).
4. Mix equal volumes of agarose solution and Sf-900-II SFM medium. The final solution therefore contains approx. 1% agarose. Apply a first agarose layer onto the plate by carefully adding 3 ml of the mixed agarose solution per well to the side of the titled plate. Allow the agarose to harden at a leveled surface. Incubate the plate in a humid environment at 27°C for 4–5 days.
5. On day 5–6, prepare a second layer of agarose similar to the first layer of agarose described above but with the addition of 2% Neutral Red Solution (GIBCO-BRL). Incubate at 27°C for 12–24 h.
6. Observe the Plaques on the next day after adding a second layer containing Neutral Red solution. Visualize the Plaques by putting the plate against natural light or white illuminating light. Count the plaque number. Determine the Virus titers using the following formula:

$$\text{pfu/ml} = \text{Plaque number} \times \text{highest dilution} \times 5.$$

7. (*The next steps are required for recombinant virus purification only*) Remove all medium from one T75 flask containing more than 90% confluent SF9 cells, add 30 ml of fresh medium, dislodge the cells, seed 1 ml of the cell suspension into each well of 24-well cell culture plate, let cells attach at 27°C for 30 min.
8. Remove all medium, and replace with fresh medium (culture medium or SF-900-II serum-free medium). Mark plaques on the back side of the agar plates from Step 5 with a color pen. Pick up the plaques using a sterile Pasteur pipette and bulb. Carefully penetrate the agarose containing a recombinant virus plaque, and transfer the agarose plug into a well of the 24-well plate; pick at least five

plaques of each transfection. Incubate the plate at 27°C in a humid environment until visible infection appears (5–7 days).

9. Harvest the supernatant, and store at 4°C. These clonally purified stocks are designated P1. Check the cells in each well to make sure that the P1 stocks from positive plaques do not contain polyhedra.
   Notes:
   i. The quality of the SF9 cells used for plaque assay and the cell density of confluent cell monolayers are very important factors for efficient plaque formation. The viability of the cells should be more than 98% and the density of the monolayer cells should be around 70%. The use of cell densities that are higher or lower than 70% will not result in efficient plaque formation.
   ii. To obtain high titer stocks and a stable recombinant virus, plaque purification is always recommended.

29.4.8 Preparation and Amplification of High-Titer Viral Stock

1. Seed a T25 Flask with 2×10^6 log phase SF9 cells in 5 ml of complete TC-100 medium. Allow the cells to attach at 27°C for 1 h, and then change the medium (serum medium or a serum-free medium will do).
2. Add 50 μl of P1 stock from the plaque purification to the T25 flask, and incubate at 27°C for 5–7 days or until the cells are completely lysed. Transfer the infected medium into a sterile 15-ml centrifuge tube, centrifuge for 1,000× *g* for 10 min at 4°C, harvest the supernatant, and store at 4°C as P2 stock.
3. Infect T75 flasks with each 200–300 μl of P2 recombinant virus stock, incubate at 27°C for 5–6 days, harvest the supernatant as step 2. Aliquot the virus stock, store part of them at −70°C for long term storage, keep the rest at 4°C as reserve stock. Baculovirus will be stable at 4°C at least for a half year, usually even more than 1 year.
4. (*Optional step instead of Step 3 for the generation of larger amounts of virus*) Infect a 250-ml suspension culture of log phase SF9 cells with 2.5 ml of P2 recombinant virus stock, and put the flask in an incubator shaker at 27°C, with 80 rpm shaking for 7–10 days. Transfer the cell suspension to a centrifuge tube, centrifuge at 4°C, 1,000× *g* for 15 min, collect the supernatant, and store at −70°C or 4°C as described in Step 3.
5. Determine the virus titer of your stocks by plaque assay (see above). The virus titer of recombinant baculovirus virus stock after amplification usually reaches 1×10^8/ml. The use of this high titer recombinant virus is optimal for high level expression of IgG antibodies.
   Note:
   Always check the IgG expression for every amplifying step by the IFA, as described above. PCR may also used to monitor the virus replication.

29.4.9 Expression of Recombinant Antibodies in Insect Cells

29.4.9.1 Non-suspension Cultures

1. Seed T75 culture flasks with about 2×10^7 SF9 cells or H5 cells per flask.
2. For optimal antibody production, infect cells with a multiplicity of infection (MOI) (MOI=1, see note i) of high-titer recombinant baculovirus. Usually, for virus stocks with a titer around 10^7–10^8 or higher, add 1–2 ml of recombinant virus into one T75 flask containing $>$ 90% confluent SF9 cells or 100% confluent H5 cells.
3. Incubate the cells at 27°C for 4–5 days. Examine the cells for viability and cytopathic effects. The cells should be enlarged in size, and large nuclei appear visible from inside the cell. The viability of total cells should be around 30–40% at the time of harvest. Higher or lower viabilities will result in lower yields of antibodies.
4. Harvest the culture supernatant. Recombinant human IgG antibody is produced in SF9 or H5 cells as a secreted protein. After centrifugation at 1,000$\times$ *g* for 15 min, keep the supernatant at 4°C for further purification and characterization.
5. Check the cells in the flask by the IFA described above. Figure 29.2 shows a typical immunofluorescence of recombinant human IgG in SF9 cells stained with FITC-conjugated anti-human IgG Fab (A) or Fc (B) antibodies.

29.4.9.2 Suspension Cultures

1. Seed approximately 2×10^6 SF9 cell/ml in a 250 or 500 ml spinner flasks, the cells should be healthy (98% viable).

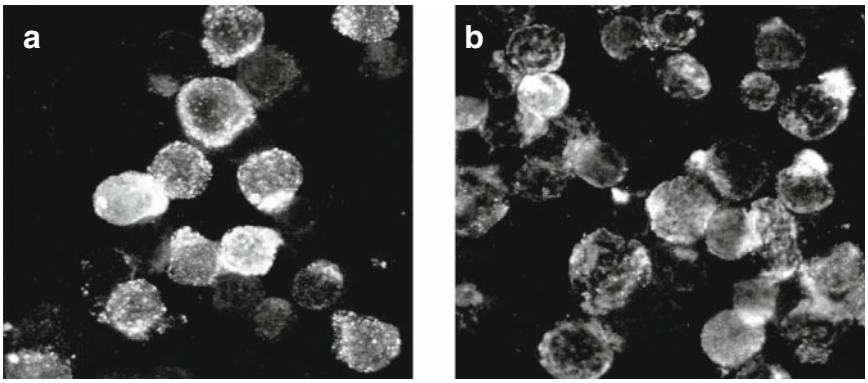


Fig. 29.2 Immunoflorescent detection of recombinant human IgG expressed in SF9 cells. (**a**) detection by FITC conjugated anti-human IgG Fc antibodies. (**b**) detection by FITC conjugated anti-human IgG Fab antibodies

2. For optimal antibody production, infect cells with a MOI (MOI=1, see note i) of high titer recombinant baculovirus. Usually, for virus stocks with a titer around 108 or higher (Note iii), add 5 ml of recombinant virus into a 250-ml flask
3. Incubate the spinner flask at 27°C a with shaking speed of 100 rpm for 4–5 days. Examine the cells for viability and cytopathic effects. The cells should be enlarged in size, and large nuclei appear visible from inside the cell. The viability of the total cells should be around 30–40% at the time of harvest. Higher or lower viabilities will result in lower yields of antibodies.
4. Harvest the culture supernatant. Recombinant human IgG antibody is produced in SF9 or H5 cells as a secreted protein. After centrifugation at 1,000× *g* for 15 min, keep the supernatant at 4°C for further purification and characterization.
5. Check the cells in the flask by the immunofluorescent assay described above. Figure 29.2 shows a typical immunofluorescence of recombinant human IgG in SF9 cells stained with FITC-conjugated anti-human IgG Fab (A) or Fc (B) antibodies.

   Notes:

   i. To obtain optimal protein production, the cells should be infected at a particular multiplicity of infection (MOI = 1). Therefore, it is essential to know the concentration of the recombinant virus to be inoculated. MOI can be calculated using the following formula:

$$\mathrm{MOI(pfu/cell)} = \frac{\text{Virus titer (pfu/ml)} \times \text{ml of inoculums}}{\text{Number of total cells to be infected}}.$$

   Accordingly, the volume of viral inoculums can be calculated from the following formula:

$$\text{ml of inoculums} = \frac{\text{MOI} \times \text{Number of total cells to be infected}}{\text{Virus titer (pfu/ml)}}$$

   ii. Both SF9 and H5 insect cells can be used to express recombinant human antibodies. Although H5 cells are particularly well suited for the expression of secreted recombinant protein, IgG expression with our vector system in H5 cells did not differ significantly from that observed using SF9 cells. The secreted IgG protein can be expressed at levels that approach 10–12 mg per liter.

   iii. For expression of IgG in suspension cultures, the virus titer must reach 10^8 or higher to avoid dilution of the cell cultures with large volumes of inoculums.

29.4.10 Affinity Purification of Recombinant Human IgG

1. Harvest 100–500 ml of the supernatants of recombinant virus infected Sf9 or H5 cells from flask or suspension culture 4–5 days after virus infection. Clear the

supernatants by centrifugation at 1,000× *g* at 4°C, and filter through 0.45 μm filters. Store it at 4°C. Warm to room temperature before loading (Step 3).

2. Equilibrate a 1 ml (or 5 ml) prepackaged protein G-sepharose affinity column (Pharmacia Biotech) to room temperature before use. Equilibrate the column with at least three volumes of start buffer (20 mM sodium phosphate, pH 7.0).
3. Apply at least 100 ml (or 500 ml) of the above filtered supernatants onto a 1-ml (or 5 ml, respectively) affinity column using a syringe or by slow pumping.
4. Wash the column with 5 column volumes of start buffer.
5. Elute with 3 column volumes of elution buffer (0.1 M glycine-HCL, pH 2.7). Collect 1ml fractions. To each fraction, add about 25–30 μl of 1 M Tris–HCL, pH 9.0, to adjust the fractions to pH 7-8.
6. Estimate the IgG concentration by determining the absorption at 260 and 280 nm in a photometer using the formula:

$$\text{IgG concentration mg/ml} = (1.55 \times \text{A280}) - (0.76 \times \text{A260}).$$

The eluted IgG can be concentrated by using centricon-10 protein concentrators (Amicon). Usually, the peak concentration of the eluted IgG fractions

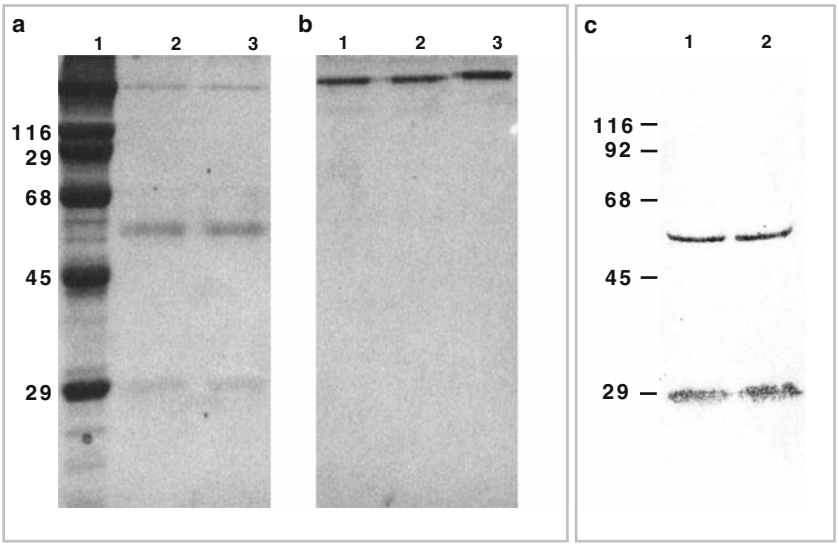


Fig. 29.3 SDS-PAGE and immunoblot analysis of recombinant human IgG produced by SF9 cells. A,B, coomassie blue stain, C, Immunoblot. Antibodies purified from the supernatants of recombinant baculovirus infected SF9 cells were analysed by SDS-PAGE using reducing (**a**) or nonreducing (**b**) conditions. Lane A-1, size Marker, lane A-2, IgG from vector pAc-L-CH3; lane A-3, IgG from vector pAc-L-Fc. Lane B-1, IgG from vector pAc-L-CH3, lane B-2, IgG from vector pAc-L-Fc. Lane B-3, Control (Human IgG franction obtained from Sigma, Deisenhofen, Germany). (**c**) Immunoblot stainig with HRP conjugated anti-human IgG (Lane C-1, IgG from vector pAc-L-CH3, lane C-2, IgG from vector pAc-L-Fc)

reaches or exceeds 200 μg/ml. Therefore, the concentration step may not be necessary for the subsequent experiments.

7. Check the purity of IgG protein by SDS-PAGE with a reducing and nonreducing condition. A typical result is shown in Fig. 29.3.

References

Barbas CF III, Lerner RA (1991) Combinatorial Immunoglobulin Libraries on the surface of phage (Phabs): rapid selection of antigen-specific Fabs. Methods: companion meth enzymol 2: 119–124

Hasemann CA, Capra JD (1990) High level production of a functional immunoglobulin heterodimer in a baculovirus expression system. Proc Natl Acad Sci USA 87:3942–3946

Jung S, Spinelli S, Schimmele B et al (2001) The importance of framework residues H6, H7 and H10 in antibody heavy chains: experimental evidence for a new structural subclassification of antibody V(H) domains. J Mol Biol 309(3):701–716

Kang AS, Burton DR, Lerner R (1991) Combinatorial immunoglobulin libraries. Methods: Companion Meth Enzymol 2:106–110

Liang MF, Mary G, Lunkvist A, Schmaljohn CS (1997) Baculovirus expression of a human G2-Specific, neutralizing IgG moclonal antibody to Puumala virus. Virology 235:252–260

Miller LK (1996) Insect virus. Fields virology. Lippincott-Raven, New York, p 533

Nesbit M, Fu ZF, McDonald-Smith J, Steplewski Z, Curtis P (1992) Production of a functional monoclonal antibody recgnizing human colorectal carcinoma cells from a baculovirus expression system. J Immunol Methods 151:201–208

Poul MA, Cerutti M, Chaabihl H, Devauchelle G, Kaczorek M, Lefranc MP (1995) Design of cassette baculovirus vectors for the production of therapeutic antibodies in sect cells Immunotechnology 1:189–196

Welschof M, Terness P, Kipriyanov S, Stanescu D, Breitling F, Dorsam H, Dübel S, Little M, Opelz G (1997) The antigen binding domain of a human IgG-anti-F(ab')2 autoantibody. Proc Natl Acad Sci USA 94:1902–1907

Weyer G, Knight S, Possee RD (1990) Analysis of very late gene expression by Autographa californica nuclear polyhedrosis virus and the further development of multiple expression fectors. J Gen Virol 71:1525–1534

Chapter 30
Expression of IgA Molecules in Mammalian Cells

Thomas Beyer, Stefan Lohse, Michael Dechant, and Thomas Valerius

30.1 Introduction

Immunoglobulin A (IgA) is the predominant antibody class in external secretions at mucosal surfaces and the second most prevalent antibody in serum, demonstrating its important biological role. Recent results also increasingly indicate the therapeutic potential of antibodies of the IgA isotype (van Egmond 2008; Woof and Mestecky 2005; Yoo and Morrison 2005). Thus, IgA molecules proved to be effective in neutralizing exotoxins and providing protection at mucosal surfaces, against both bacteria and viruses (Brandtzaeg 2007; Corthesy 2002). Additionally, they are capable of effectively triggering respiratory burst and phagocytosis by human leukocytes (Wines and Hogarth 2006). Furthermore, IgA antibodies were demonstrated to be exceptionally effective in recruiting polymorphonuclear cells (PMN), the most numerous human effector cells, for tumor therapy (van Egmond 2008). Indeed, PMN were more strongly activated by human IgA than by human IgG1 antibodies (Dechant et al. 2007).

In humans, two isotypes, IgA1 and IgA2, differing mainly in the length of their hinge regions – with IgA2 lacking a proline-rich sequence of 13 amino acids – and their glycosylation were described (see below). Three IgA2 allotypes, IgA2m(1), IgA2m(2), and IgA2(n) were described in humans, which differ in their H and L chain disulfide binding patterns. In IgA2m(2) and IgA2(n), heavy and light chains

T. Beyer, M. Dechant
Division of Nephrology and Hypertension, Christian-Albrechts-University, Schittenhelmstrasse 12, Kiel 24105, Germany
e-mail: beyer@nephro.uni-kiel.de; dechant@nephro.uni-kiel.de

S. Lohse and T. Valerius (✉)
Division of Stem Cell Transplantation and Immunotherapy, Christian-Albrechts-University, Schittenhelmstrasse 12, Kiel 24105, Germany
e-mail: s.lohse@med2.uni-kiel.de; t.valerius@med2.uni-kiel.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_30,

are linked by disulfide bonds like in IgA1 and IgG isotypes, whereas the light chains of IgA2m(1) allotype form disulfide bridges between each other (Chintalacharuvu and Morrison 1996). At the *C*-terminal end, IgA antibodies contain an additional region of 18 amino acids, termed tailpiece region, containing a penultimate cysteine residue, which is involved in dimerisation and is important for the binding of the so-called joining-(J)-chain. IgA in serum exists mainly in the monomeric form, whereas plasma cells at mucosal sites predominantly express J-chain containing IgA dimers. These dimers bind to the polymeric immunoglobulin receptor (pIgR) on serosal tissues. After transepithelial transport to luminal surfaces, the proteolytically cleaved extracellular part of pIgR remains associated with dimeric IgA, resulting in the so-called secretory IgA (Kerr 1990).

IgG antibodies generally have one N-glycan attached to Asn297, whereas only one third of circulating human IgG is additionally N-glycosylated in the Fab region (Wright and Morrison 1997). In contrast, IgA antibodies are heavily glycosylated. IgA1 antibodies contain two N-linked carbohydrates and up to six O-linked sugars in their hinge region (Woof and Kerr 2006). IgA2 molecules lack O-glycans but have two additional N-glycans at positions 166 and 337. Alloforms IgA2(m2) and IgA2(n) contain a fifth N-glycan at position 211. Depending on the carbohydrate position, the protein, and the expression system, these N-linked glycans either remain as high mannose or can be processed to complex bi- or triantennary oligosaccharides. Thus, glycoanalysis of pooled monomeric serum IgA1 revealed over 80% of N-glycans to be sialylated biantennary complex oligosaccharides (Mattu et al. 1998). Given the exceptional solvent-exposed nature of many of the carbohydrate moieties on IgA (Gomes et al. 2008; Herr et al. 2003) and their influence in natural and pathological functions of IgA antibodies (Novak et al. 2008; Yoo and Morrison 2005), expression systems' capability of posttranslational modifications, like addition of O- and N-linked carbohydrates, is of importance. Depending on the scope of application several expression systems, like yeasts, insect cells, plants, transgenic animals or mammalian expression systems, like mouse myeloma and Chinese hamster ovary (CHO) cells, have been used successfully for IgA expression (Chintalacharuvu and Morrison 1999; Yoo and Morrison 2005). CHO cells were used for expression of several approved antibodies (Chu and Robinson 2001) with glycosylation patterns similar to the natural proteins. However, the CHO-Pro5 cell line was reported to not correctly assemble IgA2 antibodies (Chintalacharuvu et al. 2007), and other CHO subclones were demonstrated to lack glycosyltransferases necessary for generating both bisecting GlcNAc and $\alpha 2 \rightarrow 6$ linked sialic acid (Yoo and Morrison 2005), but so far these modifications were not reported to be of functional relevance.

Presently, selection pressure and gene amplification of CHO transfectants are commonly maintained by either methotrexate – utilizing the transfected dihydrofolate reductase (DHFR) gene – or by methionine sulfoximine (MSX) inhibiting endogenous glutamine synthesis and utilizing the transfected glutamine synthetase (GS) gene (Cockett et al. 1990; Pu et al. 1998). The GS system has several advantages compared to DHFR. Thus, high producing transfectomas can be obtained more rapidly and intracellular glutamine production in

glutamine-free medium results in less accumulation of ammonia – a toxic by-product – simplifying culture processes especially at high cell densities (de la Cruz Edmonds et al. 2006).

Despite several approaches, so far no broadly available and universally applicable method for IgA purification was established – in contrast to, e.g., protein A based affinity purification for IgG. Precipitation of IgA with caprylic acid and ammonium sulfate is cheap and easily performed but protein denaturation can occur and purity of antibodies was described to be insufficient (Perosa et al. 1990). Similarly, affinity chromatography with thiophilic agarose is limited by insufficient purity (Belew et al. 1987; Leibl et al. 1995). Affinity chromatography with the lectin Jacalin yields highly pure IgA1 antibodies, but is limited to the IgA1 isotype (Gregory et al. 1987), whereas purification via Protein L, a bacterial protein, was demonstrated to be restricted to specific kappa light chain families (Nilson et al. 1992). Another approach is affinity chromatography using antibodies´ specificity, but is therefore also of immanently limited use (Morton et al. 1993). Attempts to use IgA binding bacterial proteins or synthetic peptides have been hampered by unfavorable binding properties (Lindahl et al. 1990), specificities (Areschoug et al. 2002; Stenberg et al. 1994; Thern et al. 1995) or limited availability (Ronnmark et al. 2002; Sandin et al. 2002).

The two step purification method described in this chapter, using human kappa light chain affinity chromatography and subsequent separation of IgA monomers, dimers or polymers by size is ubiquitously applicable and highly efficient for IgA purification. Combined with the described expression and production methods, these protocols may help overcoming problems with recombinant IgA production.

30.2 Materials

30.2.1 Cloning of Expression Vector

- IgA heavy and light chain constructs (GenBank: alpha 1: AY647978; alpha 2: AY647979)
- pEE14.4 expression vector (Lonza Biologics, Slough, UK)
- Mach1 chemically competent *E.coli* (Invitrogen, Carlsbad, CA, USA)
- Restriction endonucleases *BamHI*, *EcoRI*, *SmaI* and enzyme buffers (New England Biolabs, Frankfurt, Germany)
- DNA dephosphorylation enzyme (Roche, Basel, Switzerland)
- 1% agarose gel: dissolve 1 g of agarose in 100 ml 1× TAE buffer
- 1× TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0
- T4 DNA ligase and buffer (Roche)
- Luria-Bertani medium (LB): 5 g of NaCl, 10 g of bacto-tryptone and 5 g of yeast extract per 1 L

- LB/amp/1% glucose medium: LB medium containing 100 μg/ml ampicillin and 1% glucose
- Agarose gel electrophoresis equipment (Biorad Laboratories, Hercules, CA, USA)
- Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany)
- QIAquick Gel Extraction Kit (Qiagen)
- ABI PRISM 310 automated sequencer (Applied Biosystems, Foster City, CA, USA)

30.2.2 Expression of IgA

30.2.2.1 Pre-adaptation of CHO-K1 Cells to Serum-Free Medium in Suspension Culture

- CHO-K1 cells (Lonza Biologics)
- Tissue culture flasks, flasks for suspension culture (Sarstedt, Nuernbrecht, Germany)
- DMEM medium (Invitrogen)
- Fetal calf serum (FCS) (Invitrogen)
- Chemically defined (CD)-CHO medium (Invitrogen)
- HT-Supplement:10 mM sodium hypoxanthine, 1.6 mM thymidine (Invitrogen)
- Pen/Strep: Penicillin/Streptomycin 100× (PAA, Pasching, Austria)
- Methionine sulfoximine (Sigma, St Louis, MO, USA)

30.2.2.2 Transfection of Adapted CHO-K1 Cells

- DMEM10: DMEM medium with 10% FCS and 1% Pen/Strep
- Six-well flat bottom tissue culture plates (Greiner Bio-one, Frickenhausen, Germany)
- 96-well flat bottom tissue culture plates (Greiner Bio-one)
- Optimem (Invitrogen)
- Lipofectamine 2000 (Invitrogen)
- L-glutamine-free DMEM medium (Invitrogen)
- Dialysed FCS (Invitrogen)
- L-glutamate, asparagine, guanosine, cytidine, uridine and thymidine (Sigma)
- Non-essential amino acids (Invitrogen)
- D-Select: glutamine-free DMEM containing 10% dialysed heat-inactivated FCS, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, glutamate (6 μg/ml), asparagine (6 μg/ml), guanosine (7 μg/ml), cytidine (7 μg/ml), uridine (7 μg/ml), thymidine (2.4 μg/ml), 1% 100× non-essential amino acids and 50 μM MSX.

- CDCHO-Adapt: CD-CHO medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, 1% HT-supplement and 10 ng/ml phenol red.
- CDCHO-Select: CDCHO-Adapt containing 50 μM MSX

30.2.2.3 Screening for Positive Clones

- Nunc-Immuno Module F8 Maxisorp (Thermo Fisher Scientific, Roskilde, Denmark)
- Polyclonal anti-human κ-light chain antibody (Caltag, Buckingham, UK)
- HRP-conjugated polyclonal anti-human IgA antibody (Jackson Immuno-research, Newmarket, UK)
- Bovine serum albumin (Sigma)
- Tween 20 (Merck, Darmstadt, Germany)
- Washing buffer: 0.05% Tween 20, 3% BSA in water
- SigmaFAST o-Phenylenediamine-dihydrochloride (OPD) (Sigma)
- Sunrise absorbance reader (Tecan, Groeding, Austria)

30.2.2.4 Production

- Miniperm roller bottles (Greiner Bio-one)
- Miniperm Production & Nutrient Module (Greiner Bio-one)
- Heraeus Heracell 240 Incubator (Heraeus, Hanau, Germany)

30.2.3 Purification

- Vivacell 100 Pressure Units, 100 kDa MWCO (Sartorius, Goettingen, Germany)
- HighPrep 26/10 desalting columns (GE Healthcare, Munich, Germany)
- PBS: 10 mM phosphate buffer, 2.7 mM KCl, 0.14 M NaCl, pH 7.2, (PAA)
- Anti-human κ-light chain single domain antibody matrix (Capture Select, Naarden, The Netherlands)
- Elution buffer: 0.1 M glycine at pH 2.5
- Neutralization buffer: 0.1 M Tris at pH 8.0
- Superdex 200 26/60 columns (GE Healthcare)
- Vivaspin 15 concentrators,100 kDa MWCO (Sartorius)
- HMW Gel Filtration Calibration Kit (GE Healthcare)
- 0.2 μm sterile filters (Sartorius)
- Superdex200 16/300 column (GE Healthcare)
- AKTAprime liquid chromatography system (GE Healthcare)
- Unicorn 4.11 software (GE Healthcare)

30.2.4 Quantification

- Biuret reaction Kit BCA Protein Assay Kit (Thermo Scientific, Rockford, IL)
- UV/Vis Photo spectrometer UltraSpec 2100 (GE Healthcare)
- Capillar electrophoresis, Experion Pro260 analysis kit and Chip (Bio-Rad)

30.2.5 Determination of Antibody Integrity and Purity

- Native Page 4–16% Bis-Tris gel (Invitrogen)
- Native Page 20× Running Buffer (Invitrogen)
- Native Page 4× Sample Buffer (Invitrogen)
- 5% G-250 Sample Additive (Invitrogen)
- Capillar electrophoresis, Experion Pro260 analysis kit and Chip (Bio-Rad)

30.2.6 Characterisation

30.2.6.1 Immunoblotting

- HRP-conjugated polyclonal goat anti-human IgA antibody (Jackson Immunoresearch)
- Polyclonal goat anti-human κ-light chain (Biozol, Eching, Germany)
- Precision Plus Protein Dual Color Standard (Biorad)
- X Cell Sure Lock System (Invitrogen)
- 3–8% Tris-acetate gel (Invitrogen)
- Human myeloma IgA1 and IgA2 (Biodesign International, Saco, ME, USA)
- PVDF-membrane (GE Healthcare)
- Blotting buffer: 50 mM Tris, 0.38 M glycine, 0.1% SDS and 20% methanol
- Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA)

30.2.6.2 Flow Cytometry (FACS)

- 15 ml polystyrene tubes (Sarstedt)
- Sodium azide (Merck)
- A431 cells (ACC 91, DSMZ)
- BHK-21 cells co-transfected with FcαRI (CD89) and FcR γ-chain
- Geneticin (PAA) and methotrexate (Sigma)
- Polyclonal fluorescein isothiocyanate-(FITC)-conjugated mouse anti-human κ-light chain antibody (Caltag)
- Human myeloma IgA1 and IgA2 (Biodesign International)
- Flow cytometer, Coulter EPICS XL-MCL (Beckman Coulter)

30.3 Protocols

Methods that are not specifically described step by step are to be done according to manufacturers´ protocols.

30.3.1 Cloning of Expression Vector

1. Genes for the variable regions can be cloned as described in Boel et al. (2000).
2. Insert variable regions into pUC-HAVT20-vector to incorporate HAVT20-leader sequence (see Notes).
3. Confirm correct sequence by DNA sequencing analysis using M13 primers.
4. Subclone HAVT20-variable regions into vectors encoding human constant regions (e.g., pNUT) (see Notes and Fig. 30.1).
5. For optimal antibody expression clone expression cassettes into pEE14.4-expression vector digested with appropriate restriction enzymes (e.g., *Sma*I, *EcoR*I) and transform into *E.coli* Mach1.
6. Confirm correct sequence by DNA sequencing.

30.3.2 Expression of IgA

30.3.2.1 Pre-adaptation

To avoid recurrent time consuming periods of adapting CHO-K1 transfectomas to serum-free suspension culture conditions, cells can be adapted before transfection. The same procedure could be used to adapt CHO-K1 transfectomas using CDCHO-Select instead of CDCHO-Adapt.

1. Passage CHO-K1 cells at 3×10^5 viable cells with 9 ml CDCHO-Select and 1 ml DMEM10 in a 50 ml flask and incubate in a humidified incubator with 5% CO_2 in air at 37°C.

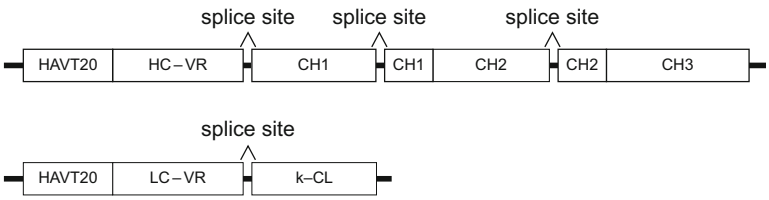


Fig. 30.1 Expression cassettes of heavy and light chains. Splice sites of the genomic DNA as well as the exons for heavy chain (HC)- or light chain (LC)-variable regions (VR) and constant regions (κ-CL, CH1-3) are indicated

2. Repeat step 1 every 4 days until cell concentration at passage is consistently double the seeding concentration.
3. Passage CHO-K1 cells at 3×10^5 viable cells with 9.9 ml CDCHO-Select and 0.1 ml DMEM10 and incubate in a humidified incubator with 5% CO_2 in air at 37°C. Repeat this step every 4 days until cell concentration at passage is consistently double the seeding concentration (see Notes).
4. Passage CHO-K1 cells at 3×10^5 viable cells into 10 ml CDCHO-Select and incubate in a humidified incubator with 5% CO_2 in air at 37°C. Repeat this step every 4 days until cell concentration at passage is consistently double the seeding concentration.
5. If CHO-K1 cells do not concurrently adapt to suspension culture during this procedure, gas flasks for 30 sec with 5% CO_2 in air and incubate at 37°C in an orbital shaking incubator at 140 rpm. Repeat this step every 4 days until all cells are suspension adapted.
6. Use suspension culture flasks from now on.

30.3.2.2 Stable Transfection of Pre-adapted CHO-K1 Cells

As positive controls use GFP-expression vector and pEE14.4 expression vector without insert, as negative control, a mock transfection should be performed.

1. Twenty-four hours before transfection seed 3.5×10^5 cells per well in a 6-well-plate and incubate with DMEM10 to permit cell adhesion.
2. Cotransfection is performed according to manufacturers' protocol with 10 µl Lipofectamine 2000 and 2 µg light chain and 2 µg heavy chain pEE14.4-expression vector constructs as well as Optimem for all washing and incubation steps.
3. Add 3 ml of DMEM10 per well after 4 h incubation.
4. After 24 h, replace medium with 4 ml of D-Select.
5. After 2 days, trypsinize adherent cells and perform limiting dilution into 96-well plates with 200 µl/well CDCHO-Select. Incubate for 6–8 weeks (see Notes).
6. To assure single cell clones a second limiting dilution step is recommended.

30.3.2.3 Screening for Positive Clones

Screening for best expressing clones is performed using a Sandwich ELISA according to standard techniques.

1. Coat 96-well-plates with 100 µl of polyclonal anti-human κ-light chain antibody at 1 µg/ml for 12 h at 4°C.
2. Wash twice with 125 µl washing buffer between all incubation steps.
3. Incubate with 100 µl supernatants for 30 min at room temperature.
4. Incubate with polyclonal anti-human IgA antibody at a dilution of 1:10.000 for 30 min at room temperature.

5. Perform staining with 125 µl staining solution and stop reaction by adding 75 µl of 3 M HCl after adequate staining of positive controls/standard dilution.
6. Readout is absorption at 492 nm.

30.3.2.4 Production

1. Fill 35 ml of CDCHO-Select and 2×10^6 cells/ml of adapted transfectomas into the production unit of a roller bottle according to manufacturers' instructions. Add 450 ml of CDCHO-Select into the supply-unit.
2. Incubate on a production module at 25 rpm in an incubator with 5% CO_2 in air at 37°C. Take 200 µl samples every second day to determine cell growth and viability and proceed to step 3 two days after cell growth is slowing down and death cell rate is increasing. (In our case approx. at day 7.)
3. Harvest cell suspension, count cells and refill the roller bottle as described in step 1 with cells from roller bottle culture. Centrifuge supernatant at 4,000 rpm for 10 min to separate antibody solution from cellular debris.

30.3.3 Purification

30.3.3.1 Concentration of Supernatant

Concentrate supernatant after sterile-filtration to shorten following processing times.

1. Filtrate supernatant to avoid clogging during concentration procedure.
2. Concentrate supernatant tenfold using Vivacell 100 Pressure Units according to manufacturers' protocol.

30.3.3.2 Desalting

Desalt concentrated supernatants to obtain buffer conditions required for antibody purification.

1. Load a 10 ml loop with the equal volume of supernatant.
2. Perform desalting using a HighPrep 26/10 desalting column and an AKTAPurifier with PBS buffer at a flow rate of 1 ml/min according to manufacturers' program and protocol.

30.3.3.3 Anti-human Kappa-Light Chain Affinity Purification

1. Pack a XK16 column with anti-human κ-light chain matrix using 5 ml of agarose matrix and sediment it with 10% ethanol at a flow rate of 0.5 ml/min.
2. Perform a blunk/blank run to avoid contamination of your probes by leaking.
3. Equilibrate packed column with 5 column volumes (CV) of PBS.

4. Load antibody solution onto the anti-kappa column at a flow rate of 1 ml/min.
5. Wash column with 10 CV of PBS.
6. Elute column-bound antibody with 4 CV of elution buffer.
7. Adjust eluted fractions to physiological pH by adding 150 µl of neutralization buffer per 1 ml eluat.
8. Concentrate and wash pooled antibody fractions thrice with 10 ml PBS each using Vivaspin 15 concentrators to change buffer conditions.

30.3.3.4 Size Exclusion Chromatography

To separate IgA monomers from dimers or higher aggregates and to control stability and purity of separated monomers size exclusion chromatography can be performed.

1. Equilibrate a Superdex 200 26/60 column with 3 CV of PBS at a flow rate of up to 5 ml/min.
2. Load a 2 ml loop with equal volume of concentrated antibody solution (>1 mg/ml).
3. Perform size exclusion run using an AKTAPurifier with PBS buffer at a flow rate of 1 ml/min, automatically collecting samples at 10 ml portions below absorption units of 100 mAU and at 2 ml portions above 100 mAU (see Notes).
4. For analytical runs use Superdex 200 16/300 columns, load up to 100 µl of antibody solution and perform the run at a flow rate of 0.3 ml/min.

30.3.4 Determination of Antibody Concentrations

1. For quantification of IgA antibody concentrations in supernatants sandwich ELISA can be used (see Sect. 3.2.3.).
2. Quantification of purified antibody can be performed by Biuret reaction using the BCA Protein Assay Kit according to manufacturers' protocol.
3. Concentration of purified IgA antibodies can also be calculated by determining absorption at 280 nm according to Beer–Lambert law for concentrations with A_{280nm} for 1 mg/ml = 1.3.
4. With capillary based electrophoresis antibody probes can be separated and concentration of individual protein bands can be calculated automatically by Experion System software on the basis of a quantity marker included in the running buffer. Buffers, probes and material have to be used or prepared as described by the manufacturer.

30.3.5 Determination of Antibody Integrity and Purity

1. To determine purity and integrity of IgA antibody use densitometric determination of electrophoresis runs under native conditions on 4–16% Bis-Tris gels.

2. To evaluate purity of IgA also use capillary based electrophoresis system, as described in Sect. 3.4. It calculates the quantity of every separated protein, thus mass percentage of every protein in the probe is determined.

30.3.6 Characterisation of Antibody

Immunoblotting and cytofluorimetric analyzes can be performed as described in standard protocols.

30.3.6.1 Immunoblotting

1. For immunoblotting of IgA antibodies 3–8% Tris Acetate Gels in the X Cell Sure Lock System can be used.
2. High molecular weight dimers or higher aggregates can be blotted on PVDF membranes using blotting buffer as described in Sect. 2.6. at 110 mA within 1.5 h.
3. Labeling of antibody is performed with either goat anti-human IgA (1:10.000) or goat anti-human κ-light chain (1:2.000) antibodies.

30.3.6.2 Flow Cytometry

1. EGF-R expressing human epidermoid carcinoma cell line A431 can be used as model for Fab-mediated binding analysis of EGF-R directed IgA antibodies.
2. BHK-21 cells co-transfected with FcαRI and FcR γ-chain can be used as model for Fc-mediated binding of IgA.
3. Incubate 1.5×10^5 cells per sample for 30 min or for 60 min if performing low affinity binding to FcαRI. To avoid cellular processes like internalization of EGF-R, cells have to be kept on ice.
4. Staining of antibodies can be performed with FITC-labeled mouse anti-human κ-light chain antibody.
5. Relative fluorescence intensities have to be calculated as ratio of mean linear fluorescence intensity of relevant to irrelevant isotype-matched antibodies.

30.4 Results

According to the methods described in this chapter κ-light chain and α1- or α2-heavy chains were subcloned into the pEE14.4-GS-expression vector and stably co-transfected in CHO-K1 cells. Using glutamine-free DMEM medium, containing

50 μM MSX to inhibit CHO's endogenous glutamine synthetase activity, IgA expression proved to be stable over more than 6 months, as analyzed by the determination of specific production rates (SPR) for several clones. As antibody purification as well as cultivation and subsequent up-scaling of transfectants were markedly improved by working with serum-free suspension cells, three methods to adapt CHO-K1 cells were investigated (Fig. 30.2). The conventional way to adapt cells to growth under suspension conditions first and subsequently to serum-free medium required 6–8 months. However, we were able to shorten this process to 3–4 months by adapting to serum-free CD-CHO medium first, as CHO-K1 cells turned out to lose adherence during this step. Importantly, no relevant decrease in SPR was measurable. Additionally, pre-adaptation of untransfected CHO-K1 cells to serum-free suspension culture and subsequent co-transfection with a slightly adjusted protocol led to comparable transfection efficiencies. SPRs of resulting clones were comparable between 0.8–2.2 picogram/cell/day (pcd) in culture flasks or between 0.13–0.34 pcd in roller bottles. The third adaptation method is the least time consuming way from transfection to monoclonal cell cultures in roller bottle bioreactors.

IgA antibody concentrations in bioreactors ranged between 20–70 μg/ml for different clones – resulting in yields of 0.7–2.5 mg antibodies per bioreactor per week. These concentrations are nearly 100 times lower than today's industrial

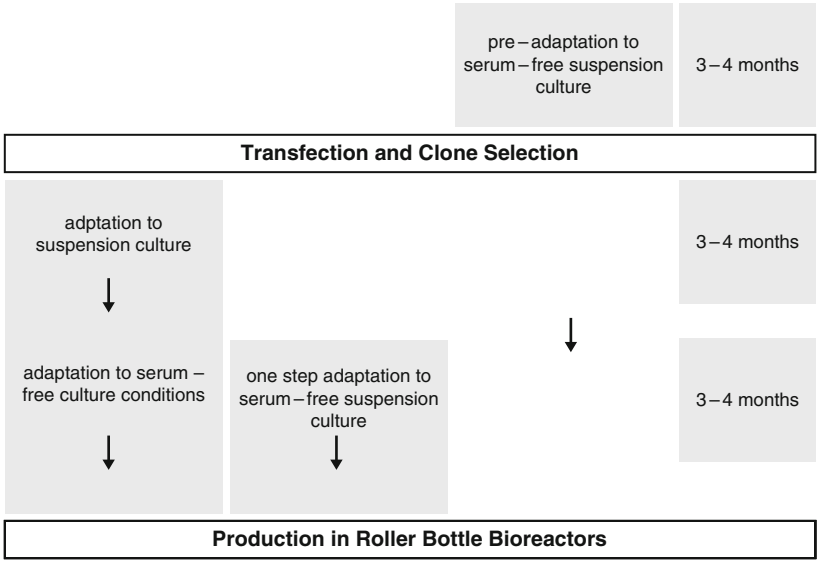


Fig. 30.2 Three different ways to adapt CHO-K1 cells to serum-free culture conditions are schematically presented. Most common is the two-step adaptation as demonstrated on the left side. Adaptation of CHO-K1 cells to CD-CHO medium, optimized for CHO cells, is also possible in a one-step process (middle). Most time can be saved by transfecting pre-adapted CHO-K1 cells avoiding the time consuming adaptation following transfection

IgG production levels (Wurm 2004), but further improvements in transfection, clone selection (Browne and Al-Rubeai 2007) and optimized culture conditions (Birch and Racher 2006) are possible and will certainly be objects of further studies.

In this chapter we also described a two step chromatographical purification protocol. First antibodies were affinity purified using an agarose matrix-bound ligand, directed against human κ-light chain constant regions followed by size exclusion chromatography. Thus, IgA antibody monomers with a purity of more than 96% were obtained, independent of the IgA isotype. Additionally, we were also able to specifically isolate dimers and higher polymers. As kappa constant regions are accessible in secretory IgA this purification method should also be applicable for secretory IgA (Bonner et al. 2008a, b). Integrity of purified IgA was analyzed by native gel electrophoresis and size exclusion chromatography (Fig. 30.3). These experiments demonstrated purified antibodies to be intact, even after 4 months of storage in PBS (Fig. 30.3). Indirect immunofluorescence analyzes confirmed specific binding of IgA monomers to their antigen (EGFR). Binding characteristics of both IgA isotypes were comparable (EC50: IgA1 15.9 µg/ml; IgA2 12.2 µg/ml) and in the same range as their IgG1 counterpart (EC50 8.9 µg/ml). Additionally, binding of both IgA1 and IgA2 isotypes to FcαRI-transfected BHK-21 cells proved to be identical. Functional tests like antibody mediated inhibition of cell proliferation and antibody-dependent cellular cytotoxicity (data not shown) affirmed antibody monomers generated and purified by the described methods to be fully functional – consistent to recently published data (Dechant et al. 2007).

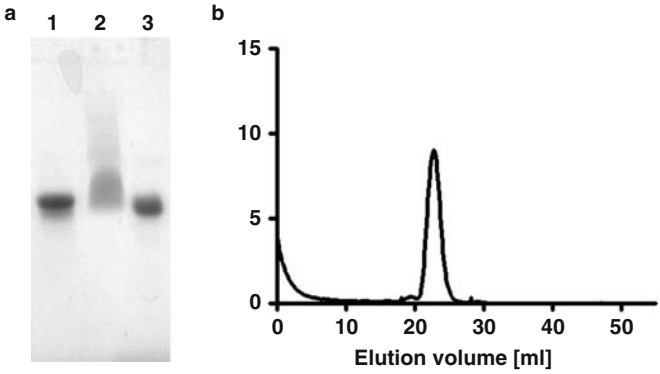


Fig. 30.3 (**a**) Native protein gel electrophoresis of purified IgA1 (lane 1) and IgA2 monomers (lane 3) compared to a monoclonal myeloma IgA2 standard (lane 2). Both purified recombinant antibodies demonstrated one single band at the size of the IgA2 standard monomers, confirming intact antibody molecules. (**b**) Size exclusion chromatography analysis of purified IgA2 monomers after 4 months of storage is depicted. No signs of aggregation or degradation were observed

30.5 Notes

1. Using hybridoma cells' endogenous secretion leader may positively influence antibody secretion.
2. Usage of genomic constant region DNA – containing natural splice sites – was demonstrated to improve antibody expression.
3. At regular intervals during adaptation to serum-free conditions stocks of the suspension adapted cells should be cryopreserved (Sect. 3.2.1).
4. It is recommended to disturb the plates as little as possible during incubation (Sect. 3.2.2).
5. High Molecular Weight Gel Filtration Calibration Kit as well as commercially available monoclonal myeloma IgA antibodies can be used to correlate the elution volume to protein size (Sect. 3.3.4).

30.6 Troubleshooting

1. Quantification of IgA1 and IgA2 antibodies in sandwich ELISA, as described above, cannot be compared directly, but have to be investigated with IgA1 or IgA2 standards separately (Sect. 3.2.3).
2. Avoid too high concentrations of IgA supernatant as this might cause antibody precipitation. Concentrations up to 700 μg/ml were feasible in roller bottle supernatants with CD-CHO medium (Sect. 3.3.1).
3. Avoid adding more Neutralization buffer than described as this might cause precipitation of antibody (Sect. 3.3.3).
4. When a cell line does not readily adapt to growth in serum-free medium, a more gradual reduction in serum concentration may be required (Sect. 3.2.1).

References

Areschoug T, Stalhammar-Carlemalm M, Karlsson I, Lindahl G (2002) Streptococcal beta protein has separate binding sites for human factor H and IgA-Fc. J Biol Chem 277(15):12642–12648

Belew M, Juntti N, Larsson A, Porath J (1987) A one-step purification method for monoclonal antibodies based on salt-promoted adsorption chromatography on a 'thiophilic' adsorbent. J Immunol Methods 102(2):173–182

Birch JR, Racher AJ (2006) Antibody production. Adv Drug Deliv Rev 58(5–6):671–685

Boel E, Verlaan S, Poppelier MJ, Westerdaal NA, Van Strijp JA, Logtenberg T (2000) Functional human monoclonal antibodies of all isotypes constructed from phage display library-derived single-chain Fv antibody fragments. J Immunol Methods 239(1–2):153–166

Bonner A, Almogren A, Furtado PB, Kerr MA, Perkins SJ (2008a) The non-planar secretory IgA2 and near-planar secretory IgA1 solution structures rationalise their different mucosal immune responses. J Biol Chem 284(8):5077–5087

Bonner A, Furtado PB, Almogren A, Kerr MA, Perkins SJ (2008b) Implications of the near-planar solution structure of human myeloma dimeric IgA1 for mucosal immunity and IgA nephropathy. J Immunol 180(2):1008–1018

Brandtzaeg P (2007) Induction of secretory immunity and memory at mucosal surfaces. Vaccine 25(30):5467–5484

Browne SM, Al-Rubeai M (2007) Selection methods for high-producing mammalian cell lines. Trends Biotechnol 25(9):425–432

Chintalacharuvu KR, Gurbaxani B, Morrison SL (2007) Incomplete assembly of IgA2m(2) in Chinese hamster ovary cells. Mol Immunol 44(13):3445–3452

Chintalacharuvu KR, Morrison SL (1996) Residues critical for H-L disulfide bond formation in human IgA1 and IgA2. J Immunol 157(8):3443–3449

Chintalacharuvu KR, Morrison SL (1999) Production and characterization of recombinant IgA. Immunotechnology 4(3–4):165–174

Chu L, Robinson DK (2001) Industrial choices for protein production by large-scale cell culture. Curr Opin Biotechnol 12(2):180–187

Cockett MI, Bebbington CR, Yarranton GT (1990) High level expression of tissue inhibitor of metalloproteinases in Chinese hamster ovary cells using glutamine synthetase gene amplification. Biotechnol (NY) 8(7):662–667

Corthesy B (2002) Recombinant immunoglobulin A: powerful tools for fundamental and applied research. Trends Biotechnol 20(2):65–71

de la Cruz Edmonds MC, Tellers M, Chan C, Salmon P, Robinson DK, Markusen J (2006) Development of transfection and high-producer screening protocols for the CHOK1SV cell system. Mol Biotechnol 34(2):179–190

Dechant M, Beyer T, Schneider-Merck T, Weisner W, Peipp M, van de Winkel JG, Valerius T (2007) Effector mechanisms of recombinant IgA antibodies against epidermal growth factor receptor. J Immunol 179(5):2936–2943

Gomes MM, Wall SB, Takahashi K, Novak J, Renfrow MB, Herr AB (2008) Analysis of IgA1 N-glycosylation and its contribution to FcalphaRI binding. Biochemistry 47(43):11285–11299

Gregory RL, Rundegren J, Arnold RR (1987) Separation of human IgA1 and IgA2 using jacalin-agarose chromatography. J Immunol Methods 99(1):101–106

Herr AB, Ballister ER, Bjorkman PJ (2003) Insights into IgA-mediated immune responses from the crystal structures of human FcalphaRI and its complex with IgA1-Fc. Nature 423 (6940):614–620

Kerr MA (1990) The structure and function of human IgA. Biochem J 271(2):285–296

Leibl H, Tomasits R, Mannhalter JW (1995) Isolation of human serum IgA using thiophilic adsorption chromatography. Protein Expr Purif 6(4):408–410

Lindahl G, Akerstrom B, Vaerman JP, Stenberg L (1990) Characterization of an IgA receptor from group B streptococci: specificity for serum IgA. Eur J Immunol 20(10):2241–2247

Mattu TS, Pleass RJ, Willis AC, Kilian M, Wormald MR, Lellouch AC, Rudd PM, Woof JM, Dwek RA (1998) The glycosylation and structure of human serum IgA1, Fab, and Fc regions and the role of N-glycosylation on Fc alpha receptor interactions. J Biol Chem 273(4):2260–2272

Morton HC, Atkin JD, Owens RJ, Woof JM (1993) Purification and characterization of chimeric human IgA1 and IgA2 expressed in COS and Chinese hamster ovary cells. J Immunol 151 (9):4743–4752

Nilson BH, Solomon A, Bjorck L, Akerstrom B (1992) Protein L from Peptostreptococcus magnus binds to the kappa light chain variable domain. J Biol Chem 267(4):2234–2239

Novak J, Julian BA, Tomana M, Mestecky J (2008) IgA glycosylation and IgA immune complexes in the pathogenesis of IgA nephropathy. Semin Nephrol 28(1):78–87

Perosa F, Carbone R, Ferrone S, Dammacco F (1990) Purification of human immunoglobulins by sequential precipitation with caprylic acid and ammonium sulphate. J Immunol Methods 128(1):9–16

Pu H, Cashion LM, Kretschmer PJ, Liu Z (1998) Rapid establishment of high-producing cell lines using dicistronic vectors with glutamine synthetase as the selection marker. Mol Biotechnol 10(1):17–25

Ronnmark J, Gronlund H, Uhlen M, Nygren PA (2002) Human immunoglobulin A (IgA)-specific ligands from combinatorial engineering of protein A. Eur J Biochem 269(11):2647–2655

Sandin C, Linse S, Areschoug T, Woof JM, Reinholdt J, Lindahl G (2002) Isolation and detection of human IgA using a streptococcal IgA-binding peptide. J Immunol 169(3):1357–1364

Stenberg L, O'Toole PW, Mestecky J, Lindahl G (1994) Molecular characterization of protein Sir, a streptococcal cell surface protein that binds both immunoglobulin A and immunoglobulin G. J Biol Chem 269(18):13458–13464

Thern A, Stenberg L, Dahlback B, Lindahl G (1995) Ig-binding surface proteins of Streptococcus pyogenes also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. J Immunol 154(1):375–386

van Egmond M (2008) Neutrophils in antibody-based immunotherapy of cancer. Expert Opin Biol Ther 8(1):83–94

Wines BD, Hogarth PM (2006) IgA receptors in health and disease. Tissue Antigens 68(2): 103–114

Woof JM, Kerr MA (2006) The function of immunoglobulin A in immunity. J Pathol 208(2): 270–282

Woof JM, Mestecky J (2005) Mucosal immunoglobulins. Immunol Rev 206:64–82

Wright A, Morrison SL (1997) Effect of glycosylation on antibody function: implications for genetic engineering. Trends Biotechnol 15(1):26–32

Wurm FM (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22(11):1393–1398

Yoo EM, Morrison SL (2005) IgA: an immune glycoprotein. Clin Immunol 116(1):3–10

Part V
Antibody Characterization

Chapter 31
Expression of Complete Antibodies in Transgenic Plants

Doreen M. Floss and Udo Conrad

31.1 Introduction

"Molecular farming" combines traditional agriculture and modern molecular biotechnology, and exploits the existing infrastructure of agricultural practice for the low-cost production of valuable proteins. The large scale production of recombinant proteins in general and also expression of proteins for pharmaceutical purposes ("molecular pharming") have been achieved in crop plants and domestic animals. With regard to the spread of microbial resistance to antibiotics and the emergence of new pathogens, passive immunization by recombinant antibodies is viewed as one of the most promising alternatives to combat infectious diseases (Casadevall et al. 2004). One example is the application of neutralizing antibodies against the Human Immunodeficiency Virus (HIV) to fight the main burden of the human society, AIDS, because even after two decades of intensive research, there exists no effective vaccine against HIV (Fauci et al. 2008). Current antibody production systems rely on mammalian cells grown in fermenters, which are expensive. Transgenic plants represent a versatile system of producing pharmaceutically active reagents because they offer general advantages in terms of production scale and economy, product safety, and ease of storage and distribution, and can be established in resource-poor areas (Teli and Timko 2004; Twyman et al. 2005; Ma et al. 2005; Boehm 2007).

Two important findings of the twentieth century are the prerequisites for modern plant biotechnology: (a) the capacity of the gram-negative soil bacterium *Agrobacterium tumefaciens* to transfer genes (Van Larebeke et al. 1974) and (b) the regeneration of plants from single cells (Barton et al. 1983). Nowadays, biolistic

D.M. Floss (✉)
Institute of Biochemistry, Christian Albrechts University, Olshausenstrasse 40, Kiel, Germany
e-mail: dfloss@biochem.uni-kiel.de

U. Conrad
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstrasse 3, Gatersleben, Germany

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_31,

transformation (Altpeter et al. 2005) and Agrobacterium-mediated gene transfer (Tzfira and Citovsky 2006) are the common methods to obtain genetically modified plants. For the generation of transgenic plants, which reliably accumulate assembled antibodies, two major strategies have been elaborated: the first involves the incorporation of both the light and heavy chain-encoding genes into a single transgene construct (e.g., During et al. 1990), and the second operates by separately transferring the two immunoglobulin genes into a pair of complementary transgenic lines. The two transgenes are reunited in a single plant after crosses of selected lines (e.g., Hiatt et al. 1989; Ma et al. 1994). Following these strategies, functional full-length antibodies have been produced in transgenic plants and other plant-based systems (Twyman et al. 2005; Stoger et al. 2005b). Human monoclonal antibodies, which posses a neutralization capacity against HIV-1, have been successfully expressed in transgenic plants (Rademacher et al. 2008; Ramessar et al. 2008, Floss et al. 2008, Floss et al. 2009a). The binding kinetics of these "plantibodies" were identical to those of their CHO cell-derived counterparts, making them suitable for prevention of HIV infections. In this view, the application of broadly-neutralizing human monoclonal antibodies (nAbs) to the vagina as a mucosal microbicide prior to intercourse is one promising strategy (Shattock and Moore 2003; www.microbicide.org).

In contrast to leaf material seeds, especially from cereals, e.g., barley, are attractive storage compartments for recombinant proteins because antibodies remain stable and functional even if mature seeds are stored at room temperature for several years (Fiedler and Conrad 1995; Stoger et al. 2005a).

The production of a full-length anti-HIV-1 antibody in tobacco seeds will be described in this chapter as an example. This human monoclonal antibody (mAb), 2F5 (Purtscher et al. 1994), binds to the HIV epitope ELDKWA (Muster et al. 1993). Its *in vivo* efficacy has been evaluated in both animal (Mascola et al. 1999, 2000; Baba et al. 2000) and clinical trials (Armbruster et al. 2002, 2004; Joos et al. 2006). The coding sequences for the antibody light (LC) and heavy chains (HC), including the native signal peptides for ER entry, were obtained from Polymun Scientific Immunbiologische Forschung GmbH (Vienna, Austria) and cloned individually into plant expression cassettes containing a seed-specific promoter. For seed-specific expression in transgenic tobacco, the LeB4 promoter (Baumlein et al. 1986) or the USP promoter (Baumlein et al. 1991) from *Vicia faba* can be used. Detection of antibody chains in plants and comparison of accumulation levels is simpler if a peptide tag sequence such as the c-myc tag (EQKLISEEDLN, Munro and Pelham 1986) is fused to the 3′end of the immunoglobulin chain genes. A KDEL ER retention signal is added to retain the recombinant proteins in the ER (Munro and Pelham 1987). Figure 31.1 represents a scheme of the expression cassettes, flanked by *Hin*dIII restriction sites, which have to be cloned in a binary vector. Here, we used the vector pCB301-Kan (Gahrtz and Conrad 2009), which is based on pCB301 (Xiang et al. 1999) and incorporates the T-DNA fragment from pBIN19 (Bevan 1984). This vector contains the single plant-expressed kanamycin resistance gene (*NPTII*) allowing the selection of transformed plants by kanamycin as well as the segregation analysis.

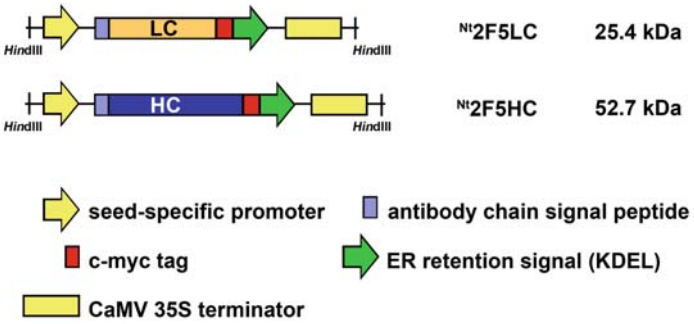


Fig. 31.1 Schematic presentation of plant expression cassettes and designation of the individual antibody chains. Expression of the recombinant proteins is driven by a seed-specific promoter. Endoplasmic reticulum retention is mediated by a *C*-terminal KDEL tag and the c-myc tag was included to facilitate detection of the recombinant proteins. ^{Nt}2F5LC, 2F5 antibody light chain; ^{Nt}2F5HC, antibody 2F5 heavy chain

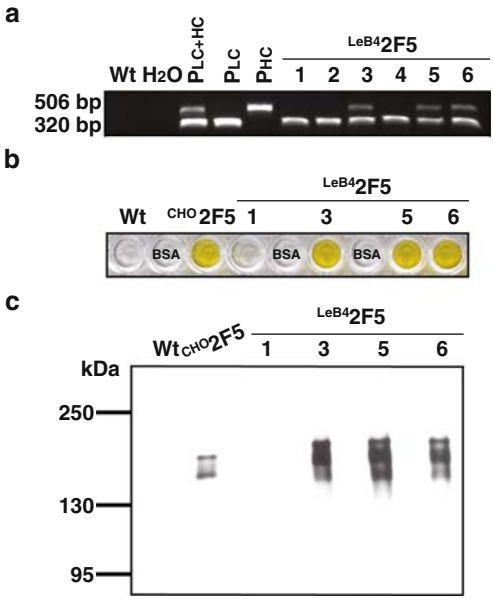


Fig. 31.2 Presence and assembly of 2F5 antibody chains in tobacco seeds. (**a**) Identification of antibody chain genes in offspring tobacco plants after crosses by multiplex PCR. Plants express the transgenes under control of the seed-specific LeB4 promoter. (**b**) Transgenic plants expressing both Ig chain genes and producing an assembled antibody as detected by protein L/protein A sandwich ELISA. (**c**) Western blot analysis of seed extracts showing 2F5 accumulation in seeds of transgenic tobacco plants. Wt: non-transformed *N. tabacum* cv. SNN; ^{LeB4}2F5, 2F5 expressed in seeds under control of LeB4 promoter; 1–6; analyzed offspring plants after cross pollination; H_2O: water control; PLC: binary vector for the light chain gene; PHC: binary vector for the heavy chain gene; ^{CHO}2F5: antibody standard produced by CHO cells; BSA: bovine serum albumin

Plants expressing antibody light or heavy chains are selected after Western blot analysis and investigated according to the segregation of the transgene. Tobacco plants with a single locus insertion are used for the crosses to create double transgenic offspring plants (F_1) expressing both immunoglobulin genes. These offspring plants containing both antibody chains are identified by multiplex PCR and by using a sandwich ELISA on mature seeds. Full-length 2F5 immunoglobulins in seeds are visualized by Western blot under non-reducing conditions (Fig. 31.2). The selection of transgenic tobacco plants that are homozygous for both Ig chain genes, a prerequisite for the large scale production of the recombinant antibody, can be easily achieved in one generation by using the doubled haploid technology (Floss et al. 2009b).

The protocols presented in this chapter are as follows:

- *Agrobacterium tumefaciens* transformation
- Isolation of plasmid DNA from *A. tumefaciens*
- Transformation of *Nicotiana tabacum* and regeneration of transgenic tobacco plants
- Isolation of genomic DNA from transformed tobacco plants
- Identification of plants expressing the individual immunoglobulin chain genes by Western blot
- Crossing of selected plant lines to obtain assembled antibodies
- Analysis of offspring plants by multiplex PCR
- Analysis of offspring plants by sandwich ELISA
- Analysis of offspring plants by Western blot under non-reducing conditions

31.2 Materials

31.2.1 Chemicals and Consumables

Unless stated otherwise consumables and chemicals were purchased from Carl Roth GmbH + Co. (Karlsruhe, Germany), Sigma-Aldrich (St. Louis, MO, USA), Schütt GmbH (Göttingen, Germany) and VWR International GmbH (Darmstadt, Germany).

31.2.2 Buffers, Media and Solutions

Buffers and solutions were prepared according to standard procedures (Sambrook and Russell 2001) using deionized water followed by sterilization by autoclaving (25 min, 121°C, 2 bar). Heat-sensitive components, e.g., antibiotics or growth factors, were prepared as stock solutions, filter-sterilized (syringe filter, CA membrane, 0.20 µm, Heinemann Labortechnik GmbH, Duderstadt, Germany) and added to the medium/buffer after cooling to 50°C.

– Antibiotics (stock solution, working concentration):

  carbenicillin (50 g/l, 50 mg/l)
  cefotaxime (200 g/l, 500 mg/l)
  rifampicin (50 g/l, 50 mg/l)

– Media for bacteria: (1.5% agar is added for agar plates)

  SOC (2% tryptone, 0.5% yeast, 10 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $MgSO_4$, 2 mM glucose)
  YEB (0.5% beef extract, 0.1% yeast, 0.5% peptone, 0.5% sucrose, 2 mM $MgSO_4$, pH 7)

– Media for plants: (0.9% agar is added for agar plates)

  MS (0.44% ready-to-use Murashige and Skoog powder including vitamins from Duchefa Biochemie, Haarlem, Netherlands; 2 or 3% sucrose, 0.05% MES),
  MG (0.44% ready-to-use Murashige and Skoog powder including vitamins from Duchefa Biochemie, Haarlem, Netherlands; 1.6% glucose, 0.05% MES)

– DNA extraction buffer for genomic DNA from tobacco leaf material (pH 7.5): 100 mM Tris, 250 mM NaCl, 25 mM EDTA, 0.5% SDS
– Seed extraction buffer (pH 8.0): 50 mM Tris, 200 mM NaCl, 5 mM EDTA, 0.1% Tween-20
– SDS-PAA gel loading buffer (pH 6.8): 72 mM Tris, 10% glycerin, 3% SDS, 0.25 μM bromphenolblue, 5% β-mercaptoethanol is used for reducing conditions
– Phosphate buffered saline (PBS, pH 7.6): 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl
– Tris buffered saline (TBS, pH 7.8): 180 mM NaCl, 20 mM Tris
– 3, 3′, 5, 5′-Tetramethylbenzidine (TMB), stock solution at 10 mg/ml in DMSO

31.2.3 Bacteria Strains

Agrobacterium tumefaciens strain C58C1 (pGV2260, Deblaere et al. 1985)

31.2.4 Plant Material

Nicotiana tabacum cv. Samsun NN (SNN)

31.2.5 Plasmids

– Binary vector pCB301-Kan for Agrobacterium-mediated transformation of tobacco leaf disks (Gahrtz and Conrad 2009) containing the expression cassettes with immunoglobulin genes (Fig. 31.1)

31.2.6 Enzymes

Restriction endonuclease *Hin*dIII was purchased from Fermentas GmbH (St. Leon-Rot, Germany). *Taq* DNA polymerase from Roche Diagnostics GmbH (Mannheim, Germany) was used for PCR reactions.

31.2.7 Reaction Kits

Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany)
ECL Western blotting analysis system (Amersham Biosciences, Piscataway, NJ, USA)

31.2.8 Synthetic Oligonucleotides

Oligonucleotides used for PCR were purchased from Metabion International AG (Martinsried, Germany).

31.2.9 Equipment

– Laminar flow cabinet (e.g., HERAsafe, Heraeus Instruments GmbH, Hanau, Germany)
– Equipment for DNA agarose gels including all necessary reagents
– CHO-derived 2F5 (^{CHO}2F5) as standard antibody (Polymun Scientific Immunbiologische Forschung GmbH, Vienna, Austria)
– SDS-polyacrylamide gel electrophoresis equipment (e.g., Bio-Rad Mini PROTEAN® 3, Bio-Rad Laboratories GmbH, Munich, Germany) including all necessary reagents to cast and to run the gels according to Sambrook and Russell (2001).
– Western blot equipment (e.g., Bio-Rad Mini Trans-Blot Cell®; Bio-Rad Laboratories GmbH, Munich, Germany).
– ELISA reader (SpectraFluorPlus Photometer, Tecan Deutschland GmbH, Crailsheim, Germany)

31.3 Methods

31.3.1 Agrobacterium tumefaciens Transformation

Electro-competent *A. tumefaciens* cells are transformed with the binary vector containing the plant expression cassette with the immunoglobulin chain gene (Fig. 31.1).

1. Inoculate 50 ml YEB medium containing carbenicillin (strain C58C1 carries resistance gene on Ti plasmid pGV2260) and rifampicin (strain C58C1 carries resistance gene on chromosomal DNA) with a single *A. tumefaciens* colony from a plate.
2. Incubate by shaking at 28°C, 180 rpm for 2 days.
3. Inoculate 400 ml YEB medium without any antibiotic with 4 ml of the culture and cultivate to an OD_{600} of approx. 0.5–0.8.
4. Harvest the cells by centrifugation at 1,750 *g*, 4°C and 15 min.
5. Resuspend the pellet in 400 ml sterile, distilled water and centrifuge (1,750 *g*, 4°C, 15 min).
6. Wash the pellet once more with 100 ml water and resuspend in 8 ml 10% glycerol.
7. Harvest the cells by centrifugation at 1,750 *g*, 4°C and 15 min.
8. Resuspend the pellet in 4 ml 10% glycerol.
9. Freeze aliquots of 50 µl in liquid nitrogen and store at −80°C.
10. Add 100–1,000 ng of plasmid DNA from the binary vector to one aliquot of competent cells and mix on ice.
11. Electroporate competent cells in an ice cold electroporation cuvette at 25 µF, 2.5 kV and 200 ohm using the Gene Pulser® (BIO-Rad Laboratories, Hercules, CA, USA).
12. Add 1 ml SOC medium to the cuvette, transfer the mixture into a sterile microcentrifuge tube and incubate by gently shaking at 28°C for 1 h.
13. Plate 50–100 µl onto selective SOC plates containing the appropriate antibiotics.

31.3.2 Isolation of Plasmid DNA from A. tumefaciens

After electroporation different *A. tumefaciens* clones are investigated for the presence of the expression cassette containing the gene of interest. Therefore, plasmid DNA is isolated and analyzed by digestion with restriction endonucleases.

1. Inoculate 2 ml SOC medium containing the appropriate antibiotics with one putative *A. tumefaciens* clone and incubate by shaking at 28°C for 20 h.
2. Harvest the bacteria by centrifugation (5,000 *g*, 3 min).
3. Resuspend the pellet in 100 µl buffer 1 (50 mM Tris, 10 mM EDTA, 100 µg/ml RNase A, pH 8.0).

4. Add 200 µl buffer 2 (200 mM NaOH, 1% SDS), gently mix and incubate for 10 min on ice.
5. Add 300 µl buffer 3 (3 M potassium acetate, pH 5.5) and mix.
6. Remove resulting cell debris by centrifugation (15,000 *g*, 15 min) and transfer the clear supernatant in a new reaction tube.
7. Add 270 µl isopropanol, mix and incubate for 10 min at room temperature.
8. Centrifuge (15,000 *g*, 15 min) for precipitation of DNA, remove the supernatant and wash the pellet with 200 µl ice cold ethanol (70%).
9. Dry the pellet and dissolve the DNA in 30 µl water.
10. Digest 15 µl DNA using the endonuclease *Hin*dIII, which flanks the expression cassette, according to manufacturer's recommendations.
11. Control the resulting fragments on an agarose gel.

31.3.3 Transformation of Nicotiana tabacum and Regeneration of Transgenic Tobacco Plants

Tobacco plant tissue culture is performed on MS medium at 21°C, a light regime of 16 h light and 8 h darkness, and a light intensity of 150 µmol m^{-2} s^{-1}. In the green house, tobacco plants are first grown in substrate 1 (Klasmann-Deilmann GmbH, Geeste-Groß Hesepe, Germany) at 22°C, 50–70% relative humidity, a light regime of 16 h light and 8 h darkness, and a light intensity of 150 µmol m^{-2} s^{-1}. Mature tobacco plants are grown in substrate 2 (Klasmann-Deilmann GmbH) at 22°C, 45% relative humidity, a light regime of 16 h light and 8 h darkness, and a light intensity of 200–220 µmol m^{-2} s^{-1}.

1. Inoculate 50 ml SOC medium containing the appropriate antibiotics with one Agrobacterium clone and incubate for 20 h at 28°C shaking at 180 rpm.
2. Dilute 10 ml of *A. tumefaciens* overnight culture with 10 ml MS medium (2% sucrose).
3. Cut leaf disks from 4- to 5-week-old tissue culture grown *N. tabacum* and incubate with the bacterial suspension for 30 min.
4. Deposit the leaf disks shortly on sterile filter paper to remove bacterial suspension completely.
5. Put the leaf disks on MS plates (3% sucrose) without antibiotics and growth factors for the co-cultivation and incubate the Petri dishes for 2 days in the dark at 24°C.
6. Transfer the leaf disks to solid MG medium containing 1 mg/ml benzylaminopurine (BAP), 0.2 mg/ml α-naphthaleneacetic acid (NAA), 500 mg/l cefotaxime (to suppress Agrobacteria) and the antibiotic depending on the selection marker.
7. Every 10 days put the leaf disks on fresh MG plates until shoots are emerging from the callus tissue.

8. Transfer shoots (1 cm in size) to solid MG medium containing 500 mg/l cefotaxime and the antibiotic depending on the selection marker in sterilized glass or plastic boxes for further growth of the shoots.
9. Cut rooted plants above the root and transfer them to solid MS medium containing cefotaxime and the antibiotic for selection.
10. Plant rooted plantlets into soil and transfer them to the green house. First 1–2 weeks, plants acclimatize in a propagator with a plastic cover before being grown in the green house.

31.3.4 Isolation of Genomic DNA from Transformed Tobacco Plants

To determine whether the transformed tobacco plants are transgenic for the immunoglobulin chain genes genomic DNA is isolated from tobacco leaves. The seed-specific promoter used, allows expression analysis only in seeds. The isolated DNA can be used for PCR or Southern blot analyses according to standard procedures (Sambrook and Russell 2001).

1. Grind two tobacco leaf disks to a fine powder (Retsch TissueLyzer, Qiagen GmbH, Hilden, Germany).
2. Add 300 µl DNA extraction buffer, homogenize the mixture and incubate for 10 min at 65°C.
3. Add 150 µl 5 M potassium acetate and remove the insoluble material by centrifugation at 15,000 *g* (20 min, 4°C).
4. Transfer the supernatant to a new reaction tube and add 0.5 ml isopropanol.
5. Precipitate the DNA by centrifugation at 15,000 *g* (20 min, 4°C).
6. Wash the DNA with 70% ethanol.
7. Resuspend the air-dried DNA pellet in 50 µl deionized water.

31.3.5 Identification of Plants Expressing the Individual Immunoglobulin Chain Genes by Western Blot

For analysis of seed-specific expression of immunoglobulin chain genes generated tobacco plants were grown to maturity in the green house. Seeds were collected after ripening and investigated by Western blot under reducing conditions (Sambrook and Russell 2001). A standard SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is carried out to separate proteins of seed extracts on 10% polyacrylamide (PAA) gels. A detailed protocol for the SDS-PAGE is described in Conrad et al. (1997). Western blot analysis is described in the chapter *Expression of antibody fragments in transgenic plants by Udo Conrad and Doreen Floss*. For Western blot analysis, seed extracts have to be prepared. About 5–60 µg total soluble seed

protein is loaded per lane. Always include a wild-type negative control and a positive control, which is a defined amount of a c-myc tagged protein. For the positive control, we routinely use 5 or 10 ng of a purified c-myc tagged single chain Fv (scFv). After transfer to nitrocellulose membrane, the individual antibody chains tagged to c-myc peptide are detected using an anti-c-myc monoclonal antibody (9E10) from hybridoma cell culture supernatant as the primary antibody. Anti-mouse Ig coupled with horseradish peroxidase (HRP) is used as secondary antibody (Amersham Biosciences, Piscataway, NJ, USA). Peroxidase activity is detected using the ECL Western blotting detection reagents and subsequent exposure of an X-ray film for 1 min.
Preparation of crude seed extracts:

1. Grind 50 mg seeds under liquid nitrogen to a fine powder.
2. Add 800 µl seed extraction buffer and homogenize.
3. Centrifuge the homogenates at 15,000 *g* for 20 min (4°C).
4. Transfer the supernatant to a new reaction tube and determine the content of total soluble protein using the Bradford assay according to the manufacturer's recommendations (Bio-Rad, Munich, Germany).
5. Before separation of the proteins by SDS-PAA electrophoresis add 2x SDS-PAA gel loading buffer (containing 5% β-mercaptoethanol) to the seed extracts to obtain a final concentration of 1 µg/µl total soluble protein (TSP) and incubate 10 min at 95°C.

31.3.6 Crossing of Selected Plant Lines to Obtain Assembled Antibodies

Seeds of the generated tobacco plants (T_0) expressing the individual antibody chain genes were analyzed according to the presence of the gene of interest by segregation analysis. Only T_1 lines showing a Mendelian segregation (3:1), consistent with a single transgenic locus of the expression cassette, were crossed to obtain plants expressing the full-length antibody.

1. Mature seeds of T_0 plants are surface sterilized in 80% ethanol for 2 min, subsequently 3× intensively washed in sterile water, 30 min soaked in sterile water for swelling, and blotted dry on sterile filter paper.
2. Put 100 surface sterilized seeds on solid MS medium (3% sucrose) containing the appropriate selection antibiotic and incubate the plate for approx. 4 weeks at 22°C.
3. Count the germinated plantlets containing the seed leaves and first leaves. Leave out non-germinated seeds because they represent insufficient germination capacity of the seed.
4. Determine the segregation pattern of the T_1 line (shoots with 2–4 leaves versus shoots with yellowish seed leaves).

5. T_1 lines showing a 3:1 segregation pattern are grown to maturity in the greenhouse.
6. Open the flower buds containing stamen (♂ part) and pistil (♀ part) with a scalpel (anthers should not produce pollen at this point of time).
7. Remove the anthers of the ♀ crossing partner (expressing antibody heavy chain).
8. Add pollen of the ♂ crossing partner (expressing antibody light chain) to the pistil of the ♀ crossing partner for the cross pollination.
9. After 4–6 weeks capsules of ♀ crossing partners can be harvested.
10. Put resulting seeds into soil and generate plants (F_1).

31.3.7 Analysis of Offspring Plants by Multiplex PCR

To determine whether the resulting F_1 plants contain both immunoglobulin genes genomic DNA is isolated as described before. This DNA serves as template for a multiplex PCR targeting the light and heavy chain immunoglobulin genes in one reaction employing standard PCR conditions (Sambrook and Russell 2001). Oligonucleotides specific for the constant parts of human antibody chain genes are selected:

LC_{FOR}: 5′-ACG AAC TGT GGC TGC ACC ATC TC-3′
LC_{REV}: 5′-CAC TCT CCC CTG TTG AAG CTC TTT G-3′
HC_{FOR}: 5′-TTC CCC CTG GCA CCC TCC TC-3′
HC_{REV}: 5′-TCC TCC CGC GGC TTT GTC TT-3′

31.3.8 Analysis of Offspring Plants by Sandwich ELISA

The presence and assembly of both antibody chains is investigated by sandwich ELISA based on antibody-protein L and antibody-protein A interactions.

1. Coat a 96-well plate (MaxiSorp™ Surface, Nunc A/S, Roskilde, Denmark) overnight with 1 µg/ml protein L (Actigen, Oslo, Norway) in PBS. Therefore, put 100 µl coating solution into each well of the microtitre plate and incubate overnight at room temperature.
2. Block the wells with 100 µl 3% bovine serum albumin (BSA) in PBS containing 0.1% Tween-20 for at least 1 h.
3. Prepare seed extracts as described above.
4. Dilute 20 µl seed extract in 100 µl 1% BSA in PBS containing 0.1% Tween-20.
5. Use the ^{CHO}2F5 (Polymun Scientific, Vienna, Austria) as a standard, and extracts from wild-type *N. tabacum* as negative control.

6. Remove the blocking solution, apply 95 µl of diluted seed extracts to the protein L coated wells and incubate 1 h at 24°C.
7. Remove the seed extracts and wash 5× with PBS containing 0.1% Tween-20.
8. Add 95 µl HRP-conjugated protein A (Amersham Biosciences, Piscataway, NJ, USA), 1:1000 diluted in 1% BSA in PBS containing 0.1% Tween-20, and incubate for 1 h at 24°C.
9. Remove the protein A solution, wash 4× with PBS containing 0.1% Tween-20 and once again with PBS (to avoid bubbles in the last step), and add 100 µl substrate solution (100 µg/ml TMB in 100 mM sodium acetate, pH 6.0 with 2 µl 30% hydrogen peroxide added to 10 ml of this solution immediately before use). Leave at room temperature and watch. A blue color should occur after 10 min.
10. Stop the reaction by adding 50 µl 1 M sulphuric acid. The blue color will turn yellow.
11. Read at 650 nm and 450 nm. Subtract OD_{650} from OD_{450}.

31.3.9 Analysis of Offspring Plants by Western Blot Under Non-reducing Conditions

The assembly of complete antibodies, produced in transgenic tobacco seeds, was analyzed by non-reducing SDS-PAGE on 6% PAA gels (Sambrook and Russell 2001). For immunoblotting, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) overnight at 18 V. Nitrocellulose membranes were blocked for 3 h at room temperature in 5% fat-free dried skimmed milk (Sprüh-Magermilchpulver J. M. Gabler-Saliter Milchwerke, Obergünzburg, Germany) dissolved in TBS. The immunological detection of assembled immunoglobulins was performed with anti-human Fc specific IgG conjugated to HRP (Sigma-Aldrich, St. Louis, MO, USA), and visualized with the ECL Western blotting analysis system (Amersham Biosciences, Piscataway, NJ, USA).

References

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breeding 15:305–327

Armbruster C, Stiegler GM, Vcelar BA, Jager W, Michael NL, Vetter N, Katinger H (2002) A phase I trial with two human monoclonal antibodies (hMAb 2F5, 2G12) against HIV-1. AIDS 16:227–233

Armbruster C, Stiegler GM, Vcelar BA, Jager W, Koller U, Jilch R, Ammann CG, Pruenster M, Stoiber H, Katinger H (2004) Passive immunization with the anti-HIV-1 human monoclonal

antibody (hMAb) 4E10 and the hMAb combination 4E10/2F5/2G12. J Antimicrob Chemother 54:915–920

Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu WD, Ayehunie S, Cavacini LA, Posner MR, Katinger H, Stiegler G, Bernacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Lu YC, Wright JE, Chou TC, Ruprecht RM (2000) Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat Med 6:200–206

Barton KA, Binns AN, Matzke AJM, Chilton MD (1983) Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell 32:1033–1043

Baumlein H, Wobus U, Pustell J, Kafatos FC (1986) The legumin gene family - structure of a B type gene of *Vicia faba* and a possible legumin gene specific regulatory element. Nucleic Acids Res 14:2707–2720

Baumlein H, Boerjan W, Nagy I, Bassuner R, Van Montagu M, Inze D, Wobus U (1991) A novel seed protein gene from *Vicia faba* is developmentally regulated in transgenic tobacco and Arabidopsis plants. Mol Gen Genet 225:459–467

Bevan M (1984) Binary Agrobacterium vectors for plant transformation. Nucleic Acids Res 12:8711–8721

Boehm R (2007) Bioproduction of therapeutic proteins in the 21st century and the role of plants and plant cells as production platforms. In: Lal S (ed) Biology of emerging viruses: Sars, Avian and Human Influenza, Metapneumovirus, Nipah, West Nile, and Ross River Virus. Blackwell Publishing, Uxford, pp 121–134

Casadevall A, Dadachova E, Pirofski L (2004) Passive antibody therapy for infectious diseases. Nat Rev Microbiol 2:695–703

Conrad U, Fiedler U, Artsaenko O, Phillips J (1997) Single-chain antibodies expressed in plants. In: Cunninham C, Porter S (eds) Methods in Biotechnology – Recombinant proteins from plants: Production and isolation of clinically useful compounds. Humana Press, Totowa, pp 103–127

Deblaere R, Bytebier B, Degreve H, Deboeck F, Schell J, Van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for Agrobacterium-mediated gene transfer to plants. Nucleic Acids Res 13:4777–4788

During K, Hippe S, Kreuzaler F, Schell J (1990) Synthesis and self-assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*. Plant Mol Biol 15:281–293

Fauci AS, Johnston MI, Dieffenbach CW, Burton DR, Hammer SM, Hoxie JA, Martin M, Overbaugh J, Watkins DI, Mahmoud A, Greene WC (2008) HIV vaccine research: the way forward. Science 321:530–532

Fiedler U, Conrad U (1995) High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. Biotechnology 13:1090–1093

Floss DM, Sack M, Stadlmann J, Rademacher T, Scheller J, Stoger E, Fischer R, Conrad U (2008) Biochemical and functional characterization of anti-HIV antibody-ELP fusion proteins from transgenic plants. Plant Biotechnol J 6:379–391

Floss DM, Sack M, Arcalis E, Stadlmann J, Quendler H, Rademacher T, Stoger E, Scheller J, Fischer R, Conrad U (2009a) Influence of elastin-like peptide fusions on the quantity and quality of a tobacco-derived human immunodeficiency virus-neutralizing antibody. Plant Biotechnol J 7:899–913

Floss DM, Kumlehn J, Conrad U, Saalbach I (2009b) Haploid technology allows for the efficient and rapid generation of homozygous antibody-accumulating transgenic tobacco plants. Plant Biotechnol J 7:1–9

Gahrtz M, Conrad U (2009) Immunomodulation of plant function by in vitro selected single-chain Fv intrabodies. In: Faye L, Gomord V (eds) Methods in Molecular Biology: Recombinant proteins from plants. Humana Press, Totowa

Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342:76–78

Joos B, Trkola A, Kuster H, Aceto L, Fischer M, Stiegler G, Armbruster C, Vcelar B, Katinger H, Guenthard HF (2006) Long-term multiple-dose pharmacokinetics of human monoclonal antibodies (MAbs) against human immunodeficiency virus type 1 envelope gp120 (MAb 2G12) and gp41 (MAbs 4E10 and 2F5). Antimicrob Agents Chemother 50:1773–1779

Ma JKC, Lehner T, Stabila P, Fux CI, Hiatt A (1994) Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. Eur J Immunol 24:131–138

Ma JKC, Chikwamba R, Sparrow P, Fischer R, Mahoney R, Twyman RM (2005) Plant-derived pharmaceuticals - the road forward. Trends Plant Sci 10:580–585

Mascola JR, Lewis MG, Stiegler G, Harris D, Van Cott TC, Hayes D, Louder MK, Brown CR, Sapan CV, Frankel SS, Lu YC, Robb ML, Katinger H, Birx DL (1999) Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J Virol 73:4009–4018

Mascola JR, Stiegler G, Van Cott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL, Lewis MG (2000) Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med 6:207–210

Munro S, Pelham HR (1986) An Hsp70-like protein in the ER: Identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46:291–300

Munro S, Pelham HR (1987) A C-terminal signal prevents secretion of luminal ER proteins. Cell 48:899–907

Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler G, Ruker F, Katinger H (1993) A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J Virol 67:6642–6647

Purtscher M, Trkola A, Gruber G, Buchacher A, Predl R, Steindl F, Tauer C, Berger R, Barrett N, Jungbauer A (1994) A broadly neutralizing human monoclonal antibody against gp41 of human immunodeficiency virus type 1. AIDS Res Hum Retroviruses 10:1651–1658

Rademacher T, Sack M, Arcalis E, Stadlmann J, Balzer S, Altmann F, Quendler H, Stiegler G, Kunert R, Fischer R, Stoger E (2008) Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. Plant Biotechnol J 6:189–201

Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G, Labrou N, Altmann F, Ma J, Stoger E, Capell T, Christou P (2008) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. Proc Natl Acad Sci USA 105:3727–3732

Sambrook J, Russell DW (2001) Molecular cloning – a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Shattock RJ, Moore JP (2003) Inhibiting sexual transmission of HIV-1 infection. Nat Rev Microbiol 1:25–34

Stoger E, Ma JKC, Fischer R, Christou P (2005a) Sowing the seeds of success: Pharmaceutical proteins from plants. Curr Opin Biotechnol 16:167–173

Stoger E, Sack M, Nicholson L, Fischer R, Christou P (2005b) Recent progress in plantibody technology. Curr Pharma Des 11:2439–2457

Teli NP, Timko MP (2004) Recent developments in the use of transgenic plants for the production of human therapeutics and biopharmaceuticals. Plant Cell Tiss Organ Cult 79:125–145

Twyman RM, Schillberg S, Fischer R (2005) Transgenic plants in the biopharmaceutical market. Expert Opin Emerg Drugs 10:185–218

Tzfira T, Citovsky V (2006) Agrobacterium-mediated genetic transformation of plants: Biology and biotechnology. Curr Opin Biotechnol 17:147–154

Van Larebeke N, Engler G, Holsters M, Van Denel S, Zaenen I, Schilpera R, Schell J (1974) Large Plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. Nature 252:169–170

Xiang CB, Han P, Lutziger I, Wang K, Oliver DJ (1999) A mini binary vector series for plant transformation. Plant Mol Biol 40:711–717

Chapter 32
Expression of Full Length Monoclonal Antibodies (mAb) in Algal Chloroplast

Miller Tran and Stephen P. Mayfield

32.1 Introduction

Antibodies have become an accepted and, in many cases, the preferred therapeutic for the treatment of human diseases. While the effectiveness of monoclonal antibodies (mabs) has been proven, their costs remain high, limiting their availability. Currently, mammalian cell cultures are used to express antibodies, and while the technology has improved, there seems to be no solution for lowering the cost associated with such therapies. Attempts have been made to express antibodies using a variety of plants in the past (Hiatt and Ma 1992; Conrad and Fiedler 1998). However, these plant derived antibodies contain differing glycosylation patterns from their mammalian expressed counterparts, thus limiting their viability as a FDA approved drug. Although antibodies were first expressed in plants in 1989, none have yet to receive FDA approval (Tekoah et al. 2004). *Chlamydomonas reinhardtii* has long been used as a model organism to understand the basic principles of photosynthesis, but it is only recently that it has been exploited as a viable tool for the expression of protein therapeutics. The single celled green algae provides many distinct advantages to the traditional expression systems, while at the same time providing the potential to drastically reduce the cost of antibody therapies (Mayfield et al. 2007). Although *C. reinhardtii* is a photosynthetic organism, it differs in many key aspects from land grown plants such as corn or tobacco. Being a single celled organism, *C. reinhardtii* is capable of being grown in large quantities in containment, limiting concerns about cross contamination with wild species (Mayfield et al. 2007). In addition to this advantage, *C. reinhardtii* grows at a much faster rate than terrestrial plants such as tobacco. Algae is capable of doubling in 8 h, and the possibilities of scaling up algae in a cost effective manner is becoming a reality (Fletcher et al. 2006; Franklin and Mayfield 2004).

M. Tran and S.P. Mayfield (✉)
The Scripps Research Institute, BCC-283 10550, N Torrey Pines Rd, La Jolla, CA 92037, USA
e-mail: mayfield@scripps.edu

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_32,

The chloroplasts of algae are capable of expressing and correctly folding many complex mammalian protein therapeutics at significant levels (Manuell et al. 2007). Unlike bacterial expression systems, *C. reinhardtii* chloroplasts contain a wide range of protein disulfide isomerases and chaperones that facilitate the formation of disulfide bridges required by functional antibodies (Kim and Mayfield 1997). Tobacco chloroplasts were initially used to show that chloroplasts could form disulfide bonds with the expression of human somatotropin (Staub et al. 2000). Tobacco and algal chloroplast have since then been shown to express and correctly fold a wide range of antibodies and vaccines molecules that require disulfide bond formation (Mayfield and Franklin 2005; Daniell et al. 2005). We have recently shown the capabilities of chloroplast to express and fold full length human IgG1 and IgA antibodies and that these correctly assembled heavy and light chain proteins bind their target antigen. (Tran et al. 2009) Chloroplasts have a number of chaperones that could be responsible for assembling the antibody proteins; however, it is still unclear which chaperone performs that function (Schroda 2004). Chloroplast expressed antibodies display the unique characteristic of being aglycosylated. There are no known post-translational modifications that occur on chloroplast expressed antibodies. This lack of post-translational modification provides *C. reinhardtii* expressed antibodies with a unique niche. Glycosylated antibodies such as rituximab are generally used for their effector functions and antibody dependent cell-mediated cytotoxicity (ADCC) (Anderson et al. 1997). Aglycosylated antibodies, while still capable of binding to their target antigen, fail to be recognized by cells containing Fc gamma RI and/or Fc gamma RII receptors (Walker et al. 1989). Antibodies expressed in the chloroplast are potentially useful as therapies wherein antigen binding or receptor blocking is required, but ADCC is not desired. With large quantities of affordable aglycosylated antibodies being produced from algal chloroplast, it would be possible for the advent of antibody prophylaxis whereby antibodies could be used for the prevention of a wide range of diseases (Wild et al. 2007). With the development of new expression vectors the amount of antibody that the chloroplast is capable of yielding has been continuously increasing. These vectors make use of the 5′ untranslated regions (UTR) of the psbA gene (Manuell et al. 2007) (Fig. 32.1). By using these vectors the psbA gene is replaced by the desired heterologous protein, causing the transformed algae to become non-photosynthetic. Knocking out the psbA gene yields some unique properties that allow for a higher throughput screening of transformants that are likely to express the antibody of interest. Algal cells that are lacking the psbA protein display high chlorophyll fluorescence (HCF) when they are illuminated by a 300-W quartz lamp that is equipped with a blue glass filter (Amybeth Cohen et al. 1993). The chloroplast contains 50 copies of its own plastid genome. To reach significant levels of expression, all 50 copies must be transformed with the desired antibody genes. Transforming all 50 copies of the plastid genome will yield the HCF phenotype that is detectable using a CCD camera that is equipped with a red filter (Fig. 32.2). This method increases the speed at which algae can be screened and the rate at which antibodies can be expressed. There are still many issues to be solved when using the algal chloroplast expression system. Of these issues,

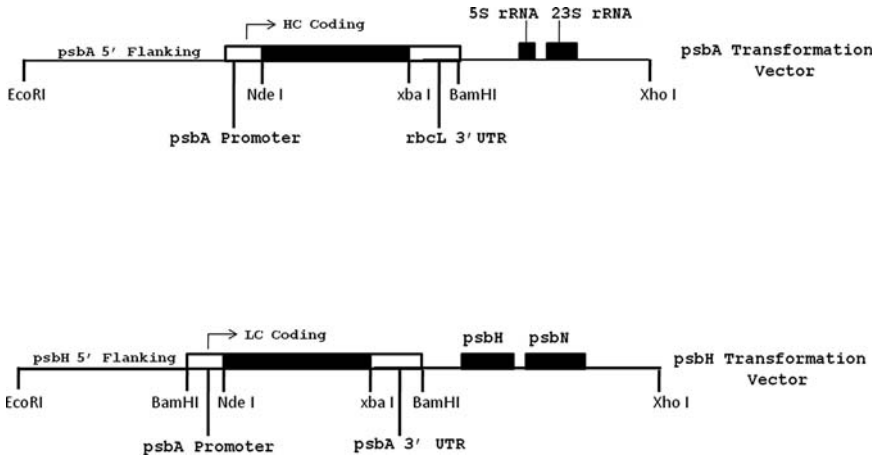


Fig. 32.1 Construct map of the psbA and psbH algal Transformation Vector

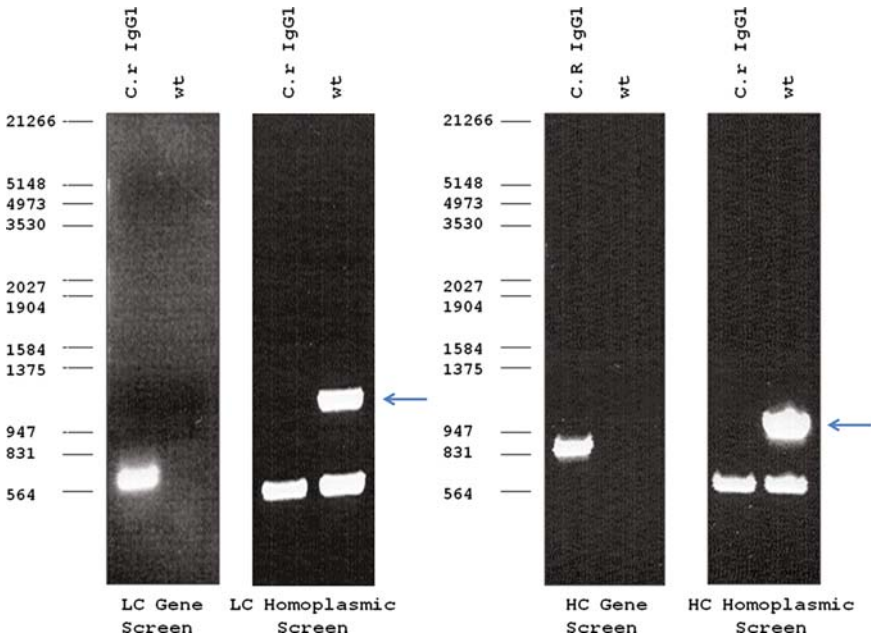


Fig. 32.2 Picture of a homoplasmic colony that is high chlorophyll fluorescent as indicated by the *arrow*

proteolysis may play a major role in determining protein yield. To date, although around 100 proteases are predicted from bioinformatic analysis to be chloroplasts localized, there are only a few proteases that have been characterized in the chloroplast (Adam 2000). Proteolysis not only reduces yield but it creates heterogeneity that

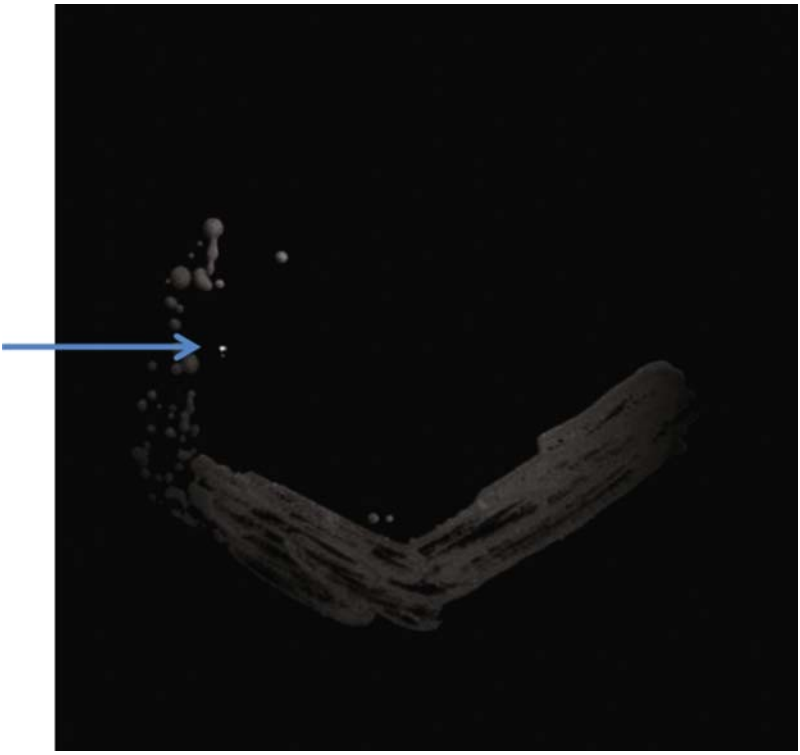


Fig. 32.3 Overview of the *C. reinhardtii* chloroplasts transformation protocol

causes issues with protein purification. However, the amounts of proteases that are normally encountered in the chloroplast are still minor when compared to the cytoplasm. Further analysis is needed with a variety of antibodies expressed in algal chloroplast to determine the degree to which antibodies are degraded. As with any new expression system there are still advancements to be made but the future remains promising for *C. reinhardtii* as a tool for the expression of aglycosylated antibodies. The following is a manual of the current protocol for algal transformation and chloroplast expression of antibodies in C *reinhardtii*. The process is outlined in Fig. 32.3.

32.2 Materials

32.2.1 Designing Genes for Expression in Chloroplast

- Gene designer software (available at www.DNA20.com)
- Chlamydomonas reinhardtii codon usage table

32.2.2 Cloning Antibody Genes into Chloroplast Expression Vector

- Restriction endonucleases NdeI and XbaI with appropriate buffers (New England Biolabs)
- T4 DNA ligase and buffer (Invitrogen)
- Topo-TA cloning kit (Invitrogen)
- Alkaline Phosphatase (CIP) (Roche)
- DH5α competent *Escherichia coli* cells
- SOC media
- LB ampicillin plates
- Maxi prep kit (Qaigen)
- psbA and psbH expression vectors (Fig. 32.1)

32.2.3 Transforming Algal Chloroplast

- Seashell Technology S04e gold DNA formulation kit
- PDS-1000HE Bolistic Particle Delivery System (Bio Rad)
- 1,350 psi Rupture disks (Bio Rad)
- Stopping Screens
- Macrocarriers
- Macrocarrier holders
- Rupture disk holder
- Helium Tank
- Vaccum pump
- 100% ethanol
- Plasmid p228 (confers Spectinomycin resistance)
- TAP plates containing 150 μg/ml of spectinomycin.

32.2.4 Screening Transformed Algae

- 10× Tris-EDTA pH 7.4 buffer
- Toothpicks
- Taq polymerase and buffer
- 12 mM $MgCl_2$
- dNTP mix (New England Biolabs)
- Primers: PsbA forward, Gene specific reverse, psbA forward and reverse screening primers, psbH forward and reverse screening primers, and control forward and reverse primers primers. (Table 32.1)
- 96-well thermal cycler

Table 32.1 A List of primers that are used to identify clones that are both gene positive and homoplasmic

Primer	Sequence
psbA forward	5′-gtgctaggtaactaacgtttgatttttt-3′
psbA Forward Screening	5′-ggaaggggaggacgtaggtacataaa-3′
psbA Reverse Screening	5′-ttagaacgtgttttgttcccaat-3′
psbH Forward Screening	5′-ctcgcctatcggctaacaag-3′
psbH Reverse Screening	5′-cacaagaagcaacccctttga-3′
Control forward	5′-ccgaactgaggttgggttta-3′
Control Reverse	5′-gggggagcgaataggattag-3′

- Agarose Gel electrophoresis equipment
- Agarose Gel Visualization Equipment
- CCD Camera equipped with a 300-W Quartz-iodine lamp that has a blue glass filter
- Red filter lens for CCD camera
- Computer equipped with Winlight 32 Imaging Software (EG&G Berthold)

32.2.5 Expression Test

- Lysis Buffer
- Sonnicator
- microfuge
- Protein Loading dye
- SDS-PAGE electrophoresis equipment
- Goat-anti human IgG AP conjugated (Southern Biotech)
- Goat-anti-human Kappa AP conjugated (Southern Biotech)
- AP Buffer
- NBT
- BCIP

32.2.6 Purification of Antibody from Algal Chloroplast

- Protein Lysis Buffer
- Sonicator
- Centrifuge
- 0.22 μm syringe filter
- Protein G resin
- IgG elution buffer
- Tris-HCL pH 8.0
- Protease inhibitor tablet (Roche)

32.3 Methods

32.3.1 Designing Genes for Expression in Chloroplast

1. Open Gene designer software (available at www.DNA20.com), Click on "File".
2. Click on "New" to design a new gene for chloroplast transformations. A design screen should appear.
3. Under "New Objects" on the left of the screen click on "New AA Segment". A Box should appear. Name your protein and enter in the amino acid sequence. Click "Ok".
4. In the Design box click on "Sequence View".
5. At the bottom of the design box there is a pull down menu entitled "Codon usage table" Pull down the menu and select "Chloroplast *C. reinhardtii*".
6. It is important to set the threshold at 10%. This will ensure optimal codon usage. (*Note 1*)
7. Click on "Actions" at the top of the screen and select "Back Translate".
8. Do not change settings in the box that appears and click on "Translate". An optimal DNA sequence should be selected.
9. To save DNA sequence, Click on "File" → "Export" and then "fasta". This will export the sequence and allow it to be viewed with the help of the Windows word program.
10. Every gene should be designed with a NdeI site at the 5′ end of the gene (This has an ATG for the start of translation) and a XbaI site at the 3′ end of the gene for ease of vector contruction. (*Note 2*).

32.3.2 Cloning Antibody Genes into Chloroplast Expression Vector

1. Prior to ligation into the expression vector, genes should be Topo-cloned and sequenced to ensure no sequence error.
2. Once Genes have been sequenced from the Topo-TA vector PCR2.1, they can be digested with the restriction endonucleases NdeI and Xba I.
3. The pasbA and psbH Expression vectors (Fig. 32.1) should also be digested with NdeI and XbaI. Following Digestation the vectors should be CIP treated to prevent any self-ligation of the vector.
4. Run the digest of the gene and the vectors on an agarose gel and gel-purify the vector backbone and the gene from the PCR2.1 vector.
5. Estimate the concentration of the vector and gene from the agarose gel, and prepare the ligation mix with a molar ration of vector:insert of 1:3 in a reaction volume on 10 μl. Incubate overnight at 16°C. (The Heavy chain gene should be ligated into the psbA vector and the LC gene should be ligated into the psbH vector).

6. Heat kill the ligation at 60°C for 10 min.
7. Add 5 μl of each ligation to separate 50 μl tube of E. *coli* competent cells.
8. Let ligation mixtures stand on ice for 30 min.
9. Heat shock ligation mixtures at 42°C for 30 sec and then place on ice for 10 min.
10. Add 250 μl of SOC Media to each ligation tube and shake at 37°C for 1 h.
11. Plate out the ligation mixture on a LB-amp plate. (Expression Vectors have a pBS(SK-) backbone that should allow for amp resistance in E.*coli)*.
12. Place the plates in a 37°C incubator overnight.
13. PCR screen colonies for the presence of the gene using the psbA forward primer and a gene specific reverse primer.
14. Select positive colonies and do a maxi DNA prep.
15. You should now have a psbA transformation vector with your heavy chain gene ligated into it along with a psbH transformation vector, with the light chain gene ligated into it. These will be used for the chloroplast transformations. (*Note 3*)

32.3.3 Transforming Algal Chloroplast

32.3.3.1 Transformation Growth Protocol

1. Inoculate a 50 ml starter culture of wt 137 mt(+) *C. reinhardtii* cells from a plate.
2. Allow Cells to grow in moderate light (3,000 lux) until log phase growth between 1×10^6 and 3×10^6 cells/ml in.
3. From the starter culture inoculate a 250 ml flask with 2×10^4 cells/ml.
4. Allow cells to grow to early to mid log phase growth between 8×10^5 cells/ml and 2×10^6 cells/ml. (At 1×10^6 cells/ml there will be enough for 12transformations). (*Note 4*)
5. Spin down cells and resuspend with TAP media to a concentration of 3×10^7 cells/ml.
6. Plate ~ 1.5×10^7 cells on each TAP/Spectinomycin plate. (TAP plates should contain 150 μg/ml of specintomycin).
7. Continue to DNA-Gold formulation protocol and ballistic transformation protocol.

32.3.3.2 DNA-Gold Formulation Protocol

1. Add 50 μl of Seashell technology binding buffer into a 1.7 m eppindorf.
2. Add 5 μg of each transformation plasmid (psbA and psbH) into the binding buffer. Add 4 μg of the selection plasmid p228 to the mixture.
3. To the DNA binding buffer mixture, add 60 μl (3 mg) of seashell S04e carrier gold.

4. Let stand on ice for 1 min.
5. Add 100 μl of precipitation buffer and let stand on ice for 1 min.
6. Mildly vortex and spin (10,000 rpm in eppendorf microfuge for 10 sec) to settle the gold particles.
7. Remove the supernatant and wash the gold pellet with 500 μl of cold 100% ethanol. Spin briefly in microfuge, remove supernatant and add 50 μl of ice cold 100% ethanol.
8. Resuspend the pellet with a brief 1–2 sec burst using a probe tip of a sonnicator.
9. Place Sterilized macrocarrier membrane into the macrocarrier membrane holder.
10. Immediately transfer 10 μl to the macrocarrier membrane. Keep aggregation to a minimum by sonnicating immediately before apply gold to the carrier membrane. Since there is a total of 50 μl the transformation can be attempted five times on five different macrocarriers.

32.3.3.3 Particle Bombardment of Algal Chloroplast

1. Turn on the PDS-1000He particle delivery system and open the helieum tank completely. The helium tank should have a psi reading of approximately 2,000 psi. Be sure to sterilize the chamber with ethanol.
2. Turn on the vacuum pump that is connected to the PDS-1000He particle delivery system.
3. Place a rupture disk into the grey rupture disk holder and secure tightly into the PDS-1000He particle delivery system.
4. Place a stopping screen on/at the bottom of the macrocarrier launch assembly unit.
5. Place macrocarrier holder containing the gold coated macrocarrier into the macrocarrier launch assembly unit above the stopping screen. Place the gold coated side facing down. (*Note 5*)
6. Place assembly unit into the highest slot closest to the rupture disk.
7. Place the plated cells from Sect. 32.3.3.1 on the plate holder approximately 6 cm from the assembly unit.
8. Close the chamber and turn on the vacuum until the vacuum reaches 28 in Hg. Upon reaching 28 in Hg, hold the pressure by flipping the vacuum switch from "Vac" to "Hold".
9. Hold down the fire button and DO NOT RELEASE until the rupture disk ruptures.
10. Release the Vacuum by moving the vacuum switch to the vent position, open the chamber door, and immediately cover the plate to prevent contamination.
11. Repeat steps 3–9 for additional transformations.
12. Place cells in dim light, $<$1,500 lux and wait for background to die and transformed colonies to appear. (*Note 6*)

32.3.4 Screening Transformed Algae

32.3.4.1 Screening for Gene Positive Colonies

1. Pick and patch 96 colonies onto a TAP Spectinomycin plate. Allow colonies to grow for a week.
2. After a week, touch each patch with a tip and place into 20 μl 10xTE. Do this for each patch in a 96 well PCR plate. (*Note 7*)
3. Place the PCR plate containing the samples in a PCR thermal Cycler at 95°C for 10 min.
4. Place samples at 4°C while preparing PCR mix.
5. Screen all 96 samples for the heavy chain and light chain genes using the psbA forward primer and the gene specific reverse primer. Do this in two separate reactions to ensure integration of both genes. Use only 2 μl of the Patch 10xTE mix in the PCR.
6. Once gene positive colonies are found, streak out the positive patch for more individual colonies. Allow 1–2 weeks to grow in low/soft light to avoid pressuring the gene positive cells to remain photosynthetic. (*Note 8*) (Fig. 32.4)
7. Once individual colonies appear proceed to screening for homoplasmic colonies.

32.3.4.2 Screening for Homoplasmic Colonies Using a CCD Camera (Optional)

1. Illuminate plates from previous step with a 300-W quartz-iodine lamp equipped with a blue glass filter. A CCD camera that is equipped with a red filter will then be used to visualize High chlorophyll fluorescence (HCF).
2. The video signal from the camera is transferred over to a computer that is equipped with winlight 32 imaging software.
3. Homoplasmic colonies will fluoresce more than those that are heteroplasmic. (*Note 9*)
4. HCF colonies are selected by matching the pattern of colonies in the software imaging program to those on the TAP/Spectinomycin plate. (Fig. 32.2)
5. HCF colonies are then patched out onto a separate plate and further analyzed by a PCR homoplasmic screen.

32.3.4.3 Screening for Homoplasmic by PCR

1. Touch a tip to the gene positive patch and place into 20 μl 10xTE. Be sure to patch a wt 137c mt(+) into 10xTE to be used as a control.
2. Place the algal-10xTE mixture into a PCR thermal cycler for 10 min at 94°C.
3. To screen for strains homoplasmic at the psbA locus (Where the Heavy chain gene integrates) use two sets of primers in the PCR: psba forward and reverse screening primers and control forward and reverse primers. The algal patches

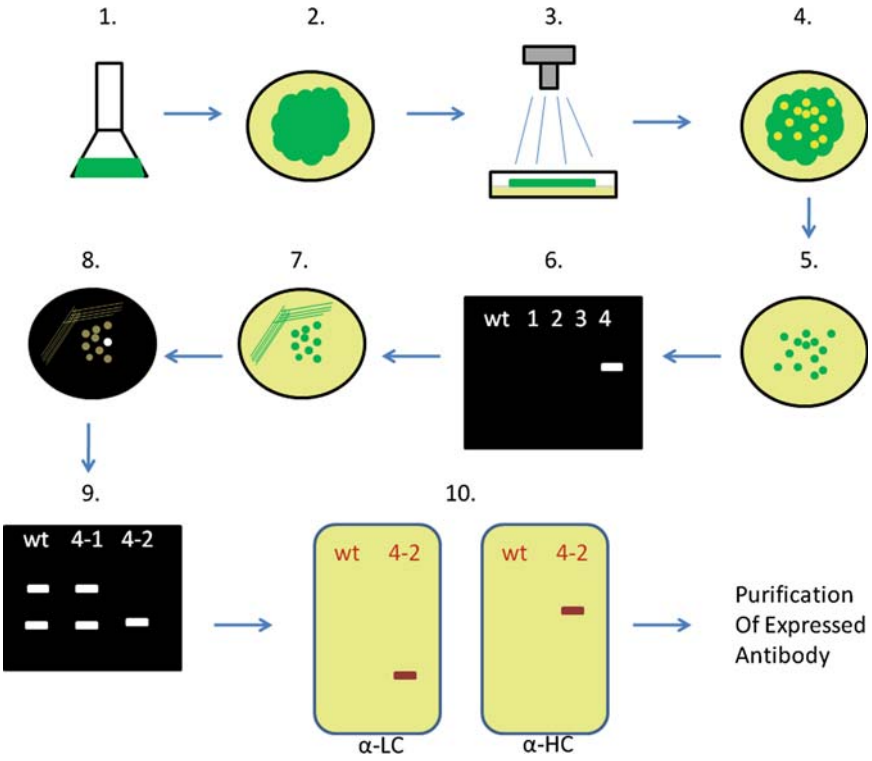


Fig. 32.4 An Example of the results of a PCR screen for both the Heavy chain and Light chain of an antibody with the corresponding homplasmic screen at both the psbA and psbH locus. The arrows indicate the wt band that is loss in a homoplasmic transformant

that are homoplasmic should lose the wt psbA band around 1 kb when examined on an agarose gel. Be sure to PCR a wt control to visualize the loss of the wt band (Fig. 32.4)

4. To screen for strains homoplasmic at the psbH locus (Where the Light chain gene integrates) use two sets of primers in the PCR: psbH forward and reverse screening primers and control forward and reverse primers. The homoplasmic strains should lose the wt psbH band around 1KB when examined on an agarose gel. Be sure to PCR a wt control to visualize the loss of the wt band. (Fig. 32.4)
5. Proceed to determining the expression for the transformed colonies that are homoplasmic for both the heavy and light chain genes at the psbA and psbH locus.

32.3.5 Expression Test

1. To examine if the chloroplast was capable of expressing the antibody inoculate a 50 ml TAP culture with the homoplasmic strain to a density of 2×10^5 cells/ml.

2. Allow cells to grow in the light at approximately 8,000 lux for 48 h to a density of 2×10^6 cells/ml. (*Note 10*)
3. Spin down cells in a 50 ml conical and resuspend cells in 500 μl lysis buffer containing 50 mM Tris-HCL pH8.0, 400 mM NaCl and 10% sucrose.
4. Sonicate the sample 3× for 30 sec. Place the sample on ice for at least 2 min inbetween sonications.
5. Spin down the sample at 16,000G to remove cell debris and insoluble proteins.
6. Save the soluble protein lysate and examine 40 μg of total soluble protein by immunoblot.
7. Detect expression of the heavy chain using a goat α-human IgG conjugated to alkaline phosphate.
8. Detect expression of the light chain using a goat α-kappa Light chain conjugated to alkaline phosphate.
9. Transformed strains expressing both the heavy and light chain proteins of the antibody are used for further purification and analysis.

32.3.6 Purification of Antibody from Algal Chloroplast

1. Inoculate a 1 L culture with the transformed strain expressing the antibody to a density of 2×10^5 cells/ml and allow cells to grow to a density of 2×10^6 cells/ml. (approximately 2 days in 8,000 lux)
2. Innoculate 10 L of TAP media with the 1 L culture from step 1 and allow cells to grow for another 48 h to approximately 2×10^6 cells/ml.
3. Pellet cells by centrifuging at approximately 5,000 *g* for 5 min.
4. Remove excess media and weigh the cell pellet.
5. To the cell pellet add a 10× volume of Lysis buffer containing 50mM Tris-HCL pH8.0, 400 mM NaCl, 10% sucrose and one complete protease inhibitor tablet.
6. Resuspend the cell pellet in lysis buffer and place into a glass beaker on ice.
7. Sonnicate the cells for 30 sec and then sit on ice for 1 min. Repeat this step 10 times.
8. Separate Cell debris from soluble protein by centrifuging lysate at 20,000 *g* for 30 min.
9. Apply soluble lysate to a 0.22 μm syringe filter to remove any excess cell debris. (*Note 11*)
10. Apply soluble lysate to 0.5 ml of protein G resin overnight at 4°C and proceed with a batch style purification. (*Note 12)*
11. Spin down protein G at 2,000G and save flow through for analysis.
12. Wash the column with the lysis buffer.
13. Elute protein with 500 μl IgG elution buffer (Pierce) into 25 μl of 1 M tris-HCL pH 8.0.
14. Perform further analysis on chloroplast expressed antibody as determined for each specific antibody.

32.4 Notes

1. The codon usage reflects the most abundantly used codons in the chloroplast. There is still a lot of work being done to determine the optimal codons for antibody expression in the chloroplast.
2. The Nde I and Xba I site are only necessary for ligation into our currently developed vectors. They are not necessary for transcription or translation.
3. It is important that the DNA is at a concentration of at least 1.0 μg/ml.
4. Using concentrations of cells that are more or less, will drastically decrease the transformation efficiency.
5. If gold is completely dried out add 5 μl of ethanol to the gold. Transformations usually work best when the gold is slightly moist.
6. It is best to place transformed plates in dim light because the vectors being used to transform the algal cells will cause the cells to become non-photosynthetic. Placing cells in high light will put selection pressure on the cells to remain photosynthetic.
7. Be sure to use 10× TE not 1× TE as the PCRs tend to work better with 10× TE
8. Homoplasmic screens can be performed on primary transformed colonies that are gene positive.
   There have been instances where cells are homoplasmic on the original transformation plate.
9. HCF screening only works when your transformations are knocking out a gene involved in photosynthesis.
10. When checking for expression it is important to NOT grow the cultures to high density as this will restrict light penetration into the culture, thereby reducing antibody yield.
11. This step can be replaced with an ultra centrifuge spin of 100 *g* or greater.
12. Do not allow protein G to mix with cell lysate for more than 24 h as there are proteases in the chloroplast that will cause partial degradation of the expressed antibody.

Acknowledgement Bryan O'neill Sapphire Energy

References

Adam Z (2000) Biochimie 82:647–654
Amybeth Cohen CBY, Bruick RK, Mayfield SP (1993) Methods Enzymol 297:192–208
Anderson DR, Grillo-Lopez A, Varns C, Chambers KS, Hanna N (1997) Biochem Soc Trans 25:705–708
Conrad U, Fiedler U (1998) Plant Mol Biol 38:101–109
Daniell H, Chebolu S, Kumar S, Singleton M, Falconer R (2005) Vaccine 23:1779–1783
Franklin SE, Mayfield SP (2004) Curr Opin Plant Biol 7:159–165
Hiatt A, Ma JK (1992) FEBS Lett 307:71–75
Kim J, Mayfield SP (1997) Science 278:1954–1957

Manuell AL, Beligni MV, Elder JH, Siefker DT, Tran M, Weber A, McDonald TL, Mayfield SP (2007) Plant Biotechnol J 5:402–412
Mayfield SP, Franklin SE (2005) Vaccine 23:1828–1832
Mayfield SP, Manuell AL, Chen S, Wu J, Tran M, Siefker D, Muto M, Marin-Navarro J (2007) Curr Opin Biotechnol 18:126–133
Samuel P, Fletcher MM, Stephen PM (2006) Transgenic microalgae as green cell factories
Schroda M (2004) Photosynth Res 82:221–240
Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G, Russell DA (2000) Nat Biotechnol 18:333–338
Tekoah Y, Ko K, Koprowski H, Harvey DJ, Wormald MR, Dwek RA, Rudd PM (2004) Arch Biochem Biophys 426:266–278
Tran M, Zhou B, Pettersson PL, Gonzalez MJ, Mayfield SP (2009) Biotechnol and Bioeng 104:663–673
Walker MR, Lund J, Thompson KM, Jefferis R (1989) Biochem J 259:347–353
Wild MA, Kumor K, Nolan MJ, Lockman H, Bowdish KS (2007) Hum Antibodies 16:99–105

Chapter 33
Expression of IgG Antibodies in Mammalian Cells

Thomas Jostock and Jiandong Li

33.1 Transient Expression

Transient expression of IgG antibodies in mammalian cells is well established and very suitable especially for discovery and early characterization of candidates. Most popular host cells are HEK293 and derivatives there of and, with increasing importance, Chinese Hamster Ovarian (CHO) cells. Transient expression is described in detail in (Chap. 48, T Schirrmann, this volume) of this book for scFv-Fc fusion proteins. As, with small adaptations of the vector setup, the same methods can also be applied for IgG antibodies, transient expression will not be further discussed here, and the focus of the chapter is on stable expression.

33.2 Stable Expression

33.2.1 Introduction

Despite rather high costs, currently 60–70% of all recombinant biopharmaceuticals and virtually all therapeutic monoclonal antibodies are produced in mammalian cell culture processes (Wurm 2004). During the last two decades a 100-fold improvement in production yields has been achieved for industrial large-scale processes.

T. Jostock (✉)
Novartis Biologics, Protein Sciences and Production, WKL-681.1.06A, Klybeckstrasse 141, CH-4057 Basel, Switzerland
e-mail: thomas.jostock@novartis.com
J. Li
National Institute for Viral Diseases Control and Prevention,Yingxin Street 100, Xuanwu District, Beijing 100052, China
e-mail: Jiandong_li@hotmail.com

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_33,

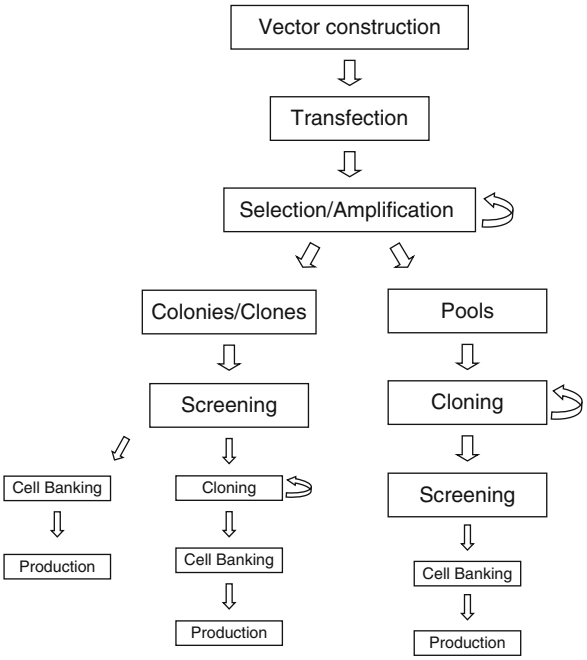


Fig. 33.1 Flow chart of stable cell line generation. The Flow chart summarizes different possible procedures for the generation of stable cell lines

In 1986, when Tissue Plaminogen Activator t-PA was the first recombinant pharmaceutical protein produced in CHO cells, the yield was about 50 mg/L. Nowadays, yields of up to 10 g/L, as reported for a recombinant antibody, are feasible in fed-batch processes, while perfusion mode cultivation can lead to even higher product concentrations of more than 20 g/L in the harvest (Lee 2008). The increase is mainly due to higher volumetric production yields based on much higher cell densities in the production process. In laboratory scale low-cell density expression experiments, the yields are usually significantly lower.

Generally, stable cell-line generation follows a common scheme of sequential steps with several different possible scenarios (Fig. 33.1): First of all, cells are transfected to introduce an expression vector. Then, cells are selected using selectable marker genes on the transfected DNA to eliminate un-transfected cells and to selectively enrich transgene over-expressing cells. After selection, single-cell cloning is done to generate monoclonal cell lines, and by screening for high productivity and production stability, candidate clones for the antibody production are identified. Further characterization steps can include an analysis of product quality and bioreactor suitability as well as growth parameters. Finally, cell banks are generated to conserve the production clones. In the following section, some general information on the available technologies for the individual steps is given.

33.2.1.1 Host Cells

Currently, the most widely used cell line for antibody production is CHO. To a lesser extent also, murine myeloma (NS0, SP2/0), BHK (baby hamster kidney), and HEK293 (human embryonic kidney) are used. Besides these more traditional host cell lines, the human retinoblastoma cell line PER.C6 recently has been shown to efficiently produce recombinant IgG at high cell densitities (Jones et al. 2003).

For laboratory scale production, CHO cells are quite suitable because of good growth and transfection behavior. Also, due to the broad distribution, a huge assortment of commercial vectors, culture media and transfection systems is available. There are several CHO cell lines with different properties available. The most common ones are CHO-K1, CHO-DXB11 and CHO-DG44. CHO-K1 are still quite close to the originally isolated hamster ovary cells (Puck 1958), while DXB11 and DG44 have undergone cycles of random mutagenesis to eliminate the endogenous DHFR (dihydrofolate reductase) genes. This genome modification was done to establish a metabolic selection and a gene-amplification system based on DHFR as the selectable marker gene. In contrast to DXB11, where one DHFR allel is deleted and one has a loss-of-function mutation, in DG44 cells both DHFR alleles are deleted (Urlaub and Chasin 1980; Urlaub et al. 1983). Thus, if DHFR based selection and gene amplification is anticipated, dhfr negative cell lines are usually used.

33.2.1.2 Vectors

Classical vector strategies to supply the host cell with the heavy and light chain encoding genes include setups with co-transfection of individual plasmids for the heavy and the light chain (Kaloff and Haas 1995; Montano and Morrison 2002; Schlatter et al. 2005). The genes thereby can have either a genome-like intron-exon structure (Kalwy et al. 2006) or a cDNA structure (Li et al. 2007a). The advantages of such a multi-plasmid strategy are comparably small vector sizes and the flexibility to vary the ratio of heavy chain to light chain encoding plasmids. The disadvantages are the lack of control over the ratio of heavy to light chain insertion into the host genome and the risk of multiple insertion sites for the different plasmids.

"Tandem" or "sandwich" vectors combining complete expression cassettes for heavy and light chain on a single plasmid are also commonly used and are proven to be suitable for generating high-producing cell lines (Schlatter et al. 2005; Kalwy et al. 2006). Such vectors offer a high probability of having equal copy numbers for both cassettes after integration and optional gene-amplification steps. However, such vectors typically reach a significant size, especially if genomic sequences for the transgenes are used. The large size can make handling of the plasmid difficult and might be a limiting factor for gene-transfer efficiencies during transfection.

One possibility to prevent this is to combine heavy and light chain in a single expression cassette with a bi-cistronic setup (Jostock et al. 2004; Li et al. 2007a, b). Internal ribosomal entry sites (IRES) can be used to efficiently drive translation initiation of the downstream cistron (Borman et al. 1994). Since both chains are

contained in a single mRNA, such bi-cistronic setups lead to a balanced expression of both polypeptides. However, the translation initiation efficiency of IRES elements usually is significantly lower as that of the 5′-cap of the mRNA, leading to an excess of translation product from the upstream cistron (Kaufman et al. 1991). Studies using mutant IRES elements that have different translation initiation efficiencies showed that this strongly affects the overall expression levels (Li et al. 2007a, b).

Recently, a new technology that allows co-expression of the heavy and the light chain from a single mRNA with a single Open Reading Frame (ORF) has been described (Fang et al. 2005). In this setup, heavy and light chain are separated by a self-processing 2A sequence motive of viral origin and a furin cleavage site. Upon translation and secretion self-processing of the 2A sequence occurs via an yet undefined mechanism that separates the heavy and light chain peptides. Furin-catalyzed proteolysis and the activity of carboxy-peptidase remove the remaining aminoacids of the motive, and the heavy and the light chain peptides are assembled to fully functional tetrameric IgG molecules (Fang et al. 2005). One of the possible advantages of the single ORF approach is the forced equimolar expression of the heavy and the light chain genes.

Usually, strong viral promoters are used to drive antibody expression in CHO cells, and the inclusion of an intron has been shown to be beneficial for transgene expression. On the 3′-end, a suitable polyadenylation signal is needed to complete the expression cassettes.

Besides the antibody expression cassettes, selectable marker genes are a key feature of vectors for stable expression. A broad range of different markers is available, and the performance of the selection system strongly depends on the host cell properties. Typically, resistance mediating marker genes to substances such as Neomycine, Puromycine, Hygromycine, or Zeocine as well as a metabolic selectable marker such as DHFR or GS (glutamine synthetase) are used (Birch and Racher 2006).

The use of chromatin opening motifs such as S/MAR or STAR elements to flank the expression cassettes of the antibody chains has recently been described to increase the ratio of high producing clones after a random integration of the vector and to support the long-term stability of transgene expression (Jostock et al. 2008). Such vectors can be useful in cases in which only limited clone-screening efforts are possible.

33.2.1.3 Transfection and Transgene Integration

Gene transfer in mammalian systems for stable expression is usually done by the CaPO4 method, lipofection, or electroporation. All three methods are suitable to transfect CHO cells, whereby lipofection usually is more efficient with adherent growing than suspension growing cells. For long-term stable expression and cell banking, integration of the expression cassette into the host cell genome is aspired. Gene integration in principle can occur in three different ways: random integration,

homologous recombination and recombinase-mediated targeted integration. Random integration approaches are widely used and comparably easy to implement, but significant clone-screening efforts might be necessary to identify highly stable and high-producing clones. Recently, commercial targeted insertion systems have become available, which can be considered as an alternative for laboratory scale production.

33.2.1.4 Selection

The selection step is very crucial for successful cell line development and needs to be optimized for each combination of vector, host cell line, selection system and culture medium. The higher the stringency of the selection system, the higher the chance to find high-producing clones and the lower the screening efforts necessary to identify such clones. With no automation equipment for clone screening in place, it is desirable to work with high stringency selection systems that allow only high-producing cells to survive the selection process. Optionally, gene amplification procedures can be applied to increase the copy number of the transgene, which can lead to a significant increase in productivity.

The Popular selection systems are G418/Neomycine, Puromycine, Hygromycine and Zeocine, all of which are based on selectable marker genes that inactivate a toxic selection reagent. With suitable vectors all these systems are applicable for CHO cells; however, the optimal concentration of the selection reagent needs to be evaluated for each combination of vector, host cell line and culture medium.

Metabolic selection systems such as DHFR or GS target metabolic key pathways such as nucleotide or amino acid synthesis. For selection, transfected cells are cultured in a medium lacking a vital metabolite. Only cells expressing the metabolic selectable marker are able to synthesize this metabolite from the precursors present in the medium due to the biologic activity of the marker. DHFR for example is an enzyme that catalyzes the generation of reduced folates, which are crucial metabolites for nucleotide synthesis. The selection stringency can be further increased by adding inhibitors of the metabolic markers. Methotrexate for example is a folate analog which inhibits the enzymatic activity of DHFR. By adding methotrexate to the cells and by increasing the concentration of methotrexate, stepwise cells that have undergone gene duplication events can be enriched leading to a higher transgene copy number. Such gene-amplification procedures can significantly increase the productivity of cell lines but are time-consuming and sometimes difficult to optimize.

Another recently developed example for a high-stringency selection system is STARselect. Here, due to a very tight linkage of selection marker and gene of interest combined with a strong attenuation of the selection marker expression, a high-proportion of high producing cells is found after selection (Jostock et al. 2008).

Generally, suitable selection conditions can strongly differ between adherent and suspension growing cells and are influenced by the media composition. With adherent cells, selection can be combined with a cloning step by seeding the cells at

a relatively low density in culture dishes, so that colonies of surviving cells appear upon selection. A similar approach is possible with adherent or suspension growing cells by seeding cells at comparably low densities in 96-well plates such that the few wells with surviving cells are likely to contain a clonal population.

Alternatively, cells are cultured as a mixed population during selection, leading to a "pool" of surviving cells, which later on can be a single cell that is cloned to generate monoclonal cell lines. Such polyclonal populations of transfected cells can yield reasonable productivitys if a stringent selection system is used. For initial production of smaller amounts of protein, such polyclonal pools can be used. However, the productivity of pools tends to decrease with increasing culture time.

33.2.1.5 Single-Cell Cloning

For continuous supply with recombinant antibody protein, monoclonal stably transfected cell lines are most suitable because of high consistency in production yield and product quality. Also, the monoclonality of the producer cell line is requested by the regulatory authorities (e.g., EMEA notes for guidance ICHQ5B and ICHQ5D). However, stability in production and consistency of product quality are not always given for all clones and can also be influenced by the antibody sequence.

Methods that are suitable for single-cell cloning of CHO cells include limiting dilution, colony picking by hand or using an automated system (e.g., ClonepixFL), and flow cytrometry. While limiting dilution cloning is nonselective, flow cytometry and automated colony picking systems allow selective cloning of high producing cells. Several approaches for efficient surface staining of high-producing cells for flow cytrometry-based detection and sorting have been developed and shown to greatly improve the clonal distribution of the productivity (Brezinsky et al. 2003; Jostock 2009).

33.2.1.6 Screening

The main parameter that is usually analyzed to identify candidate clones for stable production in a primary screening is productivity. Further screening activities include growth parameters and product quality attributes. Initial screening rounds are often performed in multi-well formats such as 96-well or 24-well plates before moving into T-flasks or shake flasks which offer better control over the seeding cell density and culture conditions. If optimized protocols for a given host cell line are in place, fed-batch production with suspension growing cells in shake flasks can result in very high yields with product concentrations of over 4 g/L in the culture medium (Jostock et al. 2008). If bioreactor production is intended, suitability screening of lead candidate clones to grow and produce under such conditions can improve the chance of successful up-scaling.

A reliable product quantification assay is crucial for successful clone screening. Suitable methods include ELISA, precipitation assays and HPLC methods.

An ELISA method for quantifying IgG is described in (Chap. 30, Beyer, Lohse, Dechant & Valerius, this volume). While ELISA and FRET assays are especially suitable for early screening activities, due to high sensitivity, comparably high throughput and low sample consumption, HPLC methods are most suitable for a more detailed clone characterization due to the high accuracy of the method.

33.2.1.7 Production Stability Testing

Besides productivity and growth behavior production stability is also a key parameter, especially if up-scaling to large culture volumes is anticipated. Production stability is assessed experimentally by sub-cultivating the candidate cell lines for the required period while regularly checking for productivity in standardized assays. Depending on the selection system and the application of the product, sub-cultivation may be done in the presence or absence of selection reagents.

33.2.1.8 Cell Banking

Cryopreservation is done to conserve and store production cell lines. Depending on the expected amount of the product needed, several layers of cell banks may be generated to secure long-lasting supply of cells for repeated production runs. Initial small banks are generated as early as possible after cloning and candidate clone selection to conserve the cells with a minimum of generations. After the final clone decision such early frozen stocks can be used to generate a large master cell bank (MCB), which usually is subject to intensive characterization including viral safety testing according to regulatory requirements (e.g., ICH Q5A). The MCB is the source of repeated generations of working cell banks (WCB) for the supply of production runs according to the actual material requirements. MCB preparation and storage under controlled conditions is often taken out in a GMP (good manufacturing practice) environment.

33.2.1.9 Laboratory-Scale Production

The production scale is mainly determined by the product amount needs and the productivity of the cell line and production process. Small amounts might be produced with adherent cells using dishes or flasks, optionally with multiple layers to increase the culture area. For larger scales and higher cell densities, suspension growing cells are favored. Optionally, adherent growing cell lines can be adapted to grow in suspension after selection and cloning but this procedure takes some time and there is no guarantee for success. Suitable culture systems for suspension cells include T-Flasks, Shake-flaks, WAVE-bags and bioreactor systems of different scales. Due to better oxygen supply, a shaken or stirred condition allows higher cell densities than static ones and controlled conditions (e.g., pH) in combination

with regular feeds of crucial metabolites can highly increase the viable cell number over time. Apart from fed-batch protocols perfusion mode can also be applied. For antibodies fed-batch processes are most common due to high product stability and the less complex equipment.

Special production media can increase cell density and yield and are available from different suppliers. Feed protocols need to be developed and optimized for each host cell line and medium, but packages of host cells, media, and feed protocols are also commercially available. Simple batch cultivation (static or shaken) is often sufficient to generate reasonable amounts of antibody for many applications.

33.2.2 Materials

The following section gives information on generally available example materials that can be used to start establishing a very basic stable expression system with a minimum of equipment requirements, as a basis for further otpimization.

33.2.2.1 Cells

Different adherent growing CHO derivatives can be obtained from ATCC, ECACC or commercial distributors. CHO-K1 cells are easy to culture, robust, and are suitable for stable cell line generation, but because of the endogenous DHFR genes, they are not predestined for DHFR-based gene amplification procedures. Suspension-growing CHO cells, pre-adapted to certain FCS-free culture media are available and might be used and cultured according to the manufacturers recommendation.

33.2.2.2 Culture Media and Solutions

Adherent CHO-K1 cells can be cultured in DMEM/F12 medium supplemented with Glucose (4.5 g/L), L-Glutamine (2 mM), and FCS (8%). Penicillin/streptomycin (100 mg/L) may be added when appropriate. Trypsin-EDTA solution is used to detach cells for passaging, and sterile phosphate-buffered saline is used for washing.

If Lipoectamine 2000 is used for transfection, OPTIMEM medium is recommended for use be during the transfection procedure.

Due to regulatory aspects serum-free, animal component-free, and chemically defined media are highly favorable if the cell line is foreseen to be used for clinical manufacturing.

33.2.2.3 Vectors

Any of the above mentioned vector setups might be used. The construction and application of mono- and bi-cistronic IgG expression vectors based on a pCMV-derivative plasmid backbone has been described (Jostock et al. 2004; Li et al. 2007a, b). Vector construction and optional linearization for transfection can be performed using standard molecular biology equipment.

A reporter vector (e.g., for GFP expression) is useful to check transfection efficiency.

The DNA quality can have a major impact on the transfection efficiency; thus the use of high quality purification kits is recommended.

33.2.2.4 Transfection Reagent

Adherent CHO-K1 cells can be transfected with high efficieny using the Lipofectamine 2000 reagent. Alternatively, other cationic liposome reagents may be used.

33.2.2.5 Selection Reagents

Selection reagents of course need to fit to the used vector. For a basic CHO-K1 selection system G418 (Li et al. 2007a, b) which can be obtained as a ready-to-use stock solution or a powder can be used.

33.2.2.6 Laboratory Equipment and Consumables

Standard cell culture equipment needs to be in place especially incubators and clean-benches. Tissue culture consumables such as dishes or flasks of different sizes for sub-cultivation and multi-well plates for cloning and clone expansion are needed.

33.2.3 Protocol

The following is an example of a protocol with minimal equipment requirements for the generation of stably transfected adherent growing CHO-K1 cells using the G418 selection system as also described in Li et al 2007a, b. It might be used as a model protocol for other selection systems as well.

33.2.3.1 Killing Curve to Determine Suitable G418 Concentration

1. Prepare a multi-well plate (e.g., 24-well) containing DMEM/F12 culture medium (e.g., 0.5 ml/well) with different concentrations of G418. A concentration range from 0 to at least 2.5 mg/L should be covered, ideally in triplicates. One can use the recommendations of the manufacturers of different selection reagents as a starting point to define the concentration range to be checked in the killing curve.
2. Trypsinize and transfer almost confluent CHO-K1 cells to fresh G418-free medium, and adjust the cell density to approximately 100,000/mL.
3. Seed 0.5 mL of the cell suspension to each of the prepared wells leading to a final cell density of 50,000 per well and a 50% reduction in the final G418 concentration.
4. Visually check the viability and survival of the cells daily to monitor the effect of the different G418 concentrations. If necessary, refresh the medium with a corresponding G418 content. The lowest concentrations that begin to lead to cell death within 3 days and kill all cells in the well within 2 weeks are suitable for selection.

33.2.3.2 Transfection and Selection

Optional: Linearize plasmid expression vectors using an appropriate restriction endonuclease and purify DNA (e.g., by alcohol precipitation).

1. Passage almost confluent CHO-K1 cells a day prior to transfection into 6-well plates at a seeding density of about 50,000 cells per well.
2. Transfect cells with appropriate expression vectors using the lipofectamine 2000 protocol according to the manufacturers instructions. Include a transfection efficiency control (e.g., GFP vector) and a mock transfection.
3. Check transfection efficiency 24–48 h after transfection before starting the selection.
4. 48 h after transfection, start selection by replacing the culture medium with a pre-warmed culture medium containing G418 at a suitable concentration as determined in a killing curve experiment. Keep the supernatant of transfected cells and measure the antibody concentration by ELISA for example, to confirm the functionality and successful transfection of the antibody expression vectors. If the selection system is used for the first time, then testing of three different G418 concentrations in parallel is recommended.
5. Visually check the cell growth and viability at least every 2–3 days, and if necessary exchange G418 containing culture medium or passage cells into Petri dishes or flasks.
6. The selection step is finished when expression vector transfected cells are growing well and mock transfected cells are dead. Cryopreservation of the surviving cell population and productivity measurement should be done to

confirm the stringency and success of the selection procedure. Depending on the productivity and the individual needs, such pools of stably transfected cells can be used for antibody production or be subjected to additional selection steps and/or cloning.

33.2.3.3 Single-Cell Cloning

Described here is a protocol for limiting dilution cloning of adherent cells, which can be done without any special equipment. For a high prpbability of obtaining monoclonal cell lines, seeding densities for the 96-well plates should be one cell per well or less; ideally it should be no more than 0.3–0.5. However, depending on the host cell line and medium, higher cell densities might be appropriate if a significant proportion of the cells does not survive the cloning procedure. Described below is a procedure for seeding one cell per well.

1. Prepare tissue culture 96-well plates by adding 0.1 mL medium containing the selection reagents in the concentration used for selection in each well. The number of plates to be prepared depends on the individual needs and possibilities. It is recommended that at least five plates be seeded. The more plates are seeded, the higher is the chance to find rare high-producing clones.
2. Trypsinize and count cells. Adjust the cell density to 10,000 cells per mL.
3. Add 0.1 mL of the cell suspension to the first column of one of the 96-well plates and make a serial 1:2 dilution over the next two columns. These are controls with higher seeding cell density (1,000, 500 and 250 cells per well, respectively) to check the accuracy of the first dilution step.
4. Dilute a 1 mL cell suspension into a 9 mL-fresh medium; the cell density will be about 1,000 cells/ml. Further dilute 0.3 mL cells suspension (about 300 cells) into 30-mL pre-warmed G418 containing culture medium. Seed 0.1 mL per well into the 96-well plates and cultivate at standard conditions. The final volume per well is 0.2 mL, and the theoretical cell density is one cell per well.
5. After 3–6 days, check the confluency of the higher cell density control wells and analyze the antibody content.
6. Regularly observe the wells to identify colonies of growing cells. Wells containing one defined colony have the highest probability of monoclonality. Media exchange usually is not necessary until screening; if the culture medium contains a pH indicator, change in color indicates wells with growing cells.
7. When wells are grown to near confluency, analyze the antibody content by ELISA for example, or any other suitable method to identify good producing clones.
8. Optional: A second cloning step might be applied to assure monoclonality of the finally selected clone.

33.3 Trouble Shooting

Problem	Possible reason	Action
Low transfection efficiency	Poor DNA quality	Prepare fresh DNA
	Nonoptimal DNA/liposome ratio	Experimentally optimize ratio or a try different method
Mock transfected cells survive selection	Selection drug inactive	Prepare fresh stock solution and test on cells
	Concentration of selection drug too low	Try higher concentrations
All transfected cells die upon selection	Concentration of selection drug too high	Try lower concentrations
	Transfection efficiency too low	Optimize transfection conditions
	Viability of cells at selection start too low	Improve transfection conditions and/or start selection later
Poor productivity of pool after transfection	Difficult to express antibody	Check transient expression level; if necessary and possible: change candidate
	Low abundance of high producing cells	Increase selection stringeny by increasing the concentration of selective drug, or generate and screen high number of clones
No colonies after limiting dilution cloning	Seeding cell density too low	Check control wells, repeat with higher seeding cell density
	Singularized cells did not survive	Try conditioned medium or additives
Growing cells in almost all wells after limiting dilution cloning	Seeding cell density too high	Check control wells, repeat with lower seeding cell density
No producing clones were identified in screening although pool productivity was reasonable	Majority of clones unstable	Repeat cloning from a different pool; if necessary change candidate antibody

References

Birch JR, Racher AJ (2006) Antibody production. Adv Drug Deliv Rev 58(5–6):671–85

Borman AM, Deliat FG, Kean KM (1994) Sequences within the poliovirus internal ribosome entry segment control viral RNA synthesis. Embo J 13(13):3149–57

Brezinsky SC, Chiang GG, Szilvasi A, Mohan S, Shapiro RI, MacLean A, Sisk W, Thill G (2003) A simple method for enriching populations of transfected CHO cells for cells of higher specific productivity. J Immunol Methods 277(1–2):141–55

Fang J, Qian JJ, Yi S, Harding TC, Tu GH, VanRoey M, Jooss K (2005) Stable antibody expression at therapeutic levels using the 2A peptide. Nat Biotechnol 23(5):584–90

Jones D, Kroos N, Anema R, van Montfort B, Vooys A, van der Kraats S, van der Helm E, Smits S, Schouten J, Brouwer K, Lagerwerf F, van Berkel P, Opstelten DJ, Logtenberg T, Bout A

(2003) High-level expression of recombinant IgG in the human cell line per.c6. Biotechnol Prog 19(1):163–8

Jostock T (2009) Reducing cycle time for cell line development. Cell line development and engineering conference. Informa, Berlin

Jostock T, Vanhove M, Brepoels E, Van Gool R, Daukandt M, Wehnert A, Van Hegelsom R, Dransfield D, Sexton D, Devlin M, Ley A, Hoogenboom H, Mullberg J (2004) Rapid generation of functional human IgG antibodies derived from Fab-on-phage display libraries. J Immunol Methods 289(1–2):65–80

Jostock T, Sewalt R, Drewello D, Lang S, Dennler S, Ortlepp M, Wichter J, Nommay A, Dragic Z, van der Vaart H, Wilms B, Knopf HP (2008) Evaluation of the STAR Technology. European Biotechnology News 7:33–36

Kaloff CR, Haas IG (1995) Coordination of immunoglobulin chain folding and immunoglobulin chain assembly is essential for the formation of functional IgG. Immunity 2(6):629–37

Kalwy S, Rance J, Young R (2006) Toward more efficient protein expression: keep the message simple. Mol Biotechnol 34(2):151–6

Kaufman RJ, Davies MV, Wasley LC, Michnick D (1991) Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus. Nucleic Acids Res 19(16):4485–90

Lee G (2008) Cell line development using a Per.C6 host cell line. Cell Line Development and Engineering Conference. Informa, San Diego, CA

Li J, Menzel C, Meier D, Zhang C, Dübel S, Jostock T (2007a) A comparative study of different vector designs for the mammalian expression of recombinant IgG antibodies. J Immunol Methods 318(1–2):113–24

Li J, Zhang C, Jostock T, Dübel S (2007b) Analysis of IgG heavy chain to light chain ratio with mutant Encephalomyocarditis virus internal ribosome entry site. Protein Eng Des Sel 20 (10):491–6

Montano RF, Morrison SL (2002) Influence of the isotype of the light chain on the properties of IgG. J Immunol 168(1):224–31

Puck TT (1958) Genetics of somatic mammalian cells. J Exp Med 108:945–955

Schlatter S, Stansfield SH, Dinnis DM, Racher AJ, Birch JR, James DC (2005) On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells. Biotechnol Prog 21(1):122–33

Urlaub G, Chasin LA (1980) Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc Natl Acad Sci USA 77(7):4216–20

Urlaub G, Kas E, Carothers AM, Chasin LA (1983) Deletion of the diploid dihydrofolate reductase locus from cultured mammalian cells. Cell 33(2):405–12

Wurm FM (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22(11):1393–8

Chapter 34
Protein A/G Chromatography

Kirstin A. Zettlitz

34.1 Introduction

Protein A or G affinity chromatography is a well established and reliable method for purifying whole antibodies, Fc-fusion proteins and Fab-fragments from serum or cell culture supernatant. The main advantage of this purification method is the fact that no special tags need to be added to the antibodies during the cloning process. Protein A (SpA) is a surface protein found in the cell wall of Staphylococcus aureus. Its importance in biochemical research is due to its ability to interact with immunoglobulins. For the use in affinity chromatography, the purified protein is immobilized by covalent binding to a solid support, generally Sepharose (Langone 1982). Protein A interacts with IgG via the Fc region, this binding site has been shown to be located both on the C_H2 and C_H3 domains of human IgG (Deisenhofer 1981). An additional binding site has been identified in the Fab portion distinct from the antigen-binding site (Johansson and Inganas 1978). The Fab binding site on the Protein A molecule is also different from the Fc site. Immunoglobulins of many mammalian species interact with Protein A, although quantitative and qualitative differences between species, subclasses, and allotypes have been observed. These findings are reflected by the different pHs and buffers needed for the elution from Protein A columns (see Table 34.1).

Other immunoglobulin-binding bacterial proteins are commonly used to purify antibodies. Protein G is expressed in group C and G Streptococcal bacteria and interacts with antibodies via the Fc region. The native molecule also binds albumin, thus recombinant Protein G lacking the albumin binding site is used in affinity chromatography to avoid albumin contamination of the purified antibodies.

K.A. Zettlitz
Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany
e-mail: kirstin.zettlitz@izi.uni-stuttgart.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_34,

Table 34.1 Binding of immunoglobulins to protein A (based on (Lindmark et al. 1983))

Immunoglobulin		Binding capacity	Elution pH
Human	IgG1	+++	3.0–4.6
	IgG2	+++	4.3–5.0
	IgG3	−	
	IgG4	+++	3.9–5.0
	IgM	±	2.5–4.0
	IgA1	−	
	IgA2	±	
Bovine	IgG1	−	
	IgG2	+	
Mouse	IgG1	+	6.0–7.0
	IgG2a	+++	4.5–5.0
	IgG2b	+++	4.5
	IgG3	+++	3.5–4.0
Rabbit	IgG	+++	3.0
Rat	IgG1	+	7.0
	IgG2a	−	
	IgG2b	−	
	IgG2c	++	3.0–4.0

Protein A/G is a recombinant fusion protein combining binding domains of both Protein A and Protein G. The additive properties of this molecule are useful in the purification of polyclonal IgG or of antibodies of an unknown subclass.

Protein L is a 36 kDa protein isolated from the bacteria Peptostreptococcus magnus which binds to immunoglobulins through interactions with the light chain. Thus Protein L is, in addition to a wider range of antibody classes than Protein A or G, suitable for the purification of single chain variable fragments and Fab fragments that contain a kappa (κ) light chain.

34.2 Materials

34.2.1 Plastic Ware

– Liquid Chromatography Columns, Luer-Lock, non-jacketed, size: 1.0 × 10 cm, bed: 8 ml, Cat. No. C3794-10EA, Sigma-Aldrich, Steinheim, Germany

34.2.2 Buffers and Solutions

– Buffer A: 0.02 M NaH_2PO_4*H_2O (2.7 g), 0.15 M NaCl (8.8 g), adjust volume to 1 L with ddH_2O, adjust pH to 8.0
– Tris-HCl: 1 M Tris (121.14 g), 100 mM Tris (12.114 g), 10 mM Tris (1.211 g), adjust volume to 1 L with ddH_2O, adjust to pH 8.0 with HCl

– 100 mM glycine (7.5 g), adjust volume to 1 L with ddH_2O, adjust to pH 3.0 with HCl
– BioRad Protein Assay, BioRad, Krefeld, Germany

34.2.3 Reagents

– Ammonium sulphate $(NH_4)_2SO_4$
– Protein A-Sepharose CL-4Ba (Cyanogen Bromide Activated), Cat. No. P-3391, Sigma-Aldrich, Steinheim, Germany

34.3 Procedure

Harvest 100–500 ml of the supernatant of IgG-producing cells (see Sect. 34.1.6). Clear the supernatant by centrifugation at 380× *g* for 5 min and store at 4°C.

34.3.1 Ammonium Sulphate Precipitation

As a first step in purification, ammonium sulphate precipitation is used to concentrate the protein. The high ionic strength of a 60% saturated solution alters the solubility of the IgGs and leads to almost complete precipitation from the supernatant.

1. Add stepwise 390 g/l of $(NH_4)_2SO_4$ (corresponding to 60% saturation) to the pooled supernatants at 4°C whilst stirring.
2. Stir for additional 30 min at 4°C.
3. Recover the protein of interest by centrifugation at 10,000× *g* for 30 min at 4°C.
4. Dissolve the protein pellet in loading buffer (1× PBS, approx. 20–30 ml).

34.3.2 Purification of IgG by Protein A

(Column Method)

1. Swell the lyophilized protein A powder in buffer A for at least 30 min at room temperature without stirring. One gram of powder swells to approximately 3–4 ml of hydrated gel with a binding capacity of 20 mg/ml (for human IgG).
2. Prepare a 1:1 suspension of resin in buffer A and pour the suspension into a liquid chromatography column (approx. 0.5–1 ml resin). Allow the column to flow as it is settling.

3. Equilibrate the column with 20 column volumes of buffer A (10–20 ml).
4. Adjust the crude antibody preparation (protein precipitated from cell culture supernatant) to pH 8.0 by adding 1/10 volume of 1 M Tris-HCl pH 8.0.
5. Load the crude antibody preparation and pass through the protein A column.
6. Wash the column with 20 column volumes (10–20 ml) of 100 mM Tris-HCl pH 8.0 and subsequently with 20 column volumes (10–20 ml) of 10 mM Tris-HCl pH 8.0 to remove unbound protein.
7. Elute bound IgG with 100 mM glycine pH 3.0, add this elution buffer stepwise, approximately 500 µl per fraction. (see Note 5)
8. Collect the eluat in 1.5 ml tubes containing 50 µl of Tris-HCl pH 8.0 and mix gently to bring the pH back to neutral (avoid bubbling and frothing as this denatures the protein).
9. Identify the IgG containing fractions by Bradford test (mix 10 µl of elution fraction and 100 µl of 1× Bradford) and dialyze them against 1× PBS overnight at 4°C (see Note 2).
10. Wash the column with additional five column volumes of elution buffer.
11. Re-equilibrate the column with 20–30 column volumes of buffer A and store with a preservative (20% EtOH) at 2–8°C. For reuse, let the column drain off (EtOH) and equilibrate with 20 column volumes buffer A (see Note 1).

34.4 Notes

1. If the binding capacity of the protein A column decreases, this may be due to steric hindrance by unspecifically bound proteins. Wash the resin with 100 mM Tris or 1 M borate buffer, 2 M NaCl pH 8.5–9.0, subsequently wash with 100 mM acetate buffer pH 4.0. Re-equilibrate with 20 column volumes of buffer A.
2. Take samples of the supernatant, the crude protein and the wash fractions and analyze by SDS-PAGE for protein containing fractions (see Fig. 34.1).

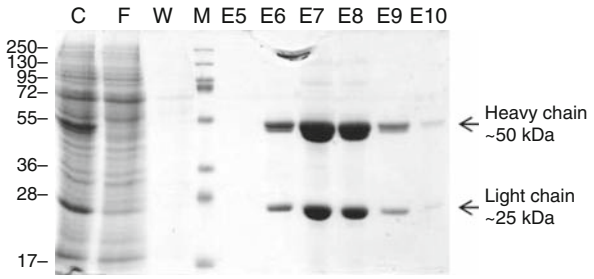


Fig. 34.1 Purification of human IgG1. 12% SDS-PAGE, reducing conditions, Coomassie-stained. C = crude, F = flow through, W = wash: 30 µl loaded; E = eluate: 10 µl loaded

3. Check the supernatant after precipitation for the remaining protein. If there is a considerable loss of protein, load cell culture supernatant (adjusted to ph 8.0) directly to the column and pass through by using an appropriate pump.
4. Serum-free production media are recommended, but purification from supernatant containing fetal bovine serum is also possible as Protein A shows no binding to bovine IgG1 and only weak binding to bovine IgG2.
5. The pH of the elution buffer is dependent on species and subclass of the concerned antibody. Elute with a pH-gradient or with alternative elution buffers to find the best conditions (e.g. 0.2 M NaH_2PO_4, 0.1 M citric acid). Refer to reference (Langone 1982) for more detailed information about Protein A binding to immunoglobulins', serum levels and recommended elution pH.
6. Tyrosine residues within the Fc region of IgG contribute to the interaction with Protein A, hence glycyltyrosine may be used for elution (0.1 M glycyltyrosine in 2% NaCl, pH 7.0).

References

Deisenhofer J (1981) Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-A resolution. Biochemistry 20(9):2361–2370

Johansson SG, Inganas M (1978) Interaction of polyclonal human IgE with protein-A from Staphylococcus aureus. Immunol Rev 41:248–260

Langone JJ (1982) Applications of immobilized protein A in immunochemical techniques. J Immunol Methods 55(3):277–296

Lindmark R, Thoren-Tolling K et al (1983) Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. J Immunol Methods 62(1):1–13

Chapter 35
Epitope Analysis Using Synthetic Peptide Repertoires Prepared by SPOT Synthesis Technology

U. Beutling and R. Frank

Abbreviations

AA	Amino acid
AAI	Antibody–antigen interaction
Abu	α-Aminobutyric acid
Acm	Acetyl-aminomethyl
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine salt
BPB	Bromophenol blue
BSA	Bovine serum albumine
CBS	Citrate-buffered saline
CDS	Colour developing solution
DCM	Dichloromethane
DIC	N,N′-Diisopropylcarbodiimide
ECL	Enhanced chemiluminescence
FITC	Fluoresceine isothiocyanate
HOBt	N-Hydroxybenzotriazole
HRP	Horse radish peroxidase
kd	Kilo dalton
MBS	Membrane blocking solution
MTBE	*tert*-Butyl methylether
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
NBT	Nitro blue tetrazolium chloride monohydrate
NMP	N-Methyl-2-pyrrolidinone
PBS	Phosphate-buffered saline

U. Beutling and R. Frank (✉)
Department of Chemical Biology, HZI (Helmholtz Centre for Infection Research), Inhoffenstrasse 7, 38124 Braunschweig, Germany
e-mail: ronald.frank@helmholtz-hzi.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_35,

PEG	Polyethylene glycol
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
PP	Polypropylene
PPI	Protein-protein interaction
PVC	Polyvinyl chloride
rpm	Revolutions per minute
SC^2	Spotting compound-support conjugates
SDS	Sodium n-dodecyl sulfate
SDS PAGE	Sodium n-dodecyl sulfate polyacrylamide gel electrophoresis
SM-A	Stripping mix A
SM-B	Stripping mix B
*t*Bu	*tert*-Butyl
TFMSA	Trifluoromethanesulfonic acid
TBS	Tris-buffered saline
TIBS	Triisobutylsilane
Trt	Triphenylmethyl

35.1 Introduction

Antibodies recognize and bind their target protein antigens via surface accessible interaction sites, the epitopes (Fig. 35.1), which can involve amino acid side chain and backbone contacts along a linear segment of the antigen amino acid chain (linear epitopes) or which can involve amino acid residues from two or more segments of the protein chain brought together by its folded secondary structure

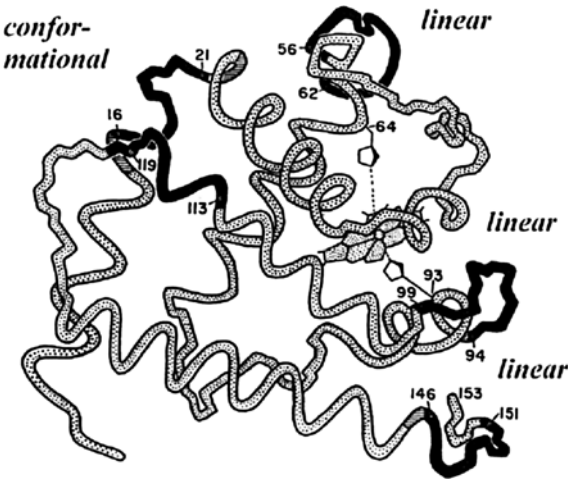


Fig. 35.1 A protein antigen (here myoglobin) presenting three linear loop-type and one conformational epitopes for interaction with cognate antibodies. (The drawing of the protein chain was adapted from Atassi (1977))

(conformational epitopes). Approaches that systematically study antibody–antigen interactions (AAI) can be directly applied to protein–protein interactions (PPIs) as well and therefore the term epitope is used here in its broadest sense for a protein interaction site and far beyond its default immunological meaning.

Linear epitopes can be copied efficiently by small peptide fragments that are readily amenable to chemical synthesis. This is also true for a significant part of the conformational epitopes when linear components of these alone show sufficient affinity or when they can be mimicked by linear "mimotope" peptides (Geysen et al. 1986; Geysen and Mason 1993). The peptide approach enables a detailed insight into the molecular recognition events and allows to rapidly decipher the chemical nature of these interactions (Winkler et al. 2000; Keitel et al. 1997). This chapter describes experimental strategies to identify and decipher epitopes exploiting the many options offered by combinatorial and parallel chemical peptide synthesis in a very convenient array format.

SPOT-Synthesis (Frank 1992) is an easy and very flexible technique for simultaneous parallel assembly of peptides on membrane supports. This method gives researchers rapid and low-cost access to a large number of peptides both as solid phase bound and solution phase products for systematic epitope analysis. Each peptide is synthesized at a distinct site (spot) on a porous membrane. The final array of cellulose-bound peptides can be directly probed for protein binding in an immunoblot type overlay process (Fig. 35.2, *left*). Membrane-bound peptide arrays manufactured by SPOT synthesis are ideally suited for rapid screening through many protein sequences; however, they can only be reused a few times. Furthermore, SPOT synthesis on porous membranes has its limitations when reducing the spot size below 1 mm and becomes costly and tedious when large numbers of copies of an identical array are required. We therefore have developed a special add-on to the SPOT synthesis process for manufacturing and application of synthetic peptide repertoires in the form of chemical mini- or, more sophisticated, microarrays printed onto, e.g., glass microscope slides (Fig. 35.2, *right*). These maintain the advantageous features of cellulose-bound probe peptides but allow massive miniaturization and multiplication (Dikmans et al. 2006).

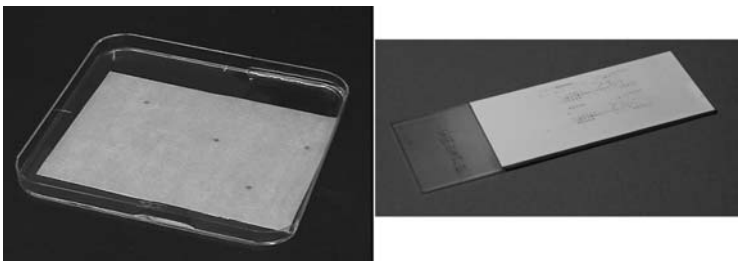


Fig. 35.2 *Left*: A cellulose membrane-bound macroarray probed in a 12 × 12 cm culture dish. *Right*: A cellulose-bound miniarray printed on a plastic-coated glass microspcope slide (original size!)

The process for manufacturing mini- or microarrays of synthetic peptides adds some more experimental steps to the whole manufacturing process and thus, requires additional effort. Therefore, it is only reasonable if a greater number of copies of the same array are required for a larger series of experiments such as screening serum collections, supernatants of hybridoma clones, genome spanning protein families (domains, kinases, etc.), recombinant protein variants, or for providing universal generic random peptide libraries to many diverse applications. The new chemical mini- and microarrays perform as reliably as the original, successful macro-SPOTs system on cellulose membranes (Fig. 35.3). Single or only a few serial experiments should still be preferably performed with the macro-array format, except if a limiting amount of sample makes miniaturization a strict prerequisite as with samples from, e.g., small model organisms like mice or worms, tissue from patients or a few sorted cells; in this case, the extra effort is certainly justified.

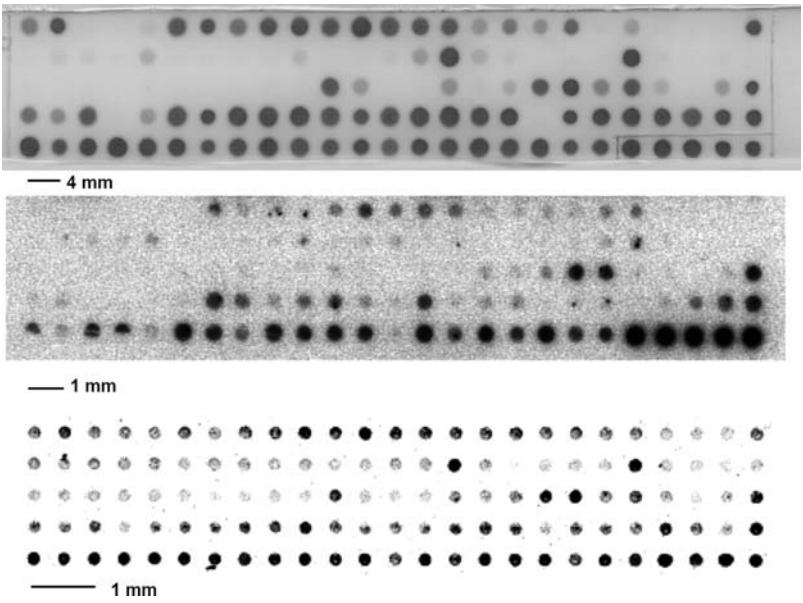


Fig. 35.3 *Top*: A macroarray of 120 peptides comprising a full single amino acid replacement set of the peptide Ac-NYGKYE-ßAla was synthesized on a conventional ACS01-cellulose membrane in lines of 25 spots each at a spot distance of 4 mm and probed with monoclonal antibody 1D3 followed by an AP-conjugated secondary antibody and color signal development with the BCIP/MTT substrates. – *Middle*: The same set of 120 peptides synthesized on an acid soluble cellulose membrane, processed through the SC^2-protocol, printed at a spot distance of 1 mm onto an untreated glass slide in the same array layout and assayed as above. – *Bottom*: The same peptide set as above spotted on an untreated glass microscope slide at a spot distance of 0.5 mm. The array was probed with mAb 1D3 followed by a Cy5-conjugated secondary antibody and recording fluorescence signals with a biochip reader

35.2 Peptide Synthesis Strategies

35.2.1 Antibody–Antigen and Protein–Protein Interactions

For pre-classifying an antigen or a protein as being recognized via a linear epitope, it is a good indication if its interaction with the cognate antibody or protein partner is detectable in a western blot analysis after denaturing SDS-PAGE; for PPIs check both orientations of the analysis (protein A denatured and probed with protein B and vice versa). The thorough investigation of an interaction domain or an entire protein using SPOT peptide arrays involves three subsequent steps (Fig. 35.4). First, 15- to 20-mer peptide fragments, covering the sequence with an offset of 3–5 amino acids, are synthesized to locate the binding site (Fig. 35.4a, b). Thus, a protein of 1,000 amino acid residues (about 120 kd in size) is covered by 200–350 peptide fragments. If the capacity of the SPOT synthesis is limited, for example when performing manual synthesis, it may be helpful to first narrow down the interaction site to a fragment or sub-domain by using other experimental methods, for example, by probing the interaction with deletion mutants applied in pull down assays. For shorter proteins or interaction domains, peptides with only one amino acid offset can be used as a starting point. When polyclonal sera are analyzed which may recognize overlapping epitopes bound by different antibodies, it can be helpful to shorten the length of the peptides to distinguish between the overlapping epitopes (Blüthner et al. 2000).

In a second step (sizing), the core binding motif is determined. An epitope peptide identified in the previous mapping experiment is further characterized by using a series of overlapping peptides having an offset of only one amino acid and a stepwise reduced size (Fig. 35.4c). Ideally, only peptides carrying the core epitope will react and one of the series will reveal one single spot which corresponds to the peptide with the minimal epitope. However, more complex results may be obtained when the core residues are not present in a single contiguous sequence. Furthermore, some proteins will bind with detectable affinity to several small "sub-epitopes."

The third step (analoging) determines the contribution of individual amino acid side chains. In the past, this task was frequently addressed by "glycine- or alanine-walks," i.e. by exchanging only one amino acid per peptide with glycine or alanine, resulting in a small set of point-mutated peptides covering the epitope. However, much more information and confidence on every amino acid position can be gained with a full replacement study, including the identification of variants with higher affinity or selectivity. For this, a set of peptides with systematic single replacements of every amino acid residue in the core peptide sequence by all other genetically coded amino acids is probed (Fig. 35.4d). Obviously, analoging can be applied directly to an initial peptide hit if capacity permits, e.g., 15mer full replacement amounts to 300 peptide spots.

Information obtained from these approaches can be used in a variety of follow-up experiments. An independent verification of the epitope identified is strictly

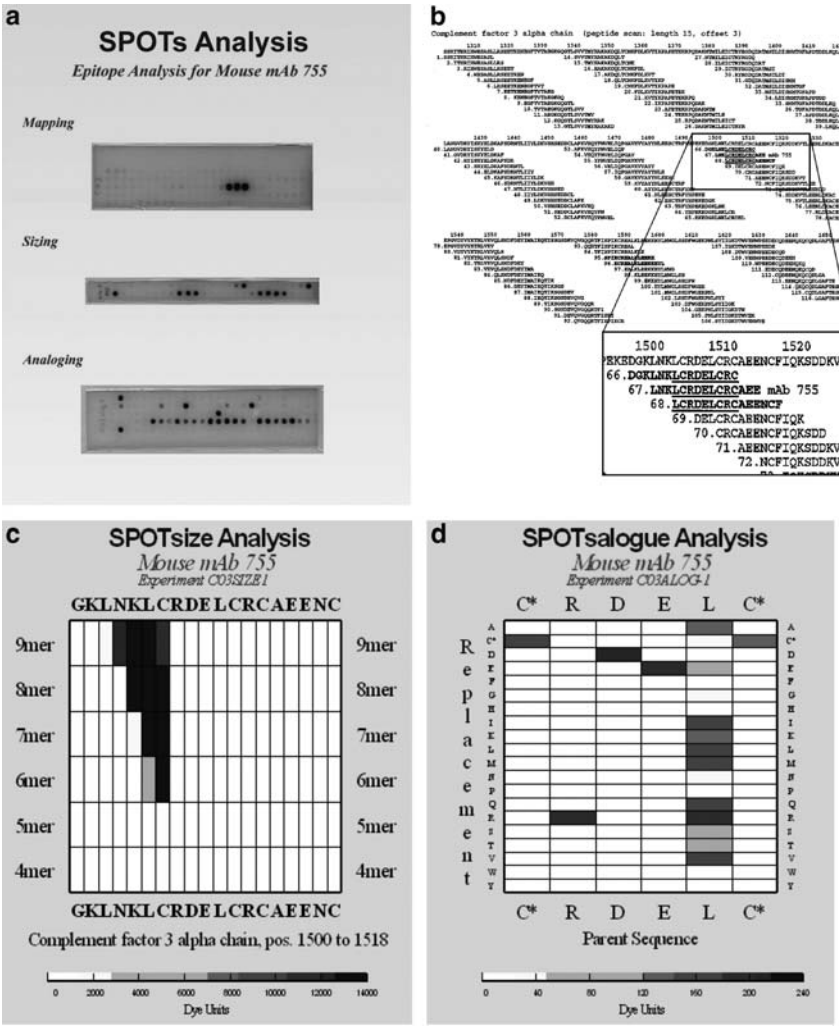


Fig. 35.4 An example for a classical epitope analysis experiment with monoclonal antibody 755 raised against the human C3a receptor α chain (Klos et al. 1988). (**a**) Overview of the three membranes that were analyzed for mapping (116 peptides), sizing (50 peptides) and analoging (120 peptides), summing up 286 peptides for the complete analysis. Order of peptides on the membranes as described in the mapping experiment. (**b**) Sketch of the overlapping peptide strategy for mapping. The three active peptides are marked in bold and their common 9mer sequence is underlined. (**c**) Spectral diagram display of the results from the sizing membrane. Signal intensity from a peptide spot is displayed at the square corresponding to the *N*-terminal residue of the respective peptide and its sequence then reads to the right: the 6mer C*RDELC* is the shortest peptide still strongly recognized. (**d**) Spectral diagram display of the results from the analoging membrane. Each square represents the spot intensity obtained from the respective analog peptide. In this particular example, all residues are important for binding and are non-replaceable except the leucine5 which tolerates quite a number of other amino acids but not all, a fact which would have escaped a "glycine or alanine walk" analysis. Dye units = arbitrary scale for relative intensities/ C* = Cys(Acm)

recommended and can be pursued by either the construction of mutant protein analogues lacking essential residues of the core epitope or by the use of the identified peptides as soluble products in competition experiments. Amino acid replacement profiles of epitopes as obtained in step 3 can also be used to search databases for potential cross-reactivity with other proteins. This is of particular importance for the identification of immunological cross-reactivity with antigens from, e.g., pathogens. Further analyses look for the contribution of post-translational modifications (e.g., phosphorylation or glycosylation) which can be studied by synthesizing sets of peptides with identical sequences, but different side chain modifications (Mukhija et al. 1998). For binding motifs located at the *N*-terminus of a protein, the contribution of the *N*-terminal amino group to binding affinity can be assessed by synthesizing peptides with amino acid residues added in front of the *N*-terminal amino acid (Kneissel et al. 1999). Additionally, using a modified linker strategy, peptides can also be presented with a free carboxy-terminal end; this could reveal effects of peptide presentation and is also required for some PPIs such as the binding to the PDZ domain (Schultz et al. 1998).

If no linear peptide binder can be identified by peptide scanning, the recognition of antigen/protein is most obviously truly conformationally defined. Assembly of branched double or triple peptide combinations on a single spot have been reported to successfully represent such epitopes (Espaniel et al. 2003). Linear peptide mimotopes may be identified by screening generic complete peptide libraries (Geysen and Mason 1993). Strategic arrays of peptide pools that cover full libraries with several billions of peptides can easily be prepared for this purpose through incorporation of amino acid mixtures (Table 35.1). An example of a peptide library array probed with a monoclonal antibody is shown in Fig. 35.5 (Frank et al. 1995). In lucky cases, the amino acid residues of a mimotope sequence may be a guide to locate segments of a conformational epitope in the primary sequence or, if available, the 3D-structure of the protein (Oggero et al. 2004). Random peptide libraries have also been applied effectively in studying peptide sequence preferences in PPIs (Rüdiger et al. 2001). Other approaches for an *a priori* delineation of peptide binders include the screening of a large series of individual, randomly selected peptides (Reineke et al. 2002).

Moreover, the chemistry allows incorporation of modified or not naturally occurring amino acids, most easily the D-enantiomers of the normal L-amino acids, as well as artificial linkages to an extend from natural peptide ligands towards peptidomimetics. An overview is given in Frank 2002a, b. See also the assembly of non-peptidic small organic molecules in Dikmans et al. (2006).

35.2.2 Other Protein Ligand Interactions

Besides the exploitation of antibody epitopes and protein-binding sites, as will be described in detail in the protocols below, a plethora of other protein interactions have been reported which can be studied with modifications of the protocol steps

Table 35.1 Strategies for the delineation of peptide sequences by screening of random peptide pools[a]

1) Iterative search starting with one or more defined positions, e.g., according to Geysen et al. (1986).		
first generation	X-X-3-4-X-X	400 pools (each 160,000 sequences)
second generation	X-2-0_3-0_4-5-X	400 pools (each 400 sequences)
third generation	1-0_2-0_3-0_4-0_5-6	400 pools (each one sequence)
2) Positional scanning with single fixed positions, one single screen according to Dooley and Houghten (1993) see also Rodriguez et al. (2004).		
1-X-X-X-X-X	20 pools (each 3.2×10^6 sequences)	
X-2-X-X-X-X	20 pools (each 3.2×10^6 sequences)	
X-X-3-X-X-X	20 pools (each 3.2×10^6 sequences)	
X-X-X-4-X-X	20 pools (each 3.2×10^6 sequences)	
X-X-X-X-5-X	20 pools (each 3.2×10^6 sequences)	
X-X-X-X-X-6	20 pools (each 3.2×10^6 sequences)	
3) Dual-positional scanning, one single screen according to Frank (1994).		
1-2-X-X-X-X	400 pools (each 160,000 sequences)	
X-2-3-X-X-X	400 pools (each 160,000 sequences)	
X-X-3-4-X-X	400 pools (each 160,000 sequences)	
X-X-X-4-5-X	400 pools (each 160,000 sequences)	
X-X-X-X-5-6	400 pools (each 160,000 sequences)	

[a]Special codes to describe the pool compositions are: 0_n = unvaried position in a particular screen occupied by single amino acid residues; 1,2,3. . . . = positions systematically varied by single amino acid residues in a particular screen; X = position occupied by a set of (e.g., all 20 L-) amino acid residues.

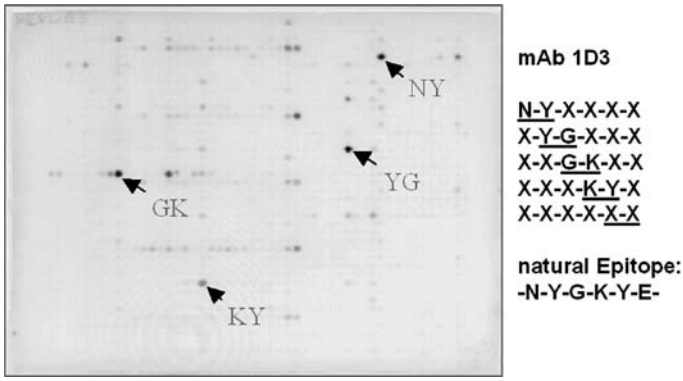


Fig. 35.5 A dual-positional-scanning peptide library experiment (corresponds to Table 35.1, entry 3) with monoclonal antibody 1D3. Signal development was carried out with an alkaline phosphatase conjugated secondary antibody and the BCIP/MTT reagents. Result of this experiment allows the determination of the epitope sequence a priori from the overlapping dipeptide signals (Frank et al. 1995). The tyrosine (Y5) and glutamic acid (E6) in the natural epitope do not contribute significantly to specific recognition as they can be replaced by almost any other residue (data from the replacement analysis not shown)

specifically adapted to the particular requirements of the respective assays. These include

- Mapping and analysis of T-cell epitopes through either MHC-binding or T-cell stimulation
- Enzyme substrate analysis and inhibitor design (Dostmann et al. 2000; Rodriguez et al. 2004)
- Protein/peptide interactions with nucleic acids
- Peptide nucleic acid (PNA) interactions with nucleic acids
- Chemical/enzymatic transformation of immobilized peptides
- Cell-based assay with cleaved, solution phase peptides.

For a review on these applications see Frank 2002b. Moreover, solid phase peptide arrays can be used for affinity-capture of the protein of interest. For example, epitope-specific antibodies can be isolated from polyclonal sera (Valle et al. 1999; Billich et al. 2002), thus, combining mono-specificity with the ease of rabbit serum preparation. More recent novel options include, e.g., the preparation of mini-protein (protein domain) arrays by the combination of solid phase synthesis and chemical ligation (Töpert et al. 2003) or the multiplexed bio-panning of phage libraries for genome wide protein interaction mapping (Bialek et al. 2003). Furthermore, peptides selected from libraries as ligands/inhibitors for proteins that usually do not bind other peptides/proteins have been developed as tools in molecular biology such as the Strep-tag peptide binding to the biotin pocket of streptavidin (Schmidt et al. 1996).

35.3 Guidelines for SPOT Peptide Synthesis

35.3.1 General Principle

The principle of the method involves for each amino acid to be added to a growing peptide chain a coupling reaction started by dispensing a small droplet of the reaction mixture onto the membrane. The droplet gets absorbed and forms a circular spot. Using a solvent of low volatility containing activated amino acid monomers, such a spot forms an open reactor for chemical conversions involving reactive functions anchored to the membrane support, comparable to conventional solid phase synthesis. A large number of separate spots can be arranged as an array on a larger membrane sheet and the intermediate areas are chemically inactivated by acetylation. Each of these spots then can be separately manipulated by manual or automated delivery of the corresponding reagent solutions (Fig. 35.5). The volume dispensed and the absorptive capacity of the membrane determine the spot size, which can be adjusted to control the scale of synthesis. The spot size also controls the minimal distance between spot positions and thereby the maximum density of the array. Synthetic steps common to all spot reactors are carried out by washing the

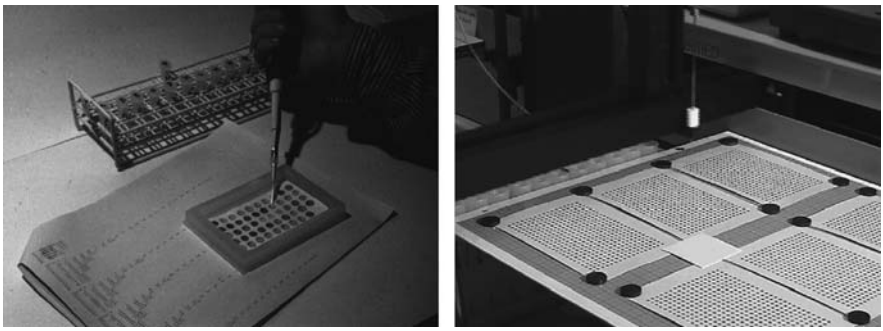


Fig. 35.6 *Left*: The minimal experimental set-up for manual SPOT-synthesis showing the reaction tray with a membrane displaying *blue* (*dark*) stained amino-spots and *yellow* (*light*) stained coupled spots, the rack of small tubes containing the activated AA derivatives, the computer-generated listing of the pipetting operations and the manually operated pipette. *Right*: Platform of the AutoSpot robot showing six cellulose membranes (8 × 12 cm) with each an array of 17 × 25 (425) spot reactors

whole membrane with respective reagents and solvents. Fully automated instruments place the membranes on a porous plate and remove the reagent and solvent excess by vacuum suction.

Because of their hydrophilic nature, cellulose membranes are particularly well suited for the presentation of immobilized peptides to a biological assay system. After SPOT synthesis of the peptide array and incubation of the membrane with an antibody or protein sample, detection of proteins bound to individual spots is done in a manner analogous to an immunoblot (Fig. 35.3 *Top*). Unspecific binding of biomolecules to the cellulose membrane itself has only rarely been reported for the assay conditions given below. Assembly of peptides on the surface of the cellulose fibres in the membrane yield quite a high local concentration which allows to capture rather low affine binders (up to several 100 μM was reported (Hoffmüller et al. 1999)).

As long as the biological assaying of these membrane-bound macroarrays does not irreversibly transform the peptide probes, they can be reused many times upon stripping off all biologicals from the assay experiment. Depending on the biological assay, this stripping, however, can be quite insufficient and thus one array may only be usable once. Furthermore, one synthesized array can be processed only serially through a set of experiments, which requires several identical synthetic arrays to proceed in parallel. Third, a conventional SPOT-type array has rather large dimensions (min. 2 mm spot distance) and therefore we call it a macroarray; this requires considerably large volumes for the assay and, thus, the amount of available sample may become limiting (e.g., blood samples from mice).

We describe an easy add-on process that overcomes these limitations of cellulose membrane-bound macroarrays produced by SPOT-synthesis by transferring a synthetic membrane-bound macroarray to a multitude of microscope slide bound mini- or microarrays. The manufacturing of the peptide macroarray follow

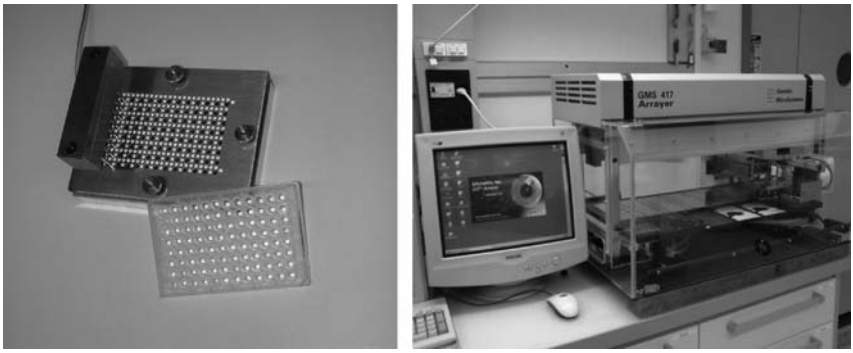


Fig. 35.7 *Left*: the 384→96 punching device used to separate peptide spots for the SC^2-process. *Right*: The model GMS 417 ring and pin Array Printer

essentially standard SPOT synthesis protocols with an array format adapted from the 384-well microtiter plates, except that a special, acid sensitive amino-cellulose membrane is used. Individual spots are separated post-assembly with the help of a 384-compatible punching device which delivers the cellulose-compound conjugate disk segments of 3 mm diameter into the wells of four 96-deepwell plates. Then, the disks are treated with a TFA cocktail containing >80% TFA plus scavengers, as used in routine solid phase peptide synthesis. This treatment solubilizes the support itself with the compounds still covalently attached and simultaneously cleaves the acid sensitive side chain protecting groups. Precipitation with ether removes the bulk of acid together with the cleavage chemistry and the dried precipitate is then dissolved in DMSO. After appropriate dilution with DMSO, minute aliquots of these solutions of compound-support conjugates are transferred (printed) and adsorbed onto the target planar surfaces, usually glass microscope slides, with the help of a suitable pipetting device. We therefore call this process "spotting compound-support conjugates": SC^2. One standard cellulose disk segment yields 0.5 mL of DMSO stock solution from which only nL to pL aliquots can be used to print up to 10^6 mini- or 10^8 microarray copies.

This SC^2-process maintains most of the beneficial properties of a cellulose-bound peptide array, in particular the low background binding and the high local peptide concentration. Thus, the new chemical mini- and microarrays perform as reliably as the original, successful macro-SPOTs system on cellulose membranes (see Fig. 35.3).

35.3.2 Brief Introduction to Solid-Phase Chemical Peptide Synthesis

Assembly of a peptide chain by chemistry starts at the *C*-terminal end (in contrast to biological synthesis, where the ribosome starts at the *N*-terminus!). But peptide

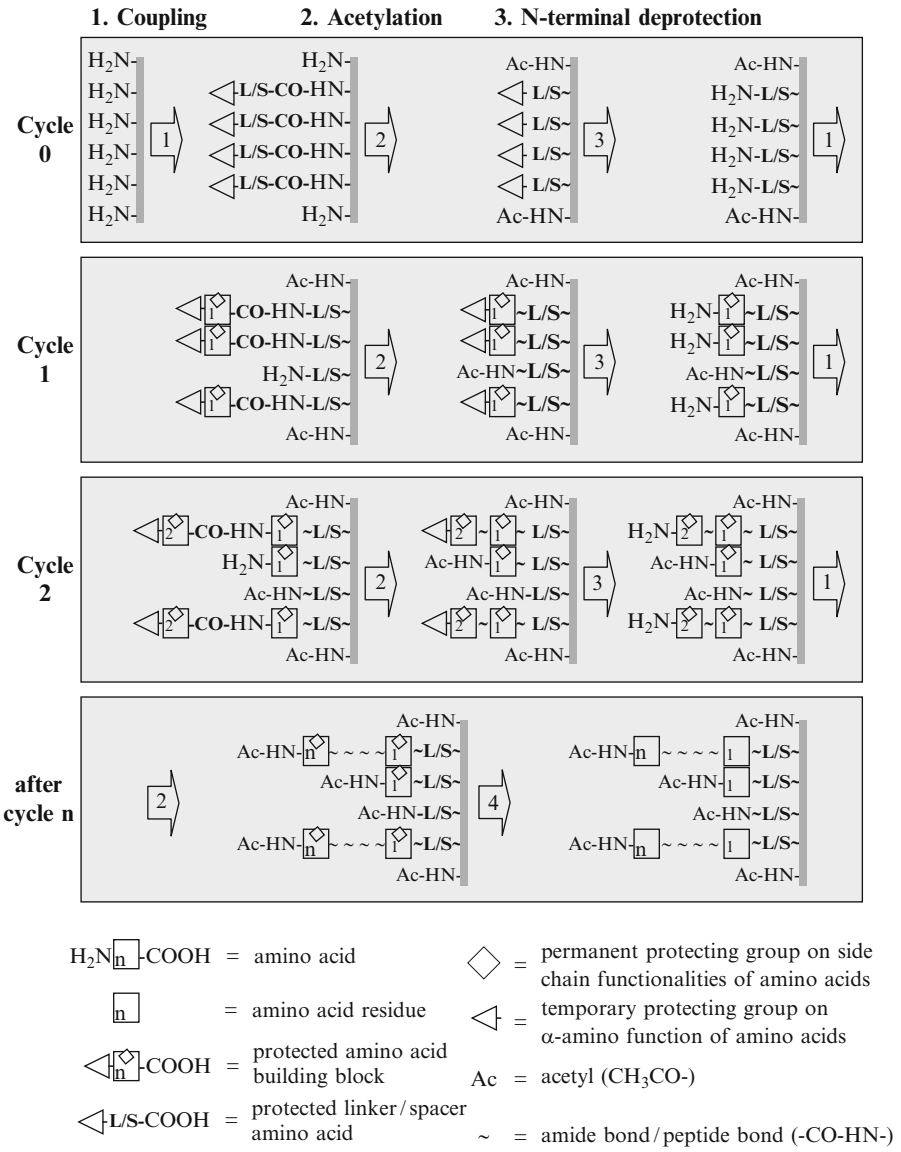


Fig. 35.8 Outline of the series of chemical transformations during peptide assembly on a solid phase. Changes introduced at each step are highlighted in *black*

chemists write a peptide sequence following the same convention; this is the *N*-terminus at the left side and the *C*-terminus at the right side.

The amino acid building blocks are specially modified amino acids that carry protecting groups which assure a directed step-by-step assembly of the peptide chain. All reactive chemical functionalities at the amino acid side chains are

blocked permanently throughout the whole assembly phase and are only removed in a final deprotection treatment. The terminal carboxylic acid function of the amino acid remains free and is chemically activated forming an active ester which then reacts in a coupling reaction to yield an amide bond (peptide bond) with a free terminal amino function of a growing peptide chain. The amino function of the amino acid is blocked by a temporary protecting group, which prevents self-coupling with its own activated carboxyl function.

Figure 35.8 outlines the basic steps in solid phase peptide assembly as they will be used in this chapter. The solid support material presents free amino functions covalently attached to its surface. The manufacturing of respective supports requires more sophisticated chemical expertise and equipment and, thus, the use of a quality checked commercial material is recommended. One cycle of amino acid addition consists of three steps which are repeated as often as amino acid residues are required for the target peptide sequence. Step 1 is the coupling of the protected amino acid building block to the free terminal amino functions presented on the support. All amino functions that have not reacted in step 1 will be blocked in step 2 by acetylation (also called: capping reaction). This prevents them from reacting in a later step of the assembly process which will result in peptide contaminants of wrong (deletion) sequences. Capping assures that inefficient coupling only gives rise to truncated peptides which are still fragments of the correct sequence! Then the *N*-terminal protecting groups at the growing peptide chains are removed in step 3 releasing the free amino function of the last coupled amino acid ready for the next coupling reaction. Cycle 0 is optional but recommended for array generation and involves the incorporation of a spacer or linker molecule preferably using the same chemistry as for amino acid coupling. A spacer amino acid increases the distance of the peptide to the support surface and will enhance access to these peptides by large protein acceptors. A linker amino acid introduces a special chemical entity with a bond that can be cleaved selectively after the assembly is completed to release the peptide from the support into solution. For more information see textbooks such as that from Chan and White (2000). After the final coupling cycle (n), the peptides are acetylated and in step 4 all side chain protecting groups are removed.

35.3.3 The SPOT-Membrane Support

The membrane supports are of specially manufactured primary amino-cellulose paper and are optimized for proper performance in synthesis and bioassay. A large variety of such synthesis membranes are commercially available. Amino-PEGylated membranes are primarily used for the preparation of immobilized peptide arrays resulting in a very stable *C*-terminal attachment of the peptides. The recently described SynthoPlan APEG CE cellulose membrane (AC-S01 grade) is particularly stable against trifluoroacetic acid used in the final deprotection step and prolonged treatment (overnight) improves the peptide quality considerably (Zander 2004).

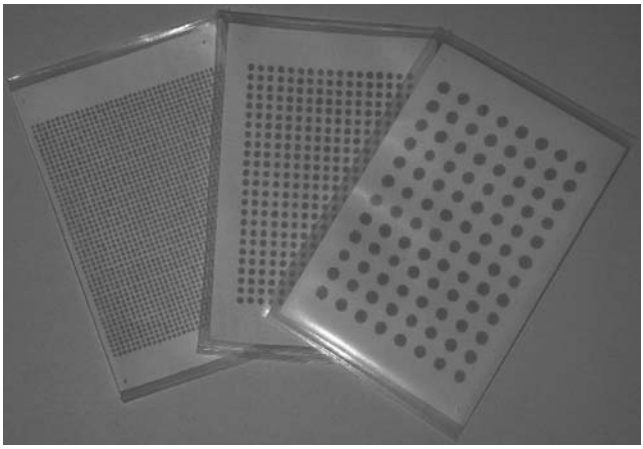


Fig. 35.9 Three array formats fitting onto an AC-S01 membrane of 8 × 12 cm: 8 × 12 spots in the conventional microtiterplate format for manual spotting (spot distance 9 mm; spot volume 0.5 μL); 17 × 25 spots (spot distance 4 mm, volume 0.1 μL); 40 × 50 spots (spot distance 2 mm, volume 30 nL)

On the other side is the SC^2-process that requires an acid soluble membrane: membrane für CelluSpots™ from Intavis Bioanalytical Instruments AG, Cologne, Germany.

High quality arrays of spots providing suitable anchor functions for peptide assembly on cellulose membranes are most easily generated by spot-wise coupling to an evenly aminated membrane of a spacer Fmoc-amino acid such as Fmoc-β-alanine (Fig. 35.8, *cycle 0*). During this derivatization cycle, the array of spot reactors is generated and all residual amino functions between spots are blocked by acetylation (step 2). This array formation process requires very accurate pipetting. During peptide assembly (cycles 1 to n), slightly larger volumes are dispensed and the wet areas then exceed those initially formed, in order to avoid incomplete couplings at the edges.

The flexibility of SPOT-synthesis enables the investigator to easily vary the number of spots, the format of the array and the scale of each peptide synthesized. The arrays on the membrane supports are freely selectable to fit the individual needs of the experiment by variation of paper quality, thickness, specific anchor, loading and spot size (Frank 1992). Figure 35.9 demonstrates some array configurations made on the recommended AC-S01 paper membrane. The standard format used in manual SPOT-synthesis was adapted to the 8 × 12 array of a microtiter plate with 96 spots. However, to fully exploit the scope of the method, use of an automated SPOT-synthesizer such as the AutoSpot or MultiPep robot (from Intavis) is recommended. The AutoSpot instrument can handle up to six standard membrane sheets simultaneously or a whole DINA4 sheet of 210 × 297 mm (Fig. 35.6 *right*). Moreover, automated spotting can be exploited to reduce the size of spots and, thus, increase the number of spots per area considerably. A 384 format (16 × 24) can be generated with, e.g., 0.1 μL spotting volume. A robust standard array format

to fit the 8 × 12 cm size of the membrane comprises 17 rows of 25 spots/row (425 spots). However, up to 2,500 spots can be generated on the same standard membrane by pipetting as little as 30 nL volumes. This instrument only performs the pipetting work; all washing steps are carried out manually. The newer MultiPep instrument can perform fully automated SPOT-synthesis and has two types of membrane platforms, one for two standard sheets and one in the DINA4 format. Alternatively, MultiSynTech GmbH, Witten, Germany, offers an auxiliary tray for fully automated SPOT-synthesis on its Syro robots.

35.3.4 Peptide Assembly

Chemical and technical performance of this type of simultaneous parallel solid phase synthesis allows for the reliable assembly of arrays of peptide sequences up to a length of 20 amino acid residues utilizing conventional mild Fmoc/*t*Bu chemistry (Fields and Noble 1990). This chemistry employs the Fmoc protecting group for the amino function of the amino acid building blocks which is removed by treatment with piperidine base. The side chain functionalities are permanently protected with *tert*-butyl type groups which are removed only at the end of the synthesis by treatment with trifluoroacetic acid. Much longer peptide sequences are reported (Töpert et al. 2001; Gail et al. 2005) but the quality of such peptides strongly depends on the particular sequence and needs to be pre-evaluated by case to case studies.

Free amino functions on the spots can be visualized by staining with bromophenol blue (Krchnák et al. 1988) after *N*-terminal deprotection (Fig. 35.8, *step 3*) and prior to the coupling reaction (step 1). This colour staining allows the visual monitoring of the proper performance of all synthesis steps such as correct dispensing, quantitative coupling and acetylation (capping) and effective removal of piperidine from the Fmoc-deblocking steps. Thus, a standard membrane used for SPOT-synthesis displays an array of light blue spots on a white background (Fig. 35.9). In low density arrays, each spot may be marked by writing a number with a pencil next to it. These numbers refer to the corresponding peptide sequences that are assembled on these spots and are a guide for rapid manual distribution of the solutions of activated amino acid derivatives at each elongation cycle (Fig. 35.6 *left*). For automated pipetting, no pencil marking is necessary as exact positioning of the membranes is assured by the perforation for the holder pins in the robot. The dry membranes are placed in a flat, chemically resistant trough or fixed on the platform of the synthesizer. As soon as the droplets of activated amino acid solutions are added to the spots, coupling proceeds with a conversion of free amino groups to amide bonds. After all amino groups have been consumed, the blue colour of the spots changes to yellow indicating a quantitative reaction. The physicochemical properties of the growing peptide chains are very different, sometimes unfavourable and can slow down or even hinder the quantitative coupling of an amino acid building block. This is an inherent problem of solid phase peptide

synthesis, however, it will be visible on the membrane when some of the spots keep their blue colour and, thus, can be monitored and documented for later interpretation of results. The solvent within the spots slowly evaporates over the reaction time. For AC-S01 membranes, after approximately 15 min a spot is dry and the reaction will stop. However, additional drops may be added onto the same position without enlargening the spots and risking overlap with their neighbours. In this way, difficult coupling reactions can be pushed towards completion by double or triple couplings. Fully automated SPOT synthesis cannot profit from bromophenol blue staining because the instruments have no electronic image monitoring. However, we recommend regularly checking the quality of a synthesis by staining, for example every first cycle of the day. Nevertheless, non reacted termini will be acetylated after each coupling step so that no false sequences will contaminate the product.

The introduction of randomized positions (X) within a peptide sequence assembled on a spot in order to prepare arrays of defined peptide mixtures (or pools) is quite reliably achieved by coupling with equimolar amino acid mixtures and applying these at a sub-molar ratio with respect to available amino functions on the spots (Kramer et al. 1993; Frank 1994). This is to allow all activated derivatives (also the slower coupling ones) to react quantitatively during a first round of spotting. All coupling reactions are then completed by three to four successive repeats of spotting. Using this coupling procedure, any position in a peptide sequence can easily be randomized without special considerations or increase in technical effort. Some current strategies for the delineation of peptide sequences by activity screening of random pools are given in Table 35.1.

35.4 Guidelines for Preparing Peptide Arrays by SPOT Synthesis

The following protocol describes the parallel chemical synthesis of short linear peptides or peptide pools as arrays on modified cellulose membranes. Peptides are synthesized starting from their *C*-termini using Fmoc-amino acid derivatives. After completion of the assembly steps, acid stable membranes are processed to cleave all side-chain protecting groups after which the peptide array is ready to be probed with the potential interaction partners. Alternatively, peptides synthesized on acid soluble membranes are separated after the assembly steps with the help of a puncher and then treated according to the SC^2-process described in (Chap. 35, see this volume).

35.4.1 Materials

– SPOT-membranes

  Acid-stable AC-S01 type amino-PEGylated membranes (manufactured by AIMS-Scientific-Products GmbH, Braunschweig, Germany) are recommended and available from AIMS itself or from Intavis AG, Cologne,

Germany. Note: The AutoSpot instrument requires a special format of the membranes with special perforation for the holder pins on the robot!

Acid-soluble membranes for CelluSpots™ are available from Intavis AG (order no. 32.105).

- Chromatography paper type 3 MM (Whatman, Maidstone, UK).
- Bromophenol blue indicator

  Prepare a stock solution of 10 mg per mL in DMF and keep at RT. This BPB stock should have an intense orange colour and should be discarded when the colour has turned to green.

- N,N-dimethylformamide (DMF)

  should be free of contaminating amines and thus of the highest affordable purity, such as the peptide synthesis grade DMF of Biosolve BV, Valkenswaard, Netherlands. Amine contamination is checked by the addition of 10 μL of BPB stock to 1 mL of DMF. If the resulting colour is yellow, then this batch can be used without further purification. Check each new batch!

- 1-Methyl-2-pyrrolidinone (NMP)

  should be of highest purity available. Amine contamination is checked as above for DMF. If the resulting colour is yellow, then the NMP can be used without further purification. Most commercial products, however, are not acceptable. To prepare a suitable quality, treat 1 L of NMP with 100 g of acidic aluminum oxide under constant vigorous shaking at RT overnight. Then, a 1 mL aliquot should give a yellow BPB test. Filter the slurry through a bed of dry silica gel (for flush chromatography, Mallinckrodt Baker BV, Deventer, Netherlands) in a closed glass filter funnel (slight nitrogen pressure can speed up the process, but is not necessary). Divide the clear liquid into 100 mL portions and store tightly closed at −20°C.

- N-Hydroxybenzotriazole (HOBt)

  Anhydrous, ISOCHEM, Vert-Le-Petit, France. Store tightly closed at room temperature in a dry place.

- N,N′-Diisopropylcarbodiimide (DIC), ≥98%
- D(+)-Biotin, 99%, from Carl ROTH, Karlsruhe, Germany (order-no: 3822.1)
- Fmoc-AA stock solutions: Fmoc-amino acid derivatives of all 20 L-amino acids as well as β-alanine and other special amino acid derivatives are available from several suppliers in sufficient quality (Novabiochem/Merck Biosciences, Schwalbach, Germany, or Bachem, Bubendorf, Switzerland). Side chain protecting groups should be Cys(Acm) or Cys(Trt), Asp(OtBu), Glu(OtBu), His (Trt), Lys(Boc), Asn(Trt), Gln(Trt), Arg(Pmc), Ser(tBu), Thr(tBu), Trp(Boc), and Tyr(tBu). It is necessary to prepare HOBt-esters of these amino acid derivatives in NMP for use throughout in spotting reactions. Dissolve 1 mMol of each Fmoc-AA in 5 mL NMP containing 0.25 M HOBt to give 0.2 M Fmoc-AA stock solutions. These stocks are kept in 10 mL plastic tubes that are closed

tightly, flush frozen in liquid nitrogen, and stored at −70°C. For use in coupling reactions with amino acid mixtures at randomized positions (X) in the peptide sequences, combine equal aliquots of Fmoc-AA stock solutions for the respective amino acids to be incorporated, dilute with three fold volume of NMP to give 50 mM solutions and store as described above.

- Special chemical derivatives:

  Free thiol functions of cysteine may be problematic because of post-synthetic uncontrolled oxidation. To avoid this, you may replace Cys by serine (Ser), alanine (Ala) or alpha-aminobutyric acid (Abu). Alternatively, choose the hydrophilic Cys(Acm) and leave protected. – For the simultaneous preparation of peptides of different size with free amino terminus, couple their terminal amino acid residues as αN-Boc derivatives so that they will not become acetylated during the normal elongation cycle. Boc is then removed during the final side chain deprotection procedure. – Negative and positive control spots for the arrays on glass slides are very helpful. We have good experiences with β-alanine (see above) as negative and biotin as positive controls. Thus, always include some of these spots in your array. Biotin at 0.2 M is rather insoluble in DMF, but dissolves upon activation with DIC, just give more time for activation.

- Acetylation-Mix

  a 2% solution of acetic anhydride (≥99.5%) in DMF.

- Piperidine-Mix

  a 20% solution of piperidine (≥99%) in DMF. Note: piperidine is toxic and should be handled only with gloves under a hood!

- Alcohol (methanol or ethanol) of a technical grade (95%).
- Deprotection-Mix

  Trifluoroacetic acid (TFA, synthesis grade), dichloromethane (DCM), triisobutylsilane (TIBS) and water in a ratio of 12% DCM, 3% TIBS, 80% TFA and 5% water (mix in this order!). Note: Trifluoroacetic acid is very harmful and volatile, and should be handled with gloves under a hood!

35.4.2 Special Equipment

All equipment used for membrane synthesis should be resistant to organic solvents. Glassware or polypropyleneware should be exclusively used in all steps involving organic solvents. Standard micropipetting tips (Gilson, Eppendorf) can be employed.

SPOT synthesis: Software for the generation of peptide lists and pipetting protocols are included in the operation software of the spotting robot. A freeware package is also available from the authors.
Flat reaction/washing troughs with a tightly closing lid made of chemically inert material (glass, teflon, polypropylene) with dimensions slightly larger than the membranes used.
A spotting robot, model AutoSpot or MultiPep peptide synthesizer with a spotting tray (Intavis AG).
1,5 mL plastic tubes (e.g., Eppendorf, safe twist) and appropriate racks as reservoirs for amino acid solutions.
A rocker table.
Two dispensers for DMF and alcohol adjustable from 5 to 50 mL. Hand-held hair dryer with non-heating option.
Appropriate bench space in a hood.
−70°C freezer.

35.4.3 Methods

All volumes given below are for one standard AC-S01/CelluSpots™ membrane paper sheet of 8 × 12 cm and have to be adjusted for more sheets, or other paper qualities and sizes. Solvents or solutions used in washing and incubation steps are gently agitated on a rocker table at room temperature if not otherwise stated and are decanted after the time indicated. During incubations and washings the troughs are closed with a lid.

35.4.3.1 Preparative Work

1. Generate a list of peptides to be prepared. You may combine more than one list. Add them one after the other to fill up a complete array. The peptides can be separated after synthesis by simply cutting the membrane into corresponding sections.
2. Select the array(s) required for the particular experiment according to number, spot size and scale. For manual spotting you should adhere to an 8 × 12 format (spot distance 9 mm; spot volume 0.5 μL for array generation in cycle 0, 0.7 μL for elongation cycles). An array of 17 rows with 25 spots each (spot distance 4 mm, volume 0.1 μL during array generation and 0.2 μL for elongation cycles) is recommended for the AutoSpot.
3. Calculate the volumes of Fmoc-AA amino acid solutions required for each derivative and cycle; consider that a triple coupling procedure may be necessary and that each vial should contain a minimum of 50 μL. For example, in your list

of peptides, alanine is required for 26 peptides at cycle 1 and you will use a 17 × 25 array. Then for A1 you will need 26 × 0.2 × 3 = 15.6 µL of Fmoc-Ala stock solution and you will take 50 µL for this vial. The available SPOT software can do this calculation for you.

4. Label a set of 1.5 mL plastic tubes with derivative and cycle code (e.g., A1) and distribute the Fmoc-amino acid stock solutions according to the calculated volumes required. Flush freeze in liquid nitrogen and store at −70°C.

35.4.3.2 Generation of the SPOT Reactor Array

1. Mark the spot positions on the membranes with pencil dots for manual synthesis and place in the reaction trough. Alternatively, fix membranes on the platform of the SPOT-robot for automated synthesis.
2. Take a 100 µL aliquot of the Fmoc-βAla stock from the freezer and bring to RT. Add 1 µL BPB stock. Add 4 µL DIC, mix, leave for 30 min and then spot aliquots (0.5 µL for 8 × 12 array or 0.1 µL for 17 × 25 array) of this solution to all positions according to the array configuration you have chosen. Let react for 60 min (cover the membranes on the spotter with glass plates). Note: For peptides longer than 20mers it is recommended to reduce the loading of the spots by applying a mixture of the Fmoc-βAla stock and a N-acetyl-alanine stock (1:9). This will avoid molecular crowding of the larger peptide mass. You may also incorporate here a cleavable linker compound instead of β-alanine in order to cleave the peptides from the spots after assembly for solution phase assays; the safety-catch Frank-linker is recommended which yields peptides in physiological buffer solutions (Hoffmann and Frank 1994, Homepage of IRIS Biotech).
3. Wash each membrane with 20 mL acetylation mix for 30 sec, once again for 2 min and finally leave overnight in acetylation mix.
4. Wash each membrane with 20 mL DMF (three times for 10 min).
5. Incubate for 5 min with 20 mL piperidine mix.
6. Wash each membrane with 20 mL DMF (three times for 10 min).
7. Incubate each membrane with 20 mL of 1% BPB stock in DMF. Exchange the solution if traces of remaining piperidine turn the DMF solution into a dark blue solution. Spots should be stained only light blue!
8. Wash each membrane with 20 ml alcohol (three times for 10 min).
9. Dry with cold air from hair dryer between a folder of 3MM paper and store sealed in a plastic bag at −20°C.

35.4.3.3 Assembly of the Peptides

1. Take the membranes from the previous step. Number the blue spot positions on the membranes with a pencil (H grade) for manual synthesis according to your peptide lists and place in separate reaction troughs. Alternatively, fix the non-numbered membranes correctly on the platform of the synthesizer. Number the

membranes with a pencil and keep this arrangement through the whole synthesis. Note: You may now mark the cutting lines using a pencil for post-synthesis segmentation of the membrane into project specific sections. If bound protein will be eluted individually from single spot positions after having probed the spot membrane with a protein solution (Valle et al. 1999; Billich et al. 2002) you should also mark the spots on those membranes used in automated synthesis. Pencil marking is quite stable during the synthesis procedure.

2. Take the set of Fmoc-amino acid stock aliquots for cycle 1 from the freezer, bring to RT and activate by addition of DIC (4 μL per 100 μL vial; ca. 0.25 M). Leave for 30 min. Then pipette aliquots of these solutions manually onto the appropriate spots on the membrane. Alternatively, place the vials with the activated Fmoc-AA solutions into the corresponding location in the rack of the spotting robot and start cycle 1. Leave for at least 15 min. Repeat the spotting twice and then let react for 2 h (cover the membranes on the spotter with glass plates). If some spots stay dark blue, you may add additional aliquots. If most spots are yellow to green, then continue. Note: Add only 1 μL DIC to 100 μL Fmoc-AA mixture stock and repeat spotting four times for the efficient introduction of randomized X positions in the peptide sequences.
3. Wash each membrane with 20 mL acetylation mix for 30 sec and once again for 2 min. Then incubate a third time for about 10 min until all remaining blue colour has disappeared.
4. Wash each membrane with 20 mL DMF (three times for 10 min).
5. Add 20 mL piperidine mix and incubate for 5 min.
6. Wash each membrane with 20 mL DMF (three times for 10 min).
7. Incubate with 20 mL of 1% BPB stock in DMF. Exchange the solution if traces of remaining piperidine turn the DMF solution into a dark blue solution. Spots should be stained only light blue! Due to the charge specific staining, BPB does not only bind to *N*-terminal amino-groups. The side chains and protecting groups of other amino acids can strongly influence the staining intensity. The visible colour of the peptides depends on the overall charge and therefore depends on the individual amino acid sequence.
8. Wash each membrane with 20 mL alcohol (three times for 10 min).
9. Dry with cold air from a hair dryer in between a folder of 3MM.
10. Start at step 2 for the next elongation cycle.

35.4.3.4 Terminal Acetylation

Synthetic peptides mimicking fragments of a longer continuous protein chain should be *N*-terminally acetylated to avoid an artificial charged terminus. Note: Alternatively, special detection labels can be attached to the *N*-termini of peptides by spotting respective derivatives. This is useful, for example, when peptides are applied as protease substrates and the enzyme activity followed through the change of the label upon cleavage of the peptide. We have successfully added biotin via its in situ formed HOBt-ester (normal activation procedure) or fluoresceine via its isothiocyanate (FITC; 0.2 M) dissolved in DMF.

Continue after the final amino acid elongation cycle from the protocol above.

1. Incubate each membrane with 20 mL acetylation mix for at least 30 min until all remaining blue colour has disappeared.
2. Wash each membrane with 20 mL DMF (three times for 10 min).
3. Wash each membrane with 20 mL alcohol (three times for 10 min).
4. Dry with cold air from a hair dryer in between a folder of 3MM.

35.4.3.5 Side Chain Deprotection of Membrane-Bound Peptide Arrays

After the peptide assembly is complete, it is necessary to remove all side chain protecting groups from the peptides. This must be performed under a hood as trifluoroacetic acid is very harmful! Note: This protocol is only applicable to acid-stable cellulose membranes such as AC-S01.

1. Prepare 40 mL of deprotection mix.
2. Place the dried membrane in the reaction trough, add deprotection mix, close the trough very tightly and agitate overnight. Note: This harsh treatment is required for complete cleavage of protecting groups (Kramer et al. 1999; Harlow and Lane 1988). Cellulose membranes less resistant than AC-S01 will not survive!
3. Wash each membrane for 5 min with 20 mL DCM (four times).
4. Wash each membrane for 5 min with 20 mL DMF (three times).
5. Wash each membrane for 5 min with 20 mL alcohol (three times).
6. Wash each membrane for 5 min with 20 mL 1 M acetic acid in water (three times). Note: This is for removal of the Boc group from tryptophane.
7. Wash each membrane with 20 mL alcohol (three times for 5 min).

The membrane sheets may now be dried with cold air and stored at −20°C or further processed as described in the next section.

35.5 Manufacturing Peptide Arrays on Glass Slides by the SC^2-Process

Compared to the synthesis of conventional peptide arrays on SPOT membranes described above (Chap. 35.4, see this volume), there are a few but important changes. Use an acid soluble cellulose membrane (e.g., CelluSpots^{TM} membrane from Intavis) for synthesizing the peptide array (see 35.4.1). The array dimensions should be 16 × 24 (384 spots at a distance of exactly 4.5 mm) to fit with the commercially available punching device (Fig. 35.7, *left*). After generation of the SPOT array with Fmoc-βAla and staining with BPB (35.4.3.2), mark on every corner of the array at least one peptide spot position with a pencil by surrounding it. These marks are used later for adjusting the membrane in the punching device. Follow the instructions described under 35.4.3.3 and 35.4.3.4 to assemble the peptides.

The dry membrane is then inserted in the punching device by placing it between two metal plates forming a plate–membrane–plate sandwich. Each metal plate has precisely drilled 3 mm holes mirroring the identical 384 spot grid of the cellulose membrane. One of four 8 × 12 tube racks is placed underneath the plates-membrane-sandwich and in this manner each cellulose–compound disk can be punched into its corresponding tube. Note: We recommend using bar-coded Matrix-Tubes instead of deepwell-microtiter plates which do seal better and can be handled also individually. You must adhere to a standard regime of correlating the four 96 daughter(′) racks to the parent 384 array; we recommend the z-pattern, that is A1 gets A′1, A2 gets A″1, B1 gets A‴1 and B2 gets A⁗1.

Then, a strong acidic solution is added to each tube to both cleave the side chain protecting groups from the peptides and simultaneously dissolve the cellulose matrix to form a homogenous solution. This acid treatment first swells the cellulose disks to double their previous thickness and usually after 0.1–24 h all cellulose-compound-disks have disintegrated into a fine-particulate suspension. Within 2–48 h most of the cellulose-compound-disks should be completely dissolved. The solved cellulose-peptide conjugates are precipitated with ether, washed with ether and finally dissolved in DMSO to give the stock solutions. These stock solutions are stored at −70°C, tightly closed to avoid trapping of water. Note: DMSO is quite hygroscopic and can take up more that 50% water from the air.

Up to 800 spots can be printed at a distance of 1 mm onto a standard microscope glass slide (2.5 × 7.5 cm) by transferring about 10 nL from respective dilutions of the stocks in DMSO using pipetting robots equipped with liquid displacement syringes and a good enough x/y-precision (e.g., Slide Spotting Robot from Intavis). Alternatively, microarray instrumentation may be exploited. The use of piezo-dispensers is not recommended as the nozzles clog too easily; this can make life hard also with the split-pin instruments. Ring-and-pin instruments (e.g. the GMS 417 Arrayer) are rather robust and reliable; we currently use this type of instrument with 500 µm solid pins to generate reliably miniarrays with a satisfying spot morphology (Fig. 35.10) or with 150 µm solid pins to generate microarrays (spot distance ~350 nm; up to 9,000 spots per microscope slide).

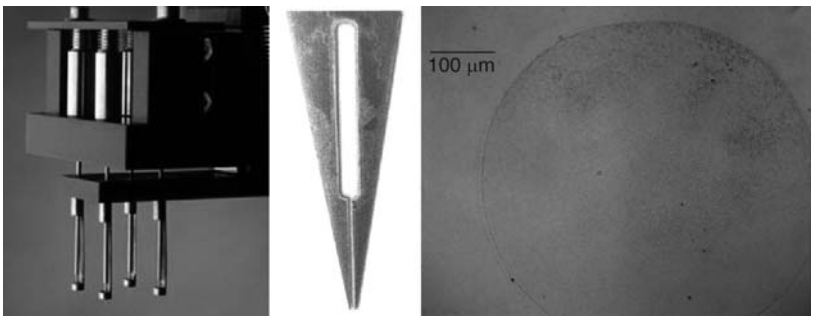


Fig. 35.10 *Left*: ring-and-pin printing head of the GMS 417 Arrayer. *Middle*: Tip of a split pin. *Right*: Magnified image of a cellulose-peptide conjugate minispot on a glass slide

The standard carrier for microarrays is the microscope glass slide. Cellulose-peptide conjugates generated by the SC^2-process readily adsorb to glass without the need of chemical fixing. However, the slides need to be very clean and of suitable homogenous hydrophilicity. Special products for the SC^2-arrays are commercially available. In this respect, it is also of interest that we successfully printed and assayed SC^2-spotting solutions on many other types of surfaces including plastic sheets made of PVC, PP and other polymers. This could be exploited to adopt SC^2-arrays to numerous alternative customized formats other than the conventional microscope slides. SC^2-arrays on special plastic coated slides are available as CelluSpots™ from Intavis AG; for home-made slides of this type contact the company.

35.5.1 Materials

- Acid soluble cellulose membrane for CelluSpots™ from Intavis Bioanalytical Instruments AG, Cologne, Germany (order no. 32.105).
- SynthoSlides for SC^2 from AIMS Scientific Products GmbH Braunschweig, Germany (order no. AG07-07/10) or coated slides for CelluSpots™ from Intavis (order no. 54.112)
- *tert*-Butyl methylether (MTBE), extra pure.
- Dimethyl sulfoxide (DMSO), 99.5% for synthesis.
- Dissolving solution

  Trifluoroacetic acid (TFA, synthesis grade), triisobutylsilane (TIBS), water and trifluoromethanesulfonic acid (TFMSA, 98%) in a ratio of 88.5% TFA, 2.5% TIBS, 5% water and 4% TFMSA. Note: Trifluoroacetic acid is very harmful and volatile, and should be handled with gloves under a hood!

35.5.2 Special Equipment

A 384-hole punching device (Intavis).

- Matrix-Tubes:

  1.4 mL 2D Barcoded TrakMates Tubes (order no. 3711) are from Matrix Technologies (now Thermo Scientific Matrix), New Hampshire, USA.

- CapMats (order no. 4431) from Matrix, not separable.
- Sepra Seal CapMats (order no. 4463) for sealing the Matrix-tubes individually.
- MixMate: benchtop mixing device from Eppendorf, Hamburg, Germany (order no. 5353 000.014).
- Temperature controlled ultrasonication bath such as the Sonorex Super 10P from Bandelin, Berlin, Germany.
- Suitable pipetting device for ether handling during the wash procedures:

Serial Mate from Matrix Technologies, New Hampshire, USA, specially equipped with an exhaust device for pipetting ether.

– Suitable pipetting device for printing the miniarrays:

Slide Spotting Robot from Intavis equipped with a 500 μL syringe and a teflon coated needle for distributing solutions in nL ranges (order no. 54.000) or GMS 417 Arrayer from Affymetrix (Santa Clara, CA, USA) equipped with 500 μm pins.

Microtiter plates made from polypropylene to fit into the sample holder of the printing device.

For slide storage, a microscope slide holder box (order no. K540.1) from Carl ROTH, Karlsruhe, Germany.

35.5.3 Methods

35.5.3.1 Preparation of Stock Solutions in DMSO of Cellulose Bound Peptides

1. Prepare 120 mL dissolving solution.
2. Pipette 300 μL of the dissolving solution in every tube containing a cellulose-compound-disk.
3. Seal tube-racks with a non separable capmat.
4. Shake/vortex racks for 1 h.
5. Sonicate racks for 1 h.
6. Repeat shaking/vortexing and sonication until all the cellulose is dissolved.
7. Add 500 μL MTBE to each tube to precipitate the cellulose-compounds. The overall volume is now 800 μL per tube. Shake/vortex the rack for 5 min at 2,000 rpm.
8. Place the tube racks for 15 min in a fridge at −20°C; a white precipitate should be visible in the tubes.
9. Centrifuge the racks in a cooled centrifuge (4°C) at 3,000 rpm for 10 min.
10. Remove the supernatant from each tube, preferably by a pipetting device. Note: Leave ca. 25 μL of the supernatant in order not to perturb the precipitated pellet.
11. Wash the remaining pellets three times with each 500 μL MTBE by repeating steps 7–10.
12. Cover the racks containing the opened tubes with a double sheet of 3 MM paper. Remove very carefully remains of ether by tilting the racks aside so that liquid can slowly drip out onto the 3 MM paper. Do not disturb the pellets!
13. Leave the tubes open to air for 1 h (max!). Note: the cellulose pellet must not totally dry out!
14. Dispense 500 μL DMSO into each tube.
15. Seal tube-racks with Sepra Seal capmats (separable).

16. Dissolve the cellulose-compound pellets by a combination of shaking/vortexing and sonication at 40°C until a clear, colourless solution for each cellulose-compound conjugate is obtained.
17. Flush freeze the stock solutions with liquid nitrogen and store at −20°C until use.

35.5.3.2 Printing Miniarrays on Microscope Glass Slides

1. Prepare 1:10 dilutions with DMSO from the cellulose-peptide stock solutions in a polypropylene microtiter plate. Arrange peptides to fit to the layout of the final array and the pipetting scheme used by the printing device. Include the positive and negative controls! Note: Do not leave the DMSO solutions standing unsealed for a period of time, longer than needed for dilution or printing, as the DMSO will trap considerable amounts of water from the air.
2. Print/spot the diluted cellulose-peptide solutions onto glass slides corresponding to the manual from the manufacturer of the printing device. The recommended type of slides enables to place spots at a distance of 1 mm by transferring about 10 nL solution. Note: When using coated slides (e.g., from Intavis) it could be necessary to spot up to 40 nL per spot to get a satisfying spot morphology. The spot distance should than be set to 1.2 mm.
3. Leave the slides sitting in the printing device until the DMSO is evaporated (approximately 60 min).
4. Place the slides in a microscope slide holder box and put the box with an open cover for 30 min in an oven at 60°C.
5. Remove the object holder box from the oven, let it cool down, close the top and store the printed slides at −4°C. Note: These slides should be usable for at least 12 months.

35.6 Guidelines for Probing the Peptide Arrays

35.6.1 General Considerations

The membrane segments from (Chap. 35.4, see this volume) or the printed slides from (Chap. 35.5, see this volume) are now ready to be probed with solutions of the protein acceptor such as an antibody or fragment thereof, antiserum, body fluid, cell extract, recombinant protein, etc.

Very much influenced by the protein sample of interest and the chosen detection method, different blocking conditions need to be explored for an optimal signal-to-noise ratio. The following blocking solutions of increasing "stringency" may be tested. (1) 3% BSA in PBS, (2) 2% (w/v) skim milk powder in TBS, (3) 2% (w/v) skim milk powder, 0.2% (v/v) Tween 20 in TBS, (4) MBS, (5) MBS with 50% (v/v) horse serum. In our hands, blocking solution (3) works best for most membrane array applications, while (1) is recommended for fluorescence detection on glass slides.

Detection of antibody/protein molecules bound to the peptide spots can be achieved in a variety of ways. Besides the standard antibody-based immunoblotting (Harlow and Lane 1988), many other labelling techniques, e.g. with radioisotopes or fluorescent dyes are also fully compatible. Biotinylation of the probe protein followed by detection with a streptavidin-AP conjugate is also a convenient option. If an antibody is the interaction partner, secondary antibodies or labelled protein A or G are available for detection. Always pre-check incubations using only the detection agents alone! Especially when enzyme labelled animal sera are used, unspecific binding or cross reaction to antibody or enzyme may give rise to false positive reactions. It is further possible that specific antibodies to a certain antigen are present in the secondary antisera, for example, when proteins are analyzed which originate from *E. coli* or other organisms naturally in contact with the donor animal. Generally, avoid detection procedures of several steps in order to lose sensitivity by washing off the protein binders. Antibodies bind bivalently and, thus, show high avidity and low off-rates (this, however, can be very unfavourable with low affinity monovalently binding proteins).

The choice of a detection system should ensure that peptide spots will not become chemically or otherwise irreversibly modified, because peptide arrays on cellulose membranes can be reused many times (more than 20 times) when treated properly. Therefore, alkaline phosphatase is recommended over horse radish peroxidase as enzyme label because the latter requires addition of hydrogen peroxide which also oxidizes the peptides; NBT-based colour development is not removable from the membrane! Therefore MTT is recommended. A comprehensive collection of relevant publications (Frank and Schneider-Mergener 2002) lists many useful detection procedures.

In case of low-affinity interactions of the antibody or protein with the peptides or in cases of significant perturbation by unspecific background from, e.g. the detection antibody, it is recommended to perform the detection of the antibodies/proteins bound to the spots after electro-transfer to a secondary (mostly nitrocellulose) membrane. This also allows application of a wider range of other detection procedures (Rüdiger et al. 2001).

Signal patterns obtained from peptide arrays on spots can be documented and quantitatively evaluated utilizing modern image analysis systems as used with other 2D analysis media such as electrophoresis gels, blotting membranes and microarrays.

Fluorescence-labelled detection reagents enable to discriminate in one experiment controls and several target proteins by applying a mixture of target-specific labelled reagents with different distinguishable fluorophores. Fluorescence detection, however, can be obscured on membranes by quenching or background fluorescence, but is very effective with arrays on glass microscope slides.

35.6.2 Materials

– Tris-buffered saline (TBS)

  8.0 g NaCl, 0.2 g KCl and 6.1 g Tris-base in 1 L water; adjust pH to 7.0 with HCl; autoclave and store at 4°C.

- T-TBS: TBS buffer plus 0.05% Tween20.
- Phosphate-buffered saline (PBS)

  8.0 g NaCl, 0.2 g KCl, 1.43 g $Na_2HPO_4 \times 2H_2O$ and 0.2 g KH_2PO_4 in 1 L water; adjust pH to 7.0 with HCl; autoclave and store at 4°C.

- Citrate buffered saline (CBS)

  8.0 g NaCl, 0.2 g KCl, and 10.51 g citric acid ($\times 1H_2O$) in 1 L water; adjust pH to 7.0 with NaOH; autoclave and store at 4°C.

- Membrane Blocking Solution (MBS)

  mix 20 mL Casein Based Blocking Buffer Concentrate (No. B6429; Sigma-Genosys Inc.), 80 mL T-TBS (pH 8.0!) and 5 g sucrose; the resulting pH will be 7.6; store at 4°C.

- Horse serum (Gibco-Invitrogen, Carlsbad, USA).
- Alkaline phosphatase (AP) conjugated detection antibodies.
- Alkaline phosphatase (AP) conjugated streptavidin.
- Colour Developing Solution (CDS)

  Dissolve 50 mg 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in 1 mL of 70% DMF in water; store at −20°C. Dissolve 60 mg 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) in 1 mL DMF; store at −20°C. Prepare CDS always fresh: To 10 mL CBS add 50 µL 1 M magnesium chloride, 40 µL BCIP and 60 µL MTT. Note: Never use NBT instead of MTT, the developed colour cannot be removed from the membrane anymore!

- Immun-Star™ Chemiluminescent Kit (No. 170-5018, Bio-Rad Laboratories, Hercules CA, USA).
- Horse radish peroxidase (HRP) conjugated detection antibodies.
- ECL™ western blotting detection reagents (Amersham Biosciences UK Ltd, Buckinghamshire, UK).
- Protan® Nitrocellulose Transfer Membrane (Schleicher and Schuell, Germany).
- Transfer buffer for western blotting

  25 mM Tris-HCl (pH 7.6), 192 mM glycine, 20% methanol, 0.03% sodium dodecyl sulfate (SDS).

- Stripping mix A (SM-A)

  8 M urea, 1% SDS in PBS; store at room temperature; add 0.5% 2-mercaptoethanol prior to use and adjust pH to 7.0 with acetic acid.

- Stripping mix B (SM-B)

  10% acetic acid, 50% ethanol and 40% water; store at room temperature.

- Fluorescence-labelled Streptavidin: Streptavidin Alexa Flour 647 conjugate from Molecular Probes (1.0 mg per mL) Note: the fluorescence label of the streptavidin should be detectable at another wavelength than the labels used for the secondary antibodies.

- Horse radish peroxidase (HRP) conjugated streptavidin for chemiluminescence detection.
- Fluorescence labelled detection antibodies (for label see above).
- BSA-blocking solution for slides (Albumine, bovine serum, Fraction V, approximately 99%; available from Sigma-Aldrich, St. Louis, MO, USA; order no. A3059): 3% BSA in PBS-Buffer, pH 7.4.

35.6.3 Special Equipment

- Polystyrene cell culture plates (12 × 12 cm) with covers (Greiner Bio-One, Frickenhausen, Germany; order no. 688102).
- Flat glass tray to hold at least one membrane.
- A sonication bath with temperature control.
- A digital recording device

  (scanner or CCD camera) for documentation of signal patterns on membranes plus analysis software for quantification of signals. In case of chemiluminescence detection, autoradiography (X-ray) films can be used.

- Plastic bags and sealing device.
- A blotting apparatus (Biometra® - Fast-Blot, Göttingen, Germany, B337593) or others.
- Thin transparent plastic wrap (Saran Wrap).
- Slide handling equipment (e.g. from Carl ROTH, Karlsruhe, Germany:
- Incubation or staining chamber (order no. HL98.1), staining box (order no. HA44.1), microscope slide holder (order no. HA49.1), cover slips (order no. H878.1), microscope slide holder box (order no.K540.1)).

35.6.4 Methods

35.6.4.1 Probing Peptide Arrays on SPOT Membranes

This basic procedure is worked out for use with AP-conjugated detection antibody and a colour signal development. HRP-labelled detection agents require hydrogen peroxide and gradually destroy the peptides! More sensitive detection can be achieved with a chemiluminescent substrate of AP (e.g. Immun-Star™). In this case follow the instructions of the supplier for steps 9–12. If radioactive labelled reagents are used adopt steps 5–12 accordingly. Prior to probing your sample with the peptide spots on the membrane, always apply this protocol first by omitting steps 5 and 6. This is necessary to assess unspecific signals from components of the detection process or remaining antibody/protein from a previous experiment on the same membrane. If the membrane itself shows background, a better blocking reagent must be chosen. In case the proteins are electro-transferred and detected on a secondary nitrocellulose membrane (method B, below), this precaution does not apply.

Protein-Binding Assay Protocol

Method A

1. Place the membrane in a polystyrene plate and wet with a few drops of alcohol. Note: This is to enhance rehydration of some hydrophobic peptide spots. The peptide locations should not be visible as white spots! If this happens, treat with alcohol in a sonication bath at room temperature until spots have disappeared.
2. Wash membrane for 10 min with 10 mL TBS (three times).
3. Incubate overnight with 10 mL MBS.
4. Wash membrane once for 10 min with 10 mL T-TBS.
5. Incubate for 2–4 h with probe antibody (or protein) diluted in 8–10 mL MBS. For monoclonal antibodies or pure proteins use approximately 4–5 μg of purified antibody per milliliter incubation volume. When using a polyclonal serum, we recommend a dilution of 1:100. Note: It is not necessary to use a large volume of protein solution for the incubation. However, make sure that the membrane is completely covered and prevent drying out by using a lid or seal in a plastic bag.
6. Wash membrane for 10 min with 10 mL T-TBS (three times).
7. Incubate for 1–2 h with AP-conjugated secondary antibody diluted in 10 mL MBS.
8. Wash membrane for 10 min with 10 mL T-TBS (twice).
9. Wash membrane for 10 min with 10 mL CBS (twice).
10. Transfer the membrane to a flat glass tray and add 10 mL of CDS. Incubate without agitation until good signals are obtained. For individual peptides on spots this usually takes 10–30 min; peptide pools may require longer incubations (2 h to overnight). Stop the reaction by washing twice with PBS (1×30 sec, 1×3 min). Keep membrane wet. For storage, leave at 4°C in a container with PBS or cover with plastic wrap. (Note: If the membrane dries out, proteins may denature and become difficult to remove). After successful documentation of signals by photography or electronic scanning, continue with membrane stripping.

Method B

. If signal to noise in Method A yields no reliable results, the electro-transfer of bound test protein onto a secondary nitrocellulose membrane may help. Here, any appropriate detection system on the nitrocellulose can be used (e.g. HRP-conjugates), as the peptides will not be affected. Proceed first as above steps 1–6.

7a. Briefly equilibrate both the peptide-membrane and a sheet of nitrocellulose, trimmed to fit the peptide membrane, in transfer buffer.

8a. Electro-transfer the proteins bound to the peptide spot membranes onto nitrocellulose for 1 h using 0.85 mA $\times$ cm^{-2}. Due to the denaturation by SDS, all proteins should have acquired a negative charge. Therefore, the nitrocellulose should be placed towards the positive electrode. Note: Depending on the

chemical properties of the protein ligands, the time required for the transfer might differ and, therefore, has to be determined empirically.

9a. Block the nitrocellulose membrane with MBS for 2 h at room temperature.

10a. Incubate the nitrocellulose membrane for 75 min with an AP- or HRP-conjugated detection antibody or AP-/HRP-streptavidin for biotinylated proteins diluted in MBS. Use dilutions comparable to those employed in immunoblots after SDS-PAGE.

11a. Wash the nitrocellulose membranes three times for 5–10 min with T-TBS, subsequently followed by washing three times for 5–10 min with TBS.

12a. Remove excess buffer from the nitrocellulose membrane by gently placing the membrane on a sheet of 3MM paper. Note: To avoid damage to the adsorbed protein do not wipe or press tissue onto the membrane.

13a. Detect the spots by using a chemiluminescence detection kit according to the instructions of the suppliers. Note: (1) If no signal can be detected after 30 min of exposure, check the detection system with a positive control from the kit. If detection reagents are functional, use less stringent blocking. If no binding occurs, this may indicate a discontinuous binding site or very low affinity binding. (2) In case of unspecific signals and a high background, increase the stringency of the blocking conditions and make sure that your primary binding partner and detection reagent (e.g. antibody) are of high purity and are used in the highest possible dilution.

Membrane Regeneration (Stripping)

A peptide spot membrane that has been processed through a protein binding assay can be used again for probing another sample if all remains from the assay can be removed completely (stripping). Principally, membranes can be regenerated up to 50 times without loss of signal intensity, because the peptides are very stably immobilized. But in some cases denatured proteins resist elution from the spots, and the membranes can only be used once or very few times for Method A (on-spot-membrane detection). This has to be checked by probing a regenerated spot membrane first with the detection system (see "protein binding assay" protocol).

1. Wash the spot membrane for 10 min with 20 mL of water (twice).
2. Incubate with 20 mL DMF until the blue colour of the spot signals has dissolved (usually about 10 min; incubate in a sonication bath at 40°C if necessary). Remove the solution and wash once again for 10 min with 20 mL DMF. Note: This step can be omitted if other than a dye precipitation detection was used.
3. Wash the spot membrane for 10 min with 20 mL of water (three times).
4. Wash the spot membrane for 10 min with 20 mL SM-A in a sonication bath at 40°C (three times).
5. Wash the spot membrane for 10 min with 20 mL SM-B (three times).

6. Wash the spot membrane for 10 min with 20 mL alcohol (three times).
7. Go to step 2 of the "protein binding assay" protocol for the next binding assay or dry the membrane with cold air from a hairdryer in between a folder of 3MM and store at −20°C sealed in a plastic bag.

35.6.4.2 Probing Peptide Arrays on Glass Slides

All washing steps are reduced to 3 min and carried out under gentle agitation on a rocker table.

1. Place the slides in the microscope slide holder of a staining box and put the holder in the staining box. Add approximately 100 mL ethanol into the box until the slide working areas are covered completely with alcohol. Wash for 3 min. Note: This is to enhance rehydration of some very hydrophobic peptide spots.
2. Wash the slides with TBS (three times).
3. Incubate overnight with BSA-blocking solution. Note: depending on the protein of interest and the detection method, different blocking conditions can be compared to obtain an optimal signal-to-noise ratio. Avoid impure, fluorescent reagents when using fluorescence detection.
4. Wash once with T-TBS.
5. Place the slides in an incubation chamber.
6. Prepare incubation solutions: per slide dilute 1 μL of the sample to be probed in 100 μL BSA-blocking solution. Pipette immediately 60 μL incubation solution on the respective slide. Prevent drying out of the slide surface. Place a glass cover slip slowly onto the surface of the droplet to spread the antibody solution. Locking of air bubbles under the cover slip must be avoided. Incubate for 2–4 h. Note: For monoclonal antibodies or purified proteins use approximately 4–5 μg per mL incubation volume. When using a polyclonal serum or cell extract, we recommend starting with a dilution of 1:100. It is not necessary to use a larger volume of protein solution for the incubation. Depending on the viscosity of the incubation solution, volumes between 60 μl and 100 μl per slide are required.
7. Remove the cover slips carefully from the slide surfaces by washing off with T-TBS. Transfer the slides back into the microscope holder and place it in the staining box.
8. Wash the slides with T-TBS (three times).
9. Prepare the labelled detection reagents such as secondary antibodies diluted in BSA-blocking solution. This second incubation solution should be spiked with the labelled streptavidin to detect the biotin-controls! Follow the instructions from entry 5. Incubate for 1–2 h. Note: Commercial secondary-antibodies that contain 1 mg per 1 mL stock solution should be diluted to 1:400 or higher in BSA-blocking solution.
10. Wash the slides with T-TBS (three times).

11. Signal read-out for fluorescence label:

    (a) Wash the slide with Milli-Q water (three times).
    (b) Dry the slide in a nitrogen stream or by centrifugation (1,000 rpm for 10 min).
    (c) Place slides into the slide reader instrument for scanning.

11a. Signal read-out for chemiluminescence detection:
Keep slides under TBS buffer until processed by a chemiluminescence procedure in a dark room or scanning device. Note: Slides must not dry in order to maintain activities of the HRP- or AP-enzymes.
A simple procedure includes:

    (a) Place a piece of X-ray film on a clean surface.
    (b) Cover with a thin transparent plastic wrap (Saran Wrap).
    (c) Add a droplet of 200 μL of a chemiluminescence substrate solution central to the covered X-ray film. Note: For the substrate solution follow the instructions of the supplier of your chemiluminescence kit.
    (d) Remove excess buffer from the slide by letting it run off from one edge onto a piece of paper towel for about 2 sec; place the slide top down onto the droplet for the time of exposition needed. Note: Avoid trapping of air bubbles. Do not use more substrate solution, otherwise the slide will swim and yield an unfocussed image.
    (e) Place the slide back to a reservoir of TBS-buffer for further expositions and process X-ray film.

35.7 Concluding Remarks

The protocols are optimized to help any researcher, even if not trained in chemistry, to prepare high quality low cost synthetic peptide arrays for epitope analysis and a variety of other biological screening experiments. These protocols worked successfully also under extreme conditions such as the tropical summer of Argentina with lab temperatures of about 35°C, the dichloromethane almost boiling and a humidity of over 90%. The authors are happy to give advice in case of problems with the procedures or changes to the procedures for other applications. We welcome comments, corrections and suggestions. Please contact us by e-mail.

Acknowledgements The protocols given here reflect the expertise of many colleagues developed over the past years. Thanks for their input, improvements to the method and extension of applications. This work is supported by grants no. 01GR0102 and 01GR0474 of the German National Genome Research Net (NGFN). We thank Dr. Heinrich Gausepohl and Dr. Ole Brandt from Intavis Bioanalytical Instruments AG for their helpful discussions to improve the SC^2-process and make it a commercial product, as well as Dr. Norbert Zander from AIMS Scientific Products for support in the development of suitable synthesis membranes and modified microscope slides.

References

Atassi MZ (1977) The complete antigenic structure of myoglobin: Approaches and conclusions for antigenic structures of proteins. In: Atassi MZ (ed) Immuno Chemistry of Proteins. Plenum Press, New York, p p77

Bialek K, Swistowski A, Frank R (2003) Anal Bioanal Chem 376:1006

Billich C, Sauder C, Frank R, Herzog S, Bechter K, Takahashi K, Peters H, Staeheli P, Schwemmle M (2002) Biol Psychiatry 51:979

Blüthner M, Mahler M, Müller DB, Dünzl H, Bautz FA (2000) J Mol Med 78:47

Chan WC, White PD (2000) (eds) Fmoc Solid Phase Peptide Synthesis: a practical approach. Oxford University Press, Oxford, UK

Dikmans A, Beutling U, Schmeisser E, Thiele S, Frank R (2006) QSAR Comb Sci 25:1069

Dooley CT, Houghten RA (1993) Life Sci 52:1509

Dostmann WRG, Taylor MS, Nickl CK, Brayden JE, Frank R, Tegge WJ (2000) Proc Natl Acad Sci 97:14772

Espaniel X, Wälchli S, Rückle T, Harrenga A, Huguenin-Reggiani M, van Huijsduijnen RH (2003) J Biol Chem 278:15162

Fields GB, Noble RL (1990) Int J Pept Protein Res 35:161

Frank R (1992) Tetrahedron 48:9217

Frank R (1994) Spot-Synthesis: An easy and flexible tool to study molecular recognition. In: Epton R (ed) Innovations and Perspectives in Solid Phase Synthesis 1994. Mayflower Worldwide Ltd, Birmingham, p p509

Frank R (2002a) Comb Chem High Throughput Screening 5:429

Frank R (2002b) J Immunol Methods 267:13

Frank R, Schneider-Mergener J (2002) SPOT-Synthesis - Scope of applications; In Koch J, Mahler M (eds) Peptide Arrays on Membrane Supports: A Laboratory Manual. Springer Verlag, Berlin, p 1

Frank R, Kieß M, Lahmann H, Behn C, Gausepohl H (1995) Combinatorial synthesis on membrane supports by the SPOT technique. In: Maia LS (ed) Peptides 1994. ESCOM, Leiden, p 479

Gail R, Frank R, Wittinghofer A (2005) J Biol Chem 280:7107

Geysen HM, Mason TJ (1993) Bioorg Med Chem Lett 3:397

Geysen HM, Rodda SJ, Mason TJ (1986) Mol Immunol 23:709

Harlow E, Lane D (1988) Antibodies – A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 319–358

Hoffmann S, Frank R (1994) Tetrahedron Lett 35:7763

Hoffmüller U, Russwurm M, Kleinjung F, Ashurst J, Oschkinat H, Volkmer-Engert R, Koesling D, Schneider-Mergener J (1999) Angew Chem Int Edit 38:2000

Homepage of IRIS BioTech http://www.iris-biotech.de/

Keitel T, Kramer A, Wessner H, Scholz C, Schneider-Mergener J (1997) Crystallographic analysis of an anti-p24 (HIV-1) monoclonal antibody cross reactivity and polyspecificity. Cell 91:811–820

Klos A, Ihrig V, Messner M, Grabbe J, Bitter-Suermann D (1988) Detection of native human complement components C3 and C5 and their primary activation peptides C3a and C5a (anaphylatoxic peptides) by ELISAs with monoclonal antibodies. J Immunol Methods 111:241–252

Kneissel S, Queitsch I, Petersen G, Behrsing O, Micheel B, Dübel S (1999) J Mol Biol 288:21

Kramer A, Volkmer-Engert R, Malin R, Reineke U, Schneider-Mergener J (1993) Peptide Res 6:314

Kramer A, Reineke U, Dong L, Hoffmann B, Hoffmüller U, Winkler D, Volkmer-Engert R, Schneider-Mergener J (1999) J Peptide Res 54:319

Krchnák V, Vágner J, Safár P, Lebl M (1988) Collect Czech Chem Commun 53:2542

Mukhija S, Germeroth L, Schneider-Mergener J, Erni B (1998) Eur J Biochem 254:433

Oggero M, Frank R, Etcheverrigaray M, Kratje R (2004) Mol Divers 8:257
Reineke U, Ivascu C, Schlief M, Landgraf C, Gericke S, Zahn G, Herzel H, Volkmer-Engert R, Schneider-Mergener J (2002) J Immunol Methods 267:37
Rodriguez M, Li SSC, Harper JW, Songyang Z (2004) J Biol Chem 279:8802
Rüdiger S, Schneider-Mergener J, Bukau B (2001) EMBO J 20:1042
Schmidt TGM, Koepke J, Frank R, Skerra A (1996) J Mol Biol 255:753
Schultz J, Hoffmüller U, Ashurst J, Krause G, Schmieder PJ, Macias M, Schneider-Mergener J, Oschkinat H (1998) Nat Struct Mol Biol 5:19
Töpert F, Pires R, Landgraf C, Oschkinat H, Schneider-Mergener J (2001) Angew Chem Int Edit 40:897
Töpert F, Knaute T, Guffler S, Pires JR, Matzdorf T, Oschkinat H, Schneider-Mergener J (2003) Angew Chem Int Ed 42:1136
Valle M, Kremer L, Martínez C, Roncal F, Valpuesta JM, Albar JP, Carrascosa JL (1999) J Mol Biol 288:899
Winkler K, Kramer A, Kuttner G, Seifert M, Scholz C, Wessner H, Schneider-Mergener J, Hohne W (2000) Changing the antigen binding specificity by single point mutations of an anti-p24 (HIV-1) antibody. J Immunol 165:4505–4514
Zander N (2004) Mol Divers 8:189

Chapter 36
Epitope Mapping by Printed Peptide Libraries

Frank Breitling, Christopher Schirwitz, Thomas Felgenhauer, Ines Block, Volker Stadler, and Ralf Bischoff

36.1 Introduction

The exact definition of an antibody's antigen-binding site can yield valuable information. When an antibody blocks, e.g., virus entry, the information, which protein is targeted, hints to a virus receptor with direct implications to a therapeutic intervention. Narrowing down the epitope to a short sequence of amino acids helps us to learn more about the molecular mechanism of virus entry and, again, might help to design a therapeutic molecule. Therefore, for the majority of antibodies – namely those that bind to proteins – it makes sense to ask:

- Which protein is bound?
- Which part of the protein is bound (*the epitope*)?
- Does the antibody bind to one linear peptide (*linear epitope*)
- Does the antibody bind to adjacent loops from several peptides (*conformational epitope*)?
- Which amino acids from the epitope are responsible for specific binding to the antigen (*the exact epitope*)?
- What else is bound by the antibody (*cross reactivity*)?
- Can we afford to determine the epitopes of many different antibodies or even antibody sera (*cost issues*)?

One prerequisite to answer these questions is the availability of many peptides that can be used to probe the antibody for specific binding. Solid phase peptide synthesis was invented more than 40 years ago, when Merrifield consecutively coupled amino acid monomers to a growing peptide immobilized on a solid support

F. Breitling (✉),
Karlsruhe Institute of Technology, Helmholtzplatz 1, 76344 Eggenstein-Leopoldshafen, Germany
C. Schirwitz, T. Felgenhauer, I. Block, V. Stadler and R. Bischoff
AG Chipbasierte Peptidbibliotheken, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany
e-mail: f.breitling@dkfz.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_36,

(Merrifield 1965). His approach was expanded later to establish the field of combinatorial chemistry, with multiple reaction spheres processed in parallel to synthesize many different peptides (Houghten et al. 1991; Reineke et al. 2001; Geysen et al. 1984). The breakthrough in the analysis of antibody epitopes came with Ronald Frank's invention of peptide arrays. Produced by the so-called SPOT synthesis, these arrays were extensively used to characterize the epitopes of many different antibodies (Frank 1992). In addition, covalently linking peptide libraries to a solid support makes them easier to handle. This technique is discussed in the previous chapter, and, therefore, will not be dealt with in this chapter.

36.1.1 High-Density Peptide Arrays

How can we define the exact epitope of an antibody, especially, if nothing else is known about the antigen (*e.g., the predominant antibodies within an autoimmune serum*)? Synthesizing an array with >10,000 stochastically chosen peptides, and staining this diversity of peptides with the antibody will answer this question for a majority of antibodies being scrutinized. The method should generate enough 3D structures to find at least one peptide that binds with low affinity to a given antibody. A systematic substitution of individual amino acids of these initial binders either yields the exact linear epitope or a mimotope (Reineke et al. 2002) (*a mimotope is a peptide that binds specifically to the antibody, but is different from the original antigen*). When compared to the databases, this information should predict cross reactions, or, eventually, the target and the causative agent of an autoimmune response. Currently this procedure is too expensive to be applied in routine (*an array with 8,000 peptides costs ~6,000 €; www.jpt.com*).

In theory, the high-density array format should drastically reduce the cost of this kind of high-throughput screenings through further miniaturization. The reason is obvious: Smaller spots reduce consumption of compounds and analyte. High peptide densities have already been achieved with a variant of the SPOT synthesis, the SC^2 method. This method produces the individual peptide-cellulose conjugates by a first SPOT synthesis in the array format. Then, the individual peptides are separated from each other and spotted in high density onto a second support, e.g., a glass slide. This procedure particularly allows for the production of multiple densely spaced peptide-array-replicas (Dikmans et al. 2006; *see previous chapter*), but it is too expensive when the task is to synthesize individual peptide arrays to determine individual antibody epitopes. A cheaper alternative might be the use of lithographic techniques that meanwhile are employed to array thousands of oligonucleotides per cm^2 (Lipshutz et al. 1999). However, lithographic methods couple only one kind of monomer at a time to spatially defined regions on the solid support. This adds up to 20×10 coupling cycles to synthesize a 10meric peptide array, compared to only 4×10 coupling cycles to generate a 10meric oligonucleotide array. This peptide-specific drawback makes it difficult to generate longer peptides in good quality (Pellois et al. 2002; Fodor et al. 1991; Mandal et al.

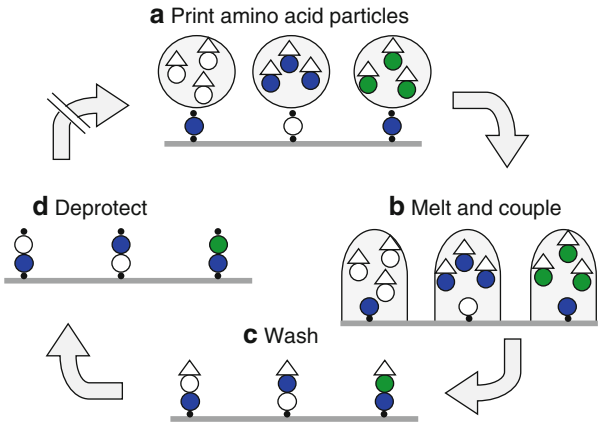


Fig. 36.1 (**a**) Solid toner particles that contain Fmoc-amino acid-OPfp esters are printed onto a solid support that is equipped with free amino groups. (**b**) The particles are melted after transfer, which results into the formation of oily reaction spheres. Previously immobilized chemically activated amino acid derivatives are then released to diffuse to the surface. The method allows for the separation of very small reaction spheres, which makes it possible to synthesize many different peptides in parallel and at high density. A cycle of synthesis is complete when (**c**) the excess of monomers is washed away and (**d**) the Fmoc protection group is cleaved. Repetitive coupling cycles generate a peptide array with only one coupling cycle per layer. Peptide length is determined by the number of printed layers

2007). However, arrays with short peptides generated by lithographic techniques are commercialized by the company LC-sciences (www.lcsciences.com).

In order to generate customized peptide arrays in good quality and at high density, high speed, and low cost, we used a modified colour laser printer to spatially address the 20 amino acids as a component of solid amino-acid-toner particles to a glass support. Once printed, an entire layer of all different amino-acid-toners is melted at once, whereby pre-activated amino acids are released from the solid matrix and start to couple to the support. Washing and deprotection steps after each printing procedure complete the cycle that, if repeated, results in the combinatorial synthesis of a peptide array (Fig. 36.1). The method uses conventional Fmoc chemistry (Chan and White 2000) and differs from the standard solid phase synthesis only in the – at room temperature – solid solvent employed that allows for the intermittent immobilization of amino acids within toner particles (Breitling et al. 2009; Stadler et al. 2008a; Breitling et al. 2008). The prototype of our "peptide laser printer" currently enables the synthesis of 160,000 peptides per 20×20 cm^2 (Fig. 36.2). A second generation "peptide laser printer" that increases the peptide density to >500,000 peptides per 20×20 cm^2 will be available in 2010. The particle-based synthesis of peptide arrays is commercialized by the company PEPperPRINT (www.pepperprint.com).

A chip based variant of this particle-based method should increase the peptide density even more, and, thus, reduce the cost for peptide arrays in the future. Instead

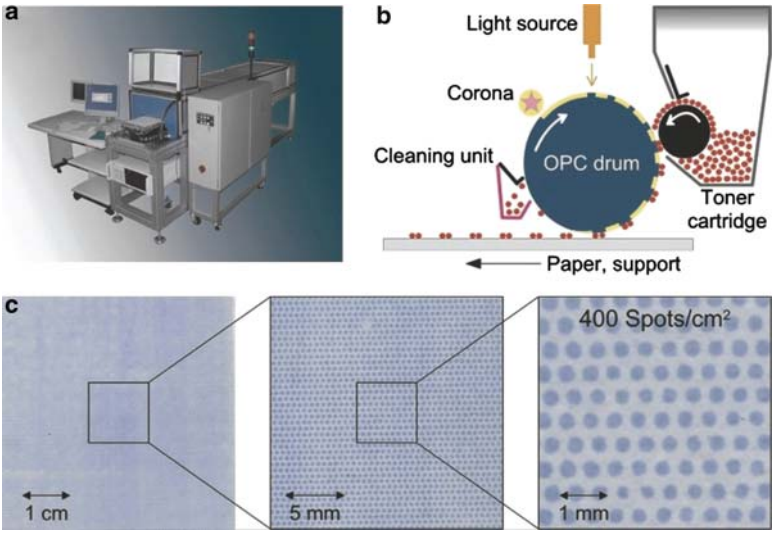


Fig. 36.2 (**a**) The peptide laser printer with 20 different printing units aligned; a mounted support can be seen at the printer inlet. (**b**) A light source (*LED row, orange*) illuminates and thereby neutralizes selected spots on an OPC drum (*blue*) that was previously uniformly charged (yellow) by a corona. Triboelectrically charged toner particles are transferred to these neutralized areas before finally a strong electric field collects them onto a solid support. (**c**) Amino-acid-toner printed on a PEGMA-coated glass slide by the peptide laser printer. Particle-embedded Fmoc-Ile-OPfp esters were released by heat and thus coupled to amino groups incorporated into the film. Residual material was washed away with DMF, and remaining free amino groups were blocked with 10% (v/v) acetic anhydride in DMF. Finally, the Fmoc protection groups were cleaved by 20% (v/v) piperidine in DMF and newly introduced free amino groups were stained with 0.1% bromophenol blue in methanol. *Figure originally published in Angewandte Chemie Int. Ed.* ***47****, 7132–7135 (2008), and reproduced by courtesy of Wiley-VCH*

of a peptide laser printer, this method uses electrical fields generated on the individual pixel electrodes of a microchip to directly address amino acid particles to very small synthesis areas (Fig. 36.3). Currently, this method allows for the synthesis of up to 40,000 peptides per cm^2 (Beyer et al. 2007). To date, the particle-based synthesis on a microchip is less matured when compared to the laser printer. Therefore, this chapter focuses on the laser printer based combinatorial synthesis.

36.2 Materials

36.2.1 Production of Amino-Acid-Toners

Materials needed to produce toner particles are listed in Table 36.1, while the schematic procedure to generate particles is depicted in Fig. 36.4:

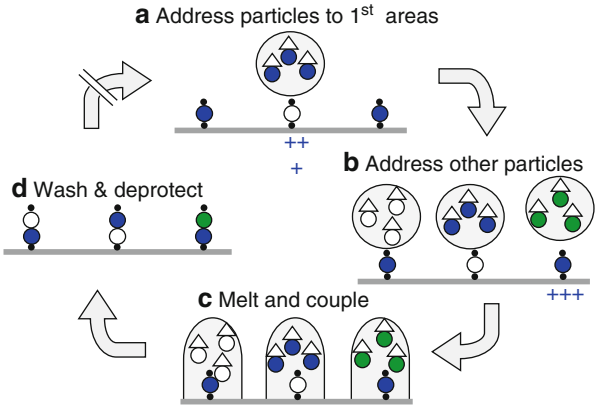


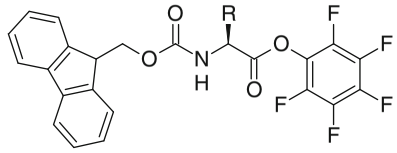
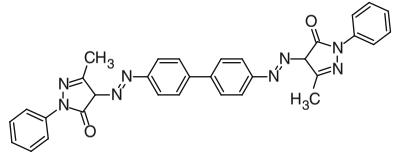
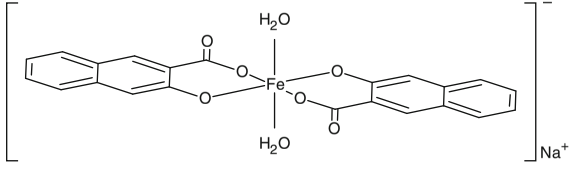
Fig. 36.3 (**a**) One type of amino acid particles is addressed to specific areas on the chip surface using electrical fields generated by individual pixel electrodes. (**b**) Voltage is applied through different patterns of pixel electrodes to consecutively position a whole layer of all 20 different amino acid particles onto the chip surface. During this procedure, the particle matrix prevents embedded and chemically activated amino acids from decomposition. (**c**) When all amino acid particles have been addressed, the whole layer is melted at once to induce the coupling reaction. (**d**) Excess monomer is washed away and the Fmoc protection group is cleaved. Repetitive coupling cycles generate a peptide array with one coupling cycle per layer, which can include all the 20 different amino acids at once

- The most important ingredients of amino acid toner particles are the activated amino acid derivatives. We use side chain protected Fmoc-amino acid pentafluorophenyl esters (OPfp) as combinatorial building blocks.
- Resins are needed to stabilize toner particles, while the trait of a solvent that is solid at room temperature is given either by diphenyle formamide, diphenyle sulfoxide, or p-tosyl sulfoxyde.
- Charge control agents pyrazolone orange (ABCR GmbH), and Fe(naphtol)$_2$ complex (Kawagishi et al. 1983) stabilize the electric charges on the surface of particles. These electric charges are generated by grinding the particles against rubber foam inside the toner cartridges.
- Amino acid microparticles are formulated, milled, sieved and characterized according to the literature (Beyer et al. 2007).

36.2.2 Peptide Laser Printer

Our peptide-laser-printer is conceptually based on the colour laser printer OKI C7400, but accommodates 20 instead of 4 printing units, and in addition a drive and mounting that allows for a repeated ±5 µm exact positioning of the solid support (Fig. 36.2a). The machine was manufactured at the Fraunhofer Institute for Production and Automation (IPA) in Stuttgart (http://rpd.ipa.fraunhofer.de/drucktechnik/led/led.php). A row of ~10,000 light emitting diodes [LEDs] per

Table 36.1 Components of amino-acid-particles

Component	Function	Relative amount (w/w)	Supplier/reference	Structure
Fmoc-L-amino-acid-OPfp-ester	Activated amino acid, monomer	10%	Merck Biosciences	
DPSO, DPF, or p-tosyl sulfoxyde	Solid solvent	20%	Merck Biosciences	
SLEC PLT 7547	Resin for physical stability	64.5%	Sekisui	SLEC PLT 7547 is a styrene-acrylic-Copolymer with no exact structure available from the supplier. CAS [25767-47-9]
Pyrazolone orange	Charge stabilizer	4.5%	ABCR GmbH	
$(Fe)_2$ naphthol complex	Charge stabilizer	1%	Kawagishi et al. (1983)	
Silica particles, Aerosil 812, hydrophobic	Anti-aggre-gation	0.04% added during milling	Degussa	

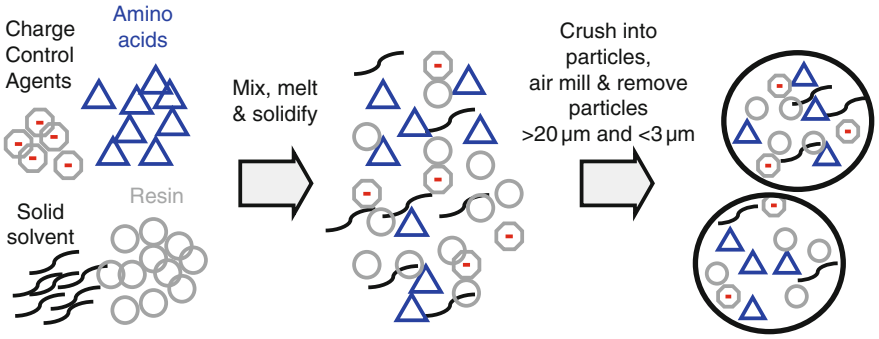


Fig. 36.4 Ingredients of amino acid toner particles are mixed, melted, and finally solidified again. The resulting toner mass is crushed into crude particles with a device similar to a coffee mill with rotating scissors. The pre-milled particles are grinded against each other in an air-jet mill to reduce particle size to 5–20 µm in diameter. During this milling procedure ~0.04% (w/w) silica particles are added in order to avoid aggregation of particles. Finally, >20 µm particles are removed with a sieve tower, while particles <3 µm are removed with a winover. Typically, 100 g of crude toner mass yield 70 g of size selected amino acid toner particles

20 cm generates a light pattern on the surface of a uniformly charged organic photo conducting [OPC] drum that rotates in ~10,000 steps per 20 cm. This results in a two dimensional light pattern, that comprises ~100 million pixels per 20×20 cm^2. The OPC material translates this light pattern into the corresponding electrostatic pattern of ~100 million pixels per 20×20 cm^2, as illuminated areas are rapidly neutralized by grounding (Borsenberger and Weiss 2002). Subsequently, charged toner particles are transferred only to those areas previously neutralized by irradiation with light, which transforms the electrostatic pattern to the corresponding particle pattern. Finally, the particles delivered by the OPC drum are collected onto a solid support by a strong electric field (4 kV/mm), where a printout is assembled from the 20 different amino-acid-toners (Fig. 36.2b). Currently, the printing resolution of this machine is limited to ~160,000 peptides per 20×20 cm^2 (Fig. 36.2c). A next generation peptide laser printer will generate >500,000 peptides per 20×20 cm^2.

36.2.3 Glass Slides Grafted with PEG Based Surfaces for Combinatorial Synthesis

– Synthesized 2-bromo-*N*-(3-triethoxysilyl)propyl isobutyramide (Stadler et al. 2007); dichloromethane (p.a.), ethanol (p.a.), methanol (p.a.), poly(ethylene glycol) methacrylate (PEGMA, $M_w \sim 360$ g/mol), copper(I) bromide (p.a.), 2,2′-bipyridyl (bipy, 98%), *N*,*N*′-diisopropyl carbodiimide (DIC, purum), and *N*-methylimidazole (NMI, p.a.) were all purchased from Sigma-Aldrich

(Steinheim, Germany); Fmoc-ß-alanine >99% was obtained from Iris Biotech (Marktredwitz, Germany).
– 100 mL Schlenk flask, with stirring bar, ultrasonic bath, vacuum desiccator, vacuum pump ($<10^{-2}$ mbar) and nitrogen (AlphaGaz^{TM} $1N_2$, AirLiquide) for silanization and polymerization under oxygen-free and dry gas phase conditions.

36.2.4 Reagents for Peptide Synthesis

– Solvents and liquids: *N*,*N*-dimethylformamide (DMF) peptide grade (Biosolve BV, Valkenswaard, The Netherlands) was dried over molecular sieve; methanol (p.a.), ethanol (p.a.), chloroform (p.a.), dichloromethane (p.a.), piperidine (>99%), acetic anhydride (p.a.), *N*,*N*-diisopropylethylamine (DIPEA, p.a.), triisobutylsilane (TIBS, puriss.) were purchased from Sigma Aldrich (Steinheim, Germany) and were used without further purification. Trifluoroacetic acid (TFA, 99%) was obtained from Acros Organics (Geel, Belgium).
– Washing chamber made from PTFE (poly tetrafluor ethylene, Teflon®) custom-built at Schmitz und Gleissle (Nußloch, Germany) with stirring bar; magnetic stirrer (model RCT basic IKAMAG safety control, VWR International); heat-resistant, gas-proof coupling chambers with two gas valves.

36.2.5 Immunostaining

– Tris-buffered saline: Prepare a 10× stock with 0.5 *M* (60.57 g) Trisma® base (Sigma Aldrich, Taufkirchen, Germany) and 1.5 *M* (87.66 g) NaCl (adjust to pH 7.4 with concentrated HCl). Prepare working solution by dilution of one part with nine parts of water. Add 0.1 v% of Tween-20 (Sigma Aldrich, Taufkirchen, Germany) to obtain TBS-T buffer for washing and antibody dilution.
– Primary antibodies: Mouse monoclonal anti-FLAG® M5 and rabbit anti-HA antibody (both from Sigma Aldrich, Taufkirchen, Germany).
– Secondary antibodies for Odyssey scanner: LI-COR IRDYE® 680 goat anti-rabbit IgG and LI-COR IRDYE® 800CW goat anti-mouse IgG (both from Licor Biosciences, Lincoln, Nebraska, USA).
– Odyssey fluorescence scanner (www.licor.com/bio/odyssey/OdysseyTour_7.jsp) and Image Analysis software (both from Licor Biosciences, Lincoln, Nebraska, USA)
– Secondary antibodies for GenePix scanner: Alexa Fluor® 647 goat anti-rabbit IgG (H+L) and Alexa Fluor® 546 goat anti-mouse IgG (H+L) (both from Invitrogen GmbH, Karlsruhe, Germany).
– GenePix 4000B fluorescence scanner and GenePix Pro 4.0 Microarray Image Analysis software (Molecular Devices).

– Antibodies for ECL readout: anti-Flag M2 antibody (conjugated with horse radish peroxidase, HRP), and biotinylated anti-Myc 9E10 antibody together with HRP coupled to streptavidin.
– The ECL system can be purchased from GE healthcare.

36.3 Protocols

36.3.1 Generation and Characterization of Amino-Acid-Toners

The production of amino acid toner particles is described in the literature (Stadler et al. 2008b). For physical stability particles comprise a resin, the trait of a solid solvent is given by a higher homologue of standard solvents. In order to stabilize electric charges on the surface of particles, charge control agents must be added, and, finally, to prevent aggregation of particles silica nanoparticles are added during the milling process (Fig. 36.4).

- Mix one of 20 different Fmoc-amino acid OPfp esters (Merck, 10% w/w), a polymer, e.g., SLEC PLT 7552 (Sekisui; 64.5% w/w), a "solid solvent," either diphenyle formamide (DPF; 20% w/w), diphenyle sulfoxide (DPSO; 20% w/w), or p-tosyl sulfoxyde (TSO; 20% w/w), the charge control agents pyrazolone orange (ABCR GmbH, 4.5% w/w), and Fe(naphtol)$_2$ complex (1% w/w). Melt mixed toner ingredients (Table 36.1) and finally solidify them.
  Thoroughly dry toner masses. Store them in a dessicator under an atmosphere of nitrogen.
- Mix homogenized and crushed toner mass with ~0.04% w/w silica particles (Degussa, Aerosil 812, hydrophobic), and slowly feed into an air jet mill where grinded particles are collected within a narrow lattice (Hosokawa alpine 100AS).
- Check physical parameters of amino-acid-particles: The mean diameter should be ~9 μm, there should be a narrow size distribution, a low tendency to aggregate, melting points of 60–80°C, a Q/m value of ~ −4 μC/g, and a similar toner transfer for the 20 different amino-acid-particles that should approximate 1 mg of transferred toner particles per cm^2. These parameters are measured with a Mastersizer (Malvern, type 2000), by differential scanning calorimetry (Netzsch, type DSC 204 F1 Phoenix), and a Q/m meter (Trek, type 210HS-2), respectively.
  The yield of particles with a size in between 5 and 25 μm should approximate 70% when referenced to toner mass.
- Light microscopy and scanning electron microscopy (SEM) pictures to determine particle morphology are taken with an Axiovert 35 light microscope (Zeiss) and an SEM (LEO; Microscope type 1530; 10 kV electron volts). The stability of OPfp esters embedded into amino-acid-particles is analyzed by HPLC.
  The characterization of amino acid toner particles was published in detail elsewhere (*Stadler et al.* 2008a).

36.3.2 Silanize Glass Surface (Fig. 36.5a)

Grafting glass slides (2.3 × 7.6 cm) with a 50–100 nm thick PEGMA coating is described in the literature (Beyer et al. 2006; Prime and Whitesides 1991; Stadler et al. 2008a, b). This method adds a third dimension for the combinatorial synthesis of peptide arrays, i.e., it increases the yield of synthesized peptides per area. The procedure for the grafting of a 3D PEG-based surface is schematically described in Fig. 36.5.

- Activate and clean the glass surfaces by treating the slides with 1 M KOH in isopropyl alcohol overnight.
- Remove glass slides from solution and wash them several times with water. Dry the slides in a stream of compressed air, then in an oven at 110°C for 1 h. Let the slides cool to room temperature. Meanwhile, directly prepare a 10 mM solution of 2-bromo-*N*-(3-triethoxysilyl)propyl isobutyramide in anhydrous dichloromethane and incubate the slides for at least 6 h to silanize the glass surfaces with a polymerization initiator (Fig. 36.5a).
  Silanization is carried out for a long time period to achieve a high conversion. The maximal recommended incubation time is 24 h.
- Dilute the reaction solution with an excess of ethanol and rinse the slides thoroughly first with ethanol, then with water.

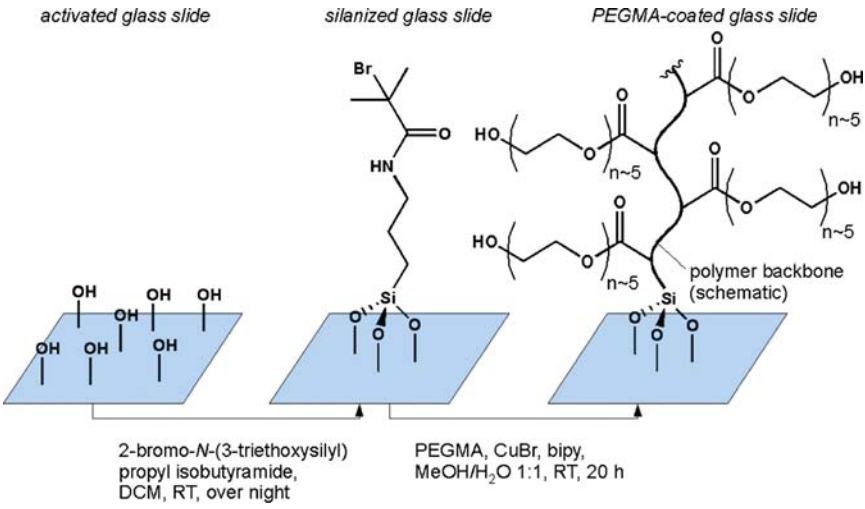


Fig. 36.5 Thoroughly cleaned and activated glass surfaces are silanized with a bromine silane to generate a surface-bound initiator for the polymerization reaction. The poly(ethylene glycol) methacrylat (PEGMA) building blocks polymerize and introduce a 3D polymeric film densely equipped with hydroxyl groups (*typically a 50–100 nm thick PEGMA-based layer accommodates 10–50 nmol per cm²*). Finally, the coupling of Fmoc protected β-alanine transforms hydroxyl groups to amino groups (not shown in the figure)

Assure appropriate disposal of halogenated organic solvents. Unless otherwise noted, solely Millipore® water (resistivity of about 18.2 MΩ cm) was used.

- Dry the slides in a stream of compressed air and place them in a pre-heated oven at 110°C for 1 h to achieve full condensation of the bromine silane with the surface. Remove the slides from the oven and allow them to cool to room temperature. Proceed with graft polymerization or store the glass slides at −20°C under nitrogen atmosphere.
  Compressed air supply should be filtered and dried to a constant low level of humidity. Silanized glasses should not be stored longer than 1 month under these conditions.

36.3.3 PEG Grafted Glass for Solid Phase Synthesis (Fig. 36.5b)

- Dissolve 141 mg 2,2′-bipyridyl (bipy, 0.90 mmol) per 10 ml water/methanol (1:1). Add 5 ml of PEGMA and 64 mg of copper(I) bromide (0.45 mmol). Immediately degas the brown reaction mixture three times and sonicate it for 3 min under nitrogen atmosphere. The amount of solution must be adjusted to the number of glass slides, i.e., the size of the reaction vessel. The slides should always be covered with an excess of solution.
  The dissolved copper(I) bromide catalyst is susceptible to oxidation. Discolouration of the solution from brown to green indicates residual oxygen, which oxidizes the catalyst and hence disrupts polymerization. Therefore, rapid but careful processing is essential as soon as the copper(I) bromide is added to the reaction mixture.
- Remove undissolved solid copper(I) bromide residues by filtration under nitrogen atmosphere. Add the reaction mixture to the silanized glass slides in an appropriate vessel and place the vessel in a desiccator.
- Carefully evacuate the desiccator three times and aerate it with nitrogen. Allow polymerization to proceed for 20 h.
  Oxygen in the Petri dish or in the desiccator must be avoided.
  Both, concentration of the monomer and polymerization time affect the resulting PEGMA film thickness. Film thickness does not noticeably increase after 20 h (Stadler et al. 2007).
- After the polymerization, wash the glass slides extensively with water until all residues of the polymerization solution are rinsed out. Incubate the glass slides 15 min in water, then 15 min in DMF.
  Unless otherwise noted, solely Millipore® water (resistivity of about 18.2 MΩ cm) was used.
- Wash the slides two times for 2 min each with methanol and dry them in a stream of compressed air. Proceed with functionalization of the polymer side chains or store the slides at −20°C under nitrogen atmosphere.
  Coated slides should not be stored longer than 1 month under these conditions.

36.3.4 Functionalize PEGMA Side Chains

- Pre-swell the PEGMA-coated surfaces in anhydrous DMF for 30 min.
- Prepare a solution of 62.26 mg Fmoc-β-alanine (0.2 M) and 37.2 µl DIC (0.24 M) per ml anhydrous DMF. After stirring for 5 min add 37.2 ml NMI (0.4 M). Replace DMF on the glass slides with this reaction mixture and incubate the slides for 16 h under nitrogen atmosphere at room temperature. Per glass slide ~1 ml of solution is needed.
  This step introduces amino groups to the PEGMA layer.
- Wash the slides three times for 2 min each with DMF.
- In order to block residual hydroxyl groups, shake the slides gently in a solution of 10% (v/v) acetic anhydride, 20% (v/v) DIPEA and 70% (v/v) DMF overnight.
  Blocking solution should be prepared immediately before use.
- Remove the solution and wash the slides five times, each time for 2 min with DMF.
- Cleave the Fmoc protection groups by incubating the glass slides for 20 min in a solution of 20% (v/v) piperidine in DMF.
  Cleavage of the Fmoc protection group can be spectrophotometrically quantified by monitoring the solution at 301 nm (Beyer et al. 2006).
- Wash the slides three times for 2 min with DMF and two times for 2 min with methanol. Dry them in a stream of compressed air. Functionalized glass slides are stored at −20°C under nitrogen atmosphere.
  An increased linker length due to β-Alanins usually provides better accessibility of the growing peptide chains to analytes, e.g., specifically binding antibodies. Therefore, it is recommended to repeat β-alanine coupling and deprotection two times to consecutively couple three β-alanine residues as the linker unit. Glasses should not be stored longer than 1 month under these conditions.

36.3.5 Particle-Based Synthesis

- Print amino acid particles to a modified slide with the peptide laser printer and transfer the slide into the coupling chamber.
  Be careful not to touch the damageable PEGMA surface. Particles keep sticking to the surface due to electrostatic charges.
- Aerate the chamber with nitrogen and place it into a pre-heated oven at 90°C for 60 min.
  The coupling reaction is initiated upon melting of the solid particle matrix which releases activated amino acids to diffuse to the PEGMA-coating of the glass and to couple to free amino groups incorporated into this coating. The nitrogen atmosphere avoids decomposition of the PEGMA surface.
- Allow the glass slides to cool to room temperature and transfer them into the washing chamber with the synthesis layer turned upwards.

- Incubate three times (1, 5 and 10 min) with a mixture of 10% (v/v) acetic anhydride, 20% (v/v) DIPEA and 70% (v/v) DMF to remove residual particle material and, at the same time, to block residual amino groups.
- Wash two times for 5 min each with DMF and two times for 5 min each with chloroform to remove adsorbed silica particles.
  Silica particles are a component part of the amino acid particles. They are added during the milling process to prevent particle aggregation. Especially washing with chloroform removes them. Otherwise they might lead to unspecific binding signals.
- Incubate the glass slides for 30 min in DMF to pre-swell the polymeric coating.
- Cleave *N*-terminal Fmoc protecting groups by exposing the glass to 20% (v/v) piperidine in DMF for 20 min.
- Remove the piperidine/DMF solution and wash five times for 5 min each with DMF and two times for 5 min each with methanol.
- Dry the glass slides in a stream of nitrogen and remove it from the washing chamber.
- Repeat amino acid particle deposition, coupling reaction as well as subsequent washing and deprotection steps until the complete peptide array is synthesized.
  The number of coupling cycles corresponds to peptide length (e.g., 10 cycles of combinatorial synthesis result in an array of 10mers). Please note that our particle-based synthesis of peptide arrays is identical to standard Merrifield synthesis/Fmoc chemistry, except for the intermittent "freezing" of the chemical reaction in solid amino acid toner particles.
- Leave the glass slides in the washing chamber in order to cleave the side chain protecting groups.
- Wash two times for 5 min each with dichloromethane.
- In the meantime, prepare a mixture of 51% (v/v) trifluoroacetic acid, 44% (v/v) dichloromethane, 3% (v/v) triisobutylsilane (TIBS) and 2% (v/v) water.
  Because of the high volatility of dichloromethane it should be added lastly to the TFA/TIBS/water mixture to avoid evaporation. CAUTION: TFA is very hygroscopic and corrosive. TFA mediated side chain deprotection can be done in PE chambers.
- Remove the dichloromethane and incubate the glass slides for 30 min in the TFA solution while continuously stirring.
  Washing chambers must be firmly closed to avoid evaporation of the reagents.
- Remove the TFA solution and wash the slides four times for 2 min each with dichloromethane and then DMF.
  TFA and DMF should not be mixed during washing steps.
- Remove the TFA solution and wash the slides four times for 2 min each with dichloromethane and then DMF.
  TFA and DMF should not be mixed during washing steps.
- Wash the glass slides two times for 2 min each with ethanol, and finally dry them in a stream of nitrogen. Proceed with immunostaining or store the glass slides at 4°C under nitrogen atmosphere
  Glass slides should not be stored longer than 1 month under these conditions.

36.3.6 Immunostaining

- Rehydrate and swell the PEGMA-coating of the slides by incubating in 1 ml of TBS-T buffer for at least 30 min.
  Since the PEGMA coating prevents nonspecific protein adsorption, no additional surface blocking with BSA or milk powder is required. However, if less protein resistant surfaces are used a blocking step must be added.
- Dilute specific antibodies (e.g., mouse anti-FLAG® and rabbit anti-HA) 1:1000 in TBS-T buffer.
- Incubate the glass slides in 1 ml of the antibody solution for 1 h at room temperature while gently rocking the samples.
- Carefully remove the buffer solution and wash six times for 5 min each in TBS-T while constantly shaking.
  Keeping the peptide array moistened is crucial to avoid Langmuir-Blodgett-like protein or antibody adsorption on the surface of the array. Nonspecific adsorption of proteins would lead to false-positive results and reduced signal-to-noise ratios in immunoassays.
- Dilute secondary antibodies (e.g., Alexa Fluor® 647 goat anti-rabbit IgG and Alexa Fluor® 546 goat anti-mouse IgG) 1:1000 in TBS-T buffer and incubate the glass slides in this solution for 1 h at room temperature. Alternatively use LI-COR IRDYE® 680 goat anti-rabbit IgG and LI-COR IRDYE® 800CW goat anti-mouse IgG for scanners in near infrared wavelength. Yet another alternative is horse radish peroxidase (HRP) conjugated secondary antibodies.
- Wash six times for 5 min each in TBS-T buffer while continuously shaking.
- Dry the glass slides in a stream of nitrogen and remove it carefully from the circuit board.
  Fluorescence-readout can be done with the GenePix scanner at appropriate wavelengths (532 nm for Alexa Fluor® 546 and 635 nm for Alexa Fluor® 647 dyes respectively). Alternatively, use the Odyssey scanner together with infrared fluorescence dyes (680 nm for LI-COR IRDYE® 680 goat anti-rabbit IgG and 800 nm for LI-COR IRDYE® 800CW goat anti-mouse IgG dyes respectively). However, another alternative is the ECL system that detects chemiluminescence generated by the enzymatic activity of horse radish peroxidase.

36.4 Results

A typical assay that resulted in the exact determination of the binding epitope of an antigen is shown in Fig. 36.6. FLAG®- and Myc-epitopes were detected by consecutively adding epitope-specific mouse monoclonal antibodies (ECL readout). We used the anti-Flag M2 antibody (directly conjugated with horse radish peroxidase HRP), as well as the biotinylated anti-Myc 9E10 antibody stainined with HRP-conjugated streptavidin afterwards. In order to stain the same glass slide consecutively with different antibodies, after one staining step residual peroxidase

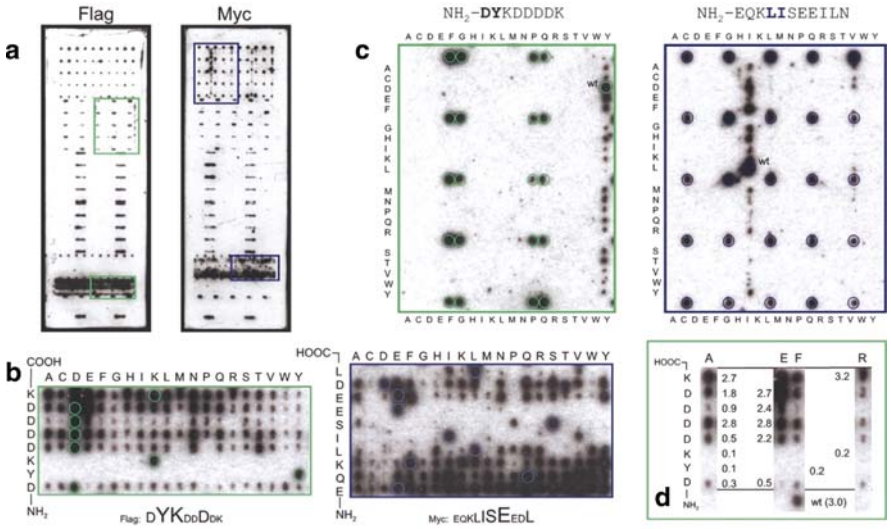


Fig. 36.6 Permutated FLAG®- and Myc-epitopes were detected by consecutively adding epitope-specific mouse monoclonal antibodies (ECL readout). (**a**) Glass slide with ~5,500 peptides stained with FLAG® or Myc antibodies. Regularly interspersed positive controls (framed spots, i.e., wild type epitopes), and some reactive peptide variants are clearly visible. Coloured frames delineate the enlargements shown below. (**b**) Determination of the exact epitopes for Flag and Myc binding. Every amino acid position in the sequence of each epitope was exchanged for all 20 different amino acids to characterize the binding requirements of epitope specific antibodies. The epitope specificity deduced from individual experiments is shown in the sequence written underneath. The dimension of amino acid symbols reflects their importance for antibody binding. Epitope specificity of 9E10 and Flag M2 antibodies matches already published results (Hilpert et al. 2001; Slootstra et al. 1997). (**c**) Epitopes permutated at two positions that are highlighted by color in the peptide sequence. Interspersed wild type sequences are encircled. The N-terminal D of the FLAG®-epitope can be substituted for other amino acids, whereas the adjacent amino acid Y is mandatory for antibody Flag M2 binding. The I in the sequence of the Myc epitope can only be substituted for V, whereas the adjacent L is less important for antibody binding. The results of these more complex permutations confirm the epitope runs shown. (**d**) Staining intensity of various FLAG® epitopes from the epitope run in (b) match already published relative affinities (Hilpert et al. 2001; Slootstra et al. 1997), which are shown next to spots stained with the Flag M2 antibody. *Figure originally published in Angewandte Chemie Int. Ed.* ***47****, 7132–7135 (2008), and reproduced by courtesy of Wiley-VCH*

was inactivated with 0.05% sodium azide in TBS. Readout of binding sites was done by exposing a film 1–2 sec in the course of ECL reaction.

36.5 Conclusions

Currently, many monoclonal antibodies exist where the exact epitope is unknown. The reason is obvious: Common peptide arrays are still quite expensive and are therefore not routinely applied in screenings. In the future, high-density peptide

arrays will be available at much lower costs thanks to particle-based synthesis. A cheap and straightforward method to determine the linear epitopes for many of the antibodies can be established by simply synthesizing densely overlapping peptides that represent the sequence of the targeted protein. In addition, the same technique should allow for the exact determination of the amino acids of an epitope that contribute to specific binding. This will be done by exchanging every amino acid of the epitope sequence with the 20 amino acids. This experiment can reveal positions of the peptide where only one or a few amino acids are accepted to allow for binding of the antibody. If this "fingerprint" is present in other proteins of a given organism, the information deposited in the databases should pinpoint cross reacting antigens, and, therefore, help to validate the information gained by staining a sample with this antibody. The technology might even validate the binding specificities for antibodies that recognize conformational epitopes. If enough peptides (>10,000) are presented on the array some of these peptides should bind the antibody by chance. These can be scrutinized as described for linear epitopes, and thus reveal the "fingerprint(s)" of mimotope(s) in addition to cross reacting antigens. We expect that our technology will be especially helpful in the characterization of complex antibody mixtures, e.g., by scanning a whole proteome of an organism with overlapping peptides to characterize polyclonal or monoclonal antibodies raised against this organism.

References

Beyer M, Felgenhauer T, Bischoff FR, Breitling F, Stadler V (2006) A novel glass slide-based peptide array support with high functionality resisting non-specific protein adsorption. Biomaterials 27:3505–3514

Beyer M, Nesterov A, Block I, König K, Felgenhauer T, Fernandez S, Leibe K, Torralba G, Hausmann M, Trunk U, Lindenstruth V, Bischoff FR, Stadler V, Breitling F (2007) Combinatorial synthesis of peptide arrays onto a computer chip's surface. Science 318:1888

Borsenberger PM, Weiss DS (2002) Photoreceptors: organic photoconductors. In: Diamond AS, Weiss DS (eds) Handbook of imaging materials, 2nd edn. Marcel Dekker, New York, pp 369–423

Breitling F, Felgenhauer T, Nesterov A, Lindenstruth V, Stadler V, Bischoff FR (2008) Particle-based combinatorial peptide synthesis. CHEMBIOCHEM 10:803–808 Concept article

Breitling F, Felgenhauer T, Nesterov A, Stadler V, Bischoff FR (2009) High-density peptide arrays. Mole Biosyst 5:224–234 Review

Chan WC, White PD (2000) Fmoc solid phase peptide synthesis. A practical approach. Oxford University Press, Oxford

Dikmans A, Beutling U, Schmeisser E, Thiele S, Frank R (2006) SC^2: A novel process for manufacturing multipurpose high-density chemical microarrays QSAR comb. Science 25:1069–1080

Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D (1991) Light-directed, spatially addressable parallel chemical synthesis. Science 251:767–773

Frank R (1992) Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron 48:9217–9232

Geysen HM, Meloen RH, Barteling SJ (1984) Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc Natl Acad Sci USA 81:3998–4002

Hilpert K, Hansen G, Wessner H, Küttner G, Welfle K, Seifert M, Höhne W (2001) Anti-c-myc antibody 9E10: epitope key positions and variability characterized using peptide spot synthesis on cellulose. Protein Eng 14:803–806

Houghten RA, Pinilla C, Blondelle SE, Appel JR, Dooley CT, Cuervo JH (1991) Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. Nature 354:84–86

Kawagishi Y, Ishida Y, Ishikawa K (1983) (Orient Chemical Ind.), patent application US 4404271A1

Lipshutz RJ, Fodor SP, Gingeras TR, Lockhart DJ (1999) High density synthetic oligonucleotide arrays. Nat Genet 21:20–24

Mandal S, Rouillard JM, Srivannavit O, Gulari E (2007) Cytophobic surface modification of microfluidic arrays for in situ parallel peptide synthesis and cell adhesion assays. Biotechnol Prog 23:972–978

Merrifield RB (1965) Automated synthesis of peptides. Science 150:178–185 Review

Pellois JP, Zhou X, Srivannavit O, Zhou T, Gulari E, Gao X (2002) Individually addressable parallel peptide synthesis on microchips. Nat Biotechnol 20:922–926

Prime KL, Whitesides GM (1991) Self-assembled organic monolayers: model systems for studying adsorption of proteins at surfaces. Science 252:1164–1167

Reineke U, Volkmer-Engert R, Schneider-Mergener J (2001) Applications of peptide arrays prepared by the SPOT-technology. Curr Opin Biotechnol 12:59–64

Reineke U, Ivascu C, Schlief M, Landgraf C, Gericke S, Zahn G, Herzel HP, Volkmer-Engert R, Schneider-Mergener J (2002) Identification of distinct antibody epitopes and mimotopes from a peptide array of 5520 randomly generated sequences. J Immunol Methods 267:37–51

Slootstra JW, Kuperus D, Plückthun A, Meloen RH (1997) Identification of new tag sequences with differential and selective recognition properties for the anti-FLAG monoclonal antibodies M1, M2 and M5. Mol Divers 2:156–164

Stadler V, Beyer M, König K, Nesterov A, Torralba G, Lindenstruth V, Hausmann M, Bischoff FR, Breitling F (2007) Multifunctional CMOS Microchip Coatings for Protein and Peptide Arrays. J. Proteome Res 6:3197–3202

Stadler V, Felgenhauer T, Beyer M, Fernandez S, Leibe K, Güttler S, Gröning M, Torralba G, Lindenstruth V, Nesterov A, Block I, Pipkorn R, Poustka A, Bischoff FR, Breitling F (2008a) Combinatorial synthesis of peptide arrays with a laser printer. Angew Chem Int Ed Engl 47:7132–7135

Stadler V, Kirmse R, Beyer M, Breitling F, Ludwig T, Bischoff FR (2008b) PEGMA/MMA Copolymer Graftings: Generation, Protein Resistance, and a Hydrophobic Domain. Langmuir 24:8151–8157

Chapter 37
Antibody Epitope Mapping Using Yeast Display

Consuelo Garcia-Rodriguez, Yu Zhou, and James D. Marks

37.1 Introduction

Many methods have been employed in the past to map antibody-binding epitopes using either linear peptides on pins or spotted on cellulose membranes (Frank and Overwin 1996), peptide fragments expressed in *Escherichia coli* (Christmann et al. 2001), yeast (Benichou and Inchauspe 1996), or bacteriophage display libraries (Mehra et al. 1986; Mullaney et al. 2001). Such approaches are limited to mAbs binding either linear epitopes, or epitopes not requiring fully folded and full length conformationally correct domains. Thus, while potentially narrowing the epitope space, such approaches are not useful for the fine mapping of mAbs recognizing conformational epitopes.

One approach for precise identification of conformational protein epitopes is alanine scanning, constructing individual alanine mutants of each residue, expressing and purifying protein, and measuring the binding affinity (Cunningham and Wells 1989). Recently, shotgun scanning mutagenesis has been described as a high throughput method for mapping paratopes and protein−protein interactions using phage display (Weiss et al. 2000; Vajdos et al. 2002). In this approach, the relative functional impact of each amino acid is determined by the relative frequency of alanine mutants vs wildtype. Precise quantitation of $\Delta\Delta G$ requires expression and purification of each alanine mutant. More recently, it has been proven possible to identify conformational antibody epitopes of complex proteins from yeast-displayed antigen libraries, including epitopes on EGF receptor, West Nile envelope protein, and botulinum nurotoxin (Chao et al. 2004; Oliphant et al. 2005; Levy et al. 2007). As the eukaryote *Saccharomyces cerevisiae* can express and display a wide range of proteins, this is evolving into a highly useful technique for epitope

C. Garcia-Rodriguez, Z. Yu, and J.D. Marks (✉)
Departments of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, CA, USA
e-mail: marksj@anesthesia.ucsf.edu

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_37,

mapping. The gene for an antigen is displayed on the yeast surface fused to the yeast surface protein AgaII and antibody binding can be quantitated by flow cytometry. For multi-domain proteins, display of the individual domains allows rapid mapping of antibody at the domain level (Cochran et al. 2004; Levy et al. 2007). An advantage of this approach is that the antigen or antigen domain does not need to be expressed and purified. Taking the approach one step further, the fine epitope of antibodies can be mapped by constructing libraries of yeast-displayed antigen mutants and selecting for loss of antibody binding by flow cytometry(Chao et al. 2004; Oliphant et al. 2005; Levy et al. 2007). Sequencing of the mutants identifies the putative location of the epitope which is confirmed using single alanine mutants. In the protocols below, we have decribed the use of yeast display to map antibody epitopes at the domain level and at the individual amino acid level.

37.2 Materials

- pfu DNA Polymerase (Stratagene)
- Taq Polymerase (New England Biolabs (NEB))
- Geneclean Turbo (MP Biomedicals, LLC, Cat. 1102-600)
- Custom DNA primers (many vendors)
- Deoxynucleotide triphosphates (NEB)
- *Nco* I, *Not* I restriction enzymes (NEB)
- SV5 Antibody (Invitrogen)
- APC conjugated Fab-specific goat-anti-human $(Fab)_2$ (Jackson Immuno Research)
- Fab preparation kit (Pierce product number 44985)
- *S. cerevisiae* EBY100 (Invitrogen)
- 1 M Lithium acetate (LiAc)
- 0.1 M LiAc
- 2 mg/mL single stranded DNA (ssDNA)
- Polyethylene glycol (PEG) 3350 (50%)
- PCR machine
- 30°C incubator
- 42°C water bath
- Plasmid vector pYD2 as described (Razai et al. 2005)
- YPD medium (recipe: To 900 mL deionized H_2O add 10 g yeast extract and 20 g peptone. Optional: Add 17 g agar/L for plates. Autoclave, cool to 55–60°C. Add 100 ml of 20% dextrose (filter sterilized). For liquid media preparation filter sterilize (0.22 μm filter) after all components have been added and dissolved.)
- SD-CAA medium (recipe: To 900 mL deionized H_2O add: 7 g yeast nitrogen base w/o amino acid, 10.19 g Na_2HPO_4•$7H_2O$ or 5.4 g Na_2HPO_4, 8.56 g NaH_2PO_4• H_2O or 7.4 g NaH_2PO_4, and 5 g CAA (DIFCO) w/o tryptophan or Ura. After all components dissolve, add 100 ml of 20% dextrose and 10 ml of 0.6% (100X) leucine. Sterilize by filtering through 0.22 μm filter.

- SG-CAA medium (recipe: To 900 mL deionized H_2O add: 7 g yeast nitrogen base w/o amino acid, 10.19 g Na_2HPO_4•$7H_2O$ or 5.4 g Na_2HPO_4, 8.56 g NaH_2PO_4• H_2O or 7.4 g NaH_2PO_4, and 5 g CAA (DIFCO) w/o tryptophan or Ura. After all components dissolve, add 100 mL of 20% galactose and 10 mL of 0.6% (100X) leucine. Sterilize by filtering through 0.22 μm filter.
- 100 mm SD-CAA plates
- Alexa-647 labeling kit (Molecular Probes)
- Alexa-488 labeling kit (Molecular Probes)
- PE conjugated anti-human Fc specific antibody (Jackson Immunoresearch)
- FACS Buffer: to 1 L 1 × PBS add 5 g BSA (final 0.5%), 1 mL 1 M $MgCl_2$ (final 1 mM) and 0.5 mL 1 M $CaCl_2$ (final 0.5 mM), sterile filter.

37.2.1 Primers

Standard PYD2 primers: pair Gap 5 and Gap 3 or pair PYDF and PYDR are used for PCR, sequencing, and/or cloning. (Fig. 37.1).

- Gap repair 5′ (Gap5): 5′-TTAAGCTTCTGCAGGCTAGTG-3′
- Gap repair 3′ (Gap3): 5′- GAGACCGAGGAGAGGGTTAGG-3′
- pYDFor: 5′-AGTAACGTTTGTCAGTAATTGC-3′
- PYDRev: 5′- GTCGATTTTGTTACATCTACAC -3′

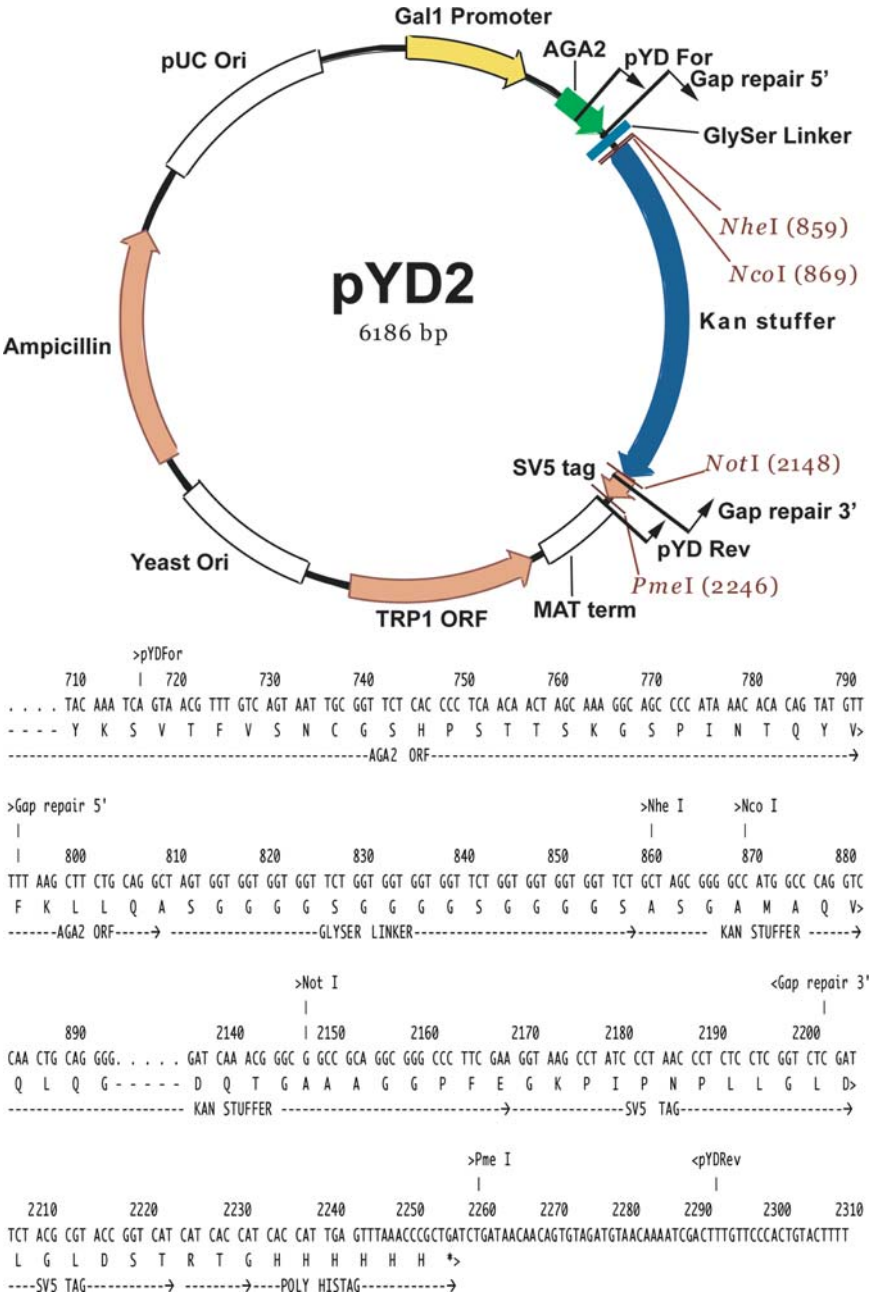
  Primers for cloning the target antigen gene by gap repair:
- Ag-Gap5 primer 5′-GTGGTTCTGCTAGCGGGGCCATGG-3----------3′
- Ag-Gap3 primer 5′-TTCGAAGGGCCCGCCT**GCGGCCGC**----------3′

The 5′ sequence of the primers Ag-Gap5 and Ag-Gap3 anneals to Nco1-Not1 digested pYD2 DNA for cloning by gap repair, the bolded sequences are the Nco1 and Not1 sites. The dashed sequence should include approximately 24 nucleotides that anneal to the 5′ and 3′ ends of the target antigen DNA.

37.3 Protocols

37.3.1 Cloning of Antigen Domains into Yeast Display Vector pYD2

Many full length receptor extracellular domains and other antigens can be successfully displayed on the surface of *S. cerevisiae*. When the full length protein does not display well, actual or putative domains deduced from either the three dimensional structure or structure prediction software can successfully be displayed (Cochran et al. 2004; Levy et al. 2007). Display of individual domains of multi-domian


Gal1 Promoter
AGA2
pYD For
Gap repair 5'
pUC Ori
GlySer Linker
NheI (859)
NcoI (869)
pYD2
6186 bp
Kan stuffer
Ampicillin
SV5 tag
NotI (2148)
Gap repair 3'
pYD Rev
PmeI (2246)
Yeast Ori
TRP1 ORF
MAT term
>pYDFor
710 720 730 740 750 760 770 780 790
. . . . TAC AAA TCA GTA ACG TTT GTC AGT AAT TGC GGT TCT CAC CCC TCA ACA ACT AGC AAA GGC AGC CCC ATA AAC ACA CAG TAT GTT
- - - - Y K S V T F V S N C G S H P S T T S K G S P I N T Q Y V>
----------AGA2 ORF----------
>Gap repair 5'
>Nhe I
>Nco I
800 810 820 830 840 850 860 870 880
TTT AAG CTT CTG CAG GCT AGT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GCT AGC GGG GCC ATG GCC CAG GTC
F K L L Q A S G G G G S G G G G S G G G G S A S G A M A Q V>
-------AGA2 ORF-----> ----------GLYSER LINKER----------> ---------- KAN STUFFER ------>
>Not I
<Gap repair 3'
890 2140 2150 2160 2170 2180 2190 2200
CAA CTG CAG GGG. . . . . GAT CAA ACG GGC G GCC GCA GGC GGG CCC TTC GAA GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT
Q L Q G - - - - - D Q T G A A A G G P F E G K P I P N P L L G L D>
---------- KAN STUFFER ----------> ----------SV5 TAG---------->
>Pme I
<pYDRev
2210 2220 2230 2240 2250 2260 2270 2280 2290 2300 2310
TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA GTTTAAACCCGCTGATCTGATAACAACAGTGTAGATGTAACAAAATCGACTTTGTTCCCACTGTACTTTT
L G L D S T R T G H H H H H H *>
----SV5 TAG----------> --------> ----POLY HISTAG---------->
Fig. 37.1 Map of yeast display vector pYD2. Antigen genes are cloned either as Nco-Not1 gene fragments or by gap repair after digestion of the vector with Nco1and Not1. Gal1 promoter = galactose promoter; Aga1=Aga1 gene; GS linker = gly-ser linker between Aga1 gene and gene of interest; Kan Stuffer = irrelevant gene sequence used to reduce cloning background, Nco I,

proteins also allows mapping of epitopes to the individual domains that they bind. For example, we have been able to map each of the three domains of botulinum neurotoxin type A (binding, translocation, or catalytic domain) that are recognized by a panel of monoclonal antibodies (Levy et al. 2007). For some neurotoxin antibodies, we have noted that their epitopes require the presence of two adjacent domains, which can also be successfully displayed on yeast (Levy et al. 2007).

We display antigens on the surface of *S. cerevisiae* as *C*-terminal fusion proteins to the AgaII cell surface protein (Boder and Wittrup 1997; Boder and Wittrup 2000; Levy et al. 2007). For antigen display, the cDNA encoding the full length antigen or antigen domain is amplified by PCR using primers that anneal to the 5′ and 3′ ends of the antigen cDNA and with 25 nucleotide overlaps with the yeast display vector pYD2 digested with the restriction enzymes NcoI and NotI. The PCR amplified antigen DNA fragment can then be cloned into NcoI-NotI-digested pYD2 vector by using gap repair (Orr-Weaver and Szostak 1983; Gietz and Schiestl 1991). Gap repair instead of digestion of the antigen cDNA fragment allows cloning of virtually all cDNA fragments including those with internal digestion sites.

37.3.1.1 PCR Amplification of Antigen Fragments

1. Make up 50 μL PCR reaction mixes containing

water	35.5 μL
20× dNTPs (5 mM each)	2.5 μL
10× pfu amplification buffer (Stratagene)	5.0 μL
Ag-Gap5 primer (10 pm/μL)	2.5 μL
Ag-Gap3 primer (10 pm/μL)	2.5 μL
Double stranded antigen gene template (10 ng)	1.0 μL
pfu DNA polymerase (Stratagene, 2.5 units)	1.0 μL

2. Heat to 94°C for 5 min in a PCR thermo-cycler.
3. Cycle 35 times to amplify the antigen genes at 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min. Times and temperatures may need adjustment depending on primer length and GC content of primer.
4. Purify the PCR amplified DNA on a 0.8% agarose gel. Extract the antigen fragment from the gel using GeneClean Turbo Kit.
5. Resuspend the antigen fragment in 20 μL of water. Determine DNA concentration by analysis on a 0.8% agarose gel with markers of known size and concentration.

Not 1= site of scFv gene cloning; TRP1 = tryptophan gene used for selection; yeast ori = yeast origin of replication; pUC ori = E. coli origin of replication. pYDFor and pYDRev are primers used for antigen gene amplification. Gap repair 5′ and 3′ are primers used for gap repair cloning of antigen genes or for sequencing

37.3.1.2 Preparation of Double Digested pYD2 Vector

1. Digest the pYD2 vector DNA with *Nco* I and *Not* I restriction enzymes in the manufacturer's NEB 3 buffer and 1× BSA solution, under conditions recommended by the manufacturer.
2. Purify the digested vector DNA on a 0.8% agarose gel. Extract the vector fragment from the gel using GeneClean Turbo Kit.
3. Resuspend purified product in 20 μL of water. Determine DNA concentration by analysis on a 0.8% agarose gel with markers of known size and concentration.
4. Mix approximately 100 ng of the amplified antigen DNA and 200 ng of NcoI-NotI digested vector pYD2 in 35 μL dH2O and keep on ice till added into TRAFO mixes (37.3.1.3).

37.3.1.3 Co-transform Yeast with Antigen Fragment and pYD2 Vector

1. Inoculate 2–5 mL of liquid YPD from a −80°C stock of EBY100 or EBY100 colonies on a plate and incubate with shaking overnight at 30°C
2. Measure OD_{600} of the overnight culture and inoculate 50–100 ml of warm YPD to a cell density of 5×10^6/ml culture (i.e., $OD_{600} = 0.25$). Incubate the culture at 30°C on a shaker at 250 rpm until it is equivalent to 4×10^7 cells/ml (i.e., OD_{600}=2). This may take 3–5 h. This culture will give sufficient cells for twenty transformations. Note that it is important to allow the cells to complete at least two divisions. Transformation efficiency (transformants/μg plasmid/10^8 cells) remains constant for 3–4 cell divisions.
3. Harvest the culture in a sterile 50 mL centrifuge tube at 3,000 × *g* (5,000 rpm) for 5 min.
4. Discard the supernatant, resuspend the cells in 25 mL of sterile water, and centrifuge again.
5. Discard the water, resuspend the cells in 1.0 mL 100 mM LiAc, and transfer the cell suspension to a 1.5 mL microfuge tube.
6. Pellet the cells at top speed for 15 s and remove the LiAc with a micropipette.
7. Resuspend the cells in about 400 μL of 100 mM LiAc to reach a final volume of 500 μL (2×10^9 cells/ml). Note that if the cell titer of the culture is greater than 2×10^9/cells ml, the volume of the LiAc should be increased to maintain the titer of this suspension at 2×10^9 cells/ml. If the titer of the culture is less than 2×10^9/cells ml, then decrease the amount of LiAc.
8. Boil a 1.0 ml of ss-DNA for 5 min. and quickly chill in ice water. Note that it is not necessary or desirable to boil the carrier DNA every time. Keep a small aliquot in your own freezer box and boil after 3–4 freeze-thaws. Keep ssDNA on ice.

9. Vortex the cell suspension and pipette 50 μL into labeled microfuge tubes. Pellet the cells and remove the LiAc with a micropipette.
10. Make up a 360 μL of transformation mixes (TRAFO mix) containing
    (a) 240 μL PEG (50% w/v)
    (b) 36 μL 1.0 M LiAc
    (c) 50 μL SS-DNA (2.0 mg/ml)
    (d) 34 μL DNA sample (0.1–10 μg)
11. Carefully add these ingredients to the yeast cell pellet in the order as listed above. Note that the order is important. The PEG should be added to the cells first, which shields the cells from the detrimental effects of the high concentration of LiAc.
12. Alternatively, premix the ingredients except the DNA, then add 326 μL of TRAFO mix on top of the cell pellet. Add 34 μL of DNA sample and mix well. Take care to deliver the correct volume as the TRAFO mix is viscous.
13. Vortex each tube vigorously until the cell pellet has been completely resuspended, which usually takes about 1 min.
14. Incubate at 30°C for 30 min.
15. Heat shock in a water bath at 42°C for 30 min.
16. Pellet cells by centrifugation at 6,000–8,000 rpm for 15 s and remove the transformation mix with a micropipette.
17. Pipette 1.0 ml of sterile water into each tube and resuspend the pellet by pipetting up and down gently. Note that we like to be as gentle as possible at this step to reach high transformation efficiency. Excessive washing is not recommended as the washes may reduce the transformant counts.
18. Transfer most of the cell suspension into SD-CAA media, plate different amounts of cells on 100 mm SD-CAA plates, and incubate for 2–4 days at 30°C. Note that when spreading yeast onto the plate, gently distribute the fluid completely with a sterile glass rod in smooth strokes. Allow the fluid to be taken up by the plate prior to incubation.

37.3.1.4 Screening for Transformants with Antigen Domains Incorporated

Transformed yeast colonies can be screened directly for successful cloning of antigen cDNA by amplifying using primers annealing to the pYD2 vector and flanking the cDNA gene. Presence of the correct insert sequence can be performed by sequencing the PCR amplified cDNA gene.

1. Pick individual colonies from a fresh plate of yeast culture with a pipette tip and resuspend in 10 μL of 0.1% SDS solution.
2. Votex vigorously for 5 min and pellet the cells by centrifugation for 5 min at 3,000 rpm. Transfer the supernatant into a new tube and keep on ice for PCR amplification.

3. Make up a 50 μL PCR reaction mix containing

water	39 μL
20× dNTPs (5 mM each)	1.5 μL
10× Taq polymerase buffer (Stratagene)	5.0 μL
pYDFor primer (10 pm/μL)	1.5 μL
pYDRev primer (10 pm/μL)	1.5 μL
DNA extract from yeast (from step 2)	1.0 μL
Taq DNA polymerase (Invitrogen, 2.5 units)	0.5 μL

4. Heat to 94°C for 5 min in a PCR thermo-cycler.
5. Cycle 35 times to amplify the antigen genes at 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min.
6. Analyze the PCR amplified DNA on a 0.8% agarose gel with markers of known size. Extract the DNA band of correct size from the gel using GeneClean Turbo Kit.
7. Resuspend the DNA fragment in 20 μL of water. Analyze the DNA sequence using commercially available service. Use primers Gap3 and Gap5, which remain inside the pYDFor and Rev primers for sequencing.

37.3.2 Display of Antigen Domains on Yeast Surface

After cloning of the antigen into the pYD2 vector, the transformed yeast cells are induced for display of the antigen on the yeast surface by switching the media from glucose containing SD-CAA to galactose containing SG-CAA. The surface display of antigen can be quantitated by flow cytometry after staining with an antibody to the *C*-terminal SV5 epitope tag.

37.3.2.1 Induction of Antigen Surface Display

1. Single colonies of transformed EBY100 are inoculated into 5 mL of SD-CAA medium and grown overnight at 30°C with shaking at 250 rpm.
2. Measure the OD_{600} of the overnight cultures, transfer 10^8 cells into a centrifuge tube, pellet the cells, resuspend in 5 mL of SG-CAA medium, and grow for 24–48 h at 18°C with shaking at 250 rpm.
3. Transfer 10^6 cells to a 1.5 mL microfuge tube, pellet cells by centrifugation at top speed for 15 s, and discard the supernatant.
4. Wash cells once by resuspending in 1 mL of FACS buffer, followed by centrifugation, aspiration of the supernatant, and resuspension in 1 mL of FACS buffer.
5. Aliquot 50 μL of the cell suspension containing 5×10^5 cells into 96-well V-bottom plates.
6. Add 50 μL of 1 μg/mL Alexa-647 labeled anti-SV5 IgG diluted in FACS buffer.

7. Incubate for 1 h at 4°C with rocking. Alexa-647 labeled anti-SV5 IgG should be previously prepared using SV5 antibody and the Alexa-647 labeling kit following the instructions provided by the manufacturer.
8. Wash cells twice with 200 μL of FACS buffer and resuspend in 150 μL of FACS buffer.
9. Measure cell fluorescence in a FACS LSRII flow cytometer or other comparable cytometer using the APC channel.
   Note that due to displayed protein partition, at best 50% of yeast will display antigen.

37.3.2.2 Evaluation of Whether an Antibody Recognizes the Yeast Displayed Antigen or Antigen Domain

1. Transfer 10^6 yeast cells to a 1.5 mL microfuge tube, and pellet cells by centrifugation at top speed for 15 s Discard the supernatant.
2. Wash cells once by resuspending in 1 mL of FACS buffer, followed by centrifugation, resuspend in 1 mL of flow cytometry buffer.
3. Aliquot 50 μL of the cell suspension containing 5×10^5 cells into 96-well V-bottom plates.
4. Add 50 μL of antibody known (or hypothesized) to bind the displayed antigen, and diluted in FACS buffer. In this example, the antibody is a human IgG antibody. Antibodies of other species and isotype would require use of a different secondary antibody (below). Incubate for 1 h at 4°C with rocking. Note that single chain Fv (scFv) and phage antibodies can also be used to stain yeast displayed antigens. In these instances, binding is detected using secondary antibodies to *C*-terminal epitope tags for scFv and anti-phage antibody for phage antibodies.
5. Wash cells twice with 200 μL of FACS buffer.
6. Resuspend cells in 100 μL of FACS buffer containing 1 μg/ml of PE-labeled anti-human Fc specific antibody and 1 μg/ml of Alexa-647 labeled anti-SV5 IgG. Incubate for 1 h at 4°C.
7. Wash cells twice with 200 μL of FACS buffer and resuspend in 150 μL of FACS buffer.
8. Measure cell fluorescence in a FACS LSRII flow cytometer or other comparable cytometer using the PE and APC channels. Note that due to displayed protein partition, at best 50% of yeast will display antigen. This population can be gated for APC staining (antigen display) and should also be the population with PE staining (antigen binding by antibody).

37.3.3 Fine Epitope Mapping Using Yeast Displayed Antigen

To identify an antibody's epitope at the level of energetically critical amino acid contacts between an antibody and its antigen, a second approach is taken. The gene

encoding the yeast displayed antigen is randomly mutagenized and a yeast displayed library constructed. The mutated domain library is then selected for loss of binding to the mAb bearing the epitope mapped. After several rounds of selection, individual clones are sequenced to analyze the location of mutations leading to loss of binding. These are then modeled on a three dimensional structure of the antigen looking for regions where the mutations cluster. Single yeast displayed alanine mutants of the putative epitope site are constructed and antibody binding determined in order to identify the epitope. By measuring the affinity of antibody for the alanine mutants compared to the wild type yeast displayed antigen, it is possiible to determine the energetic contribution of the amino acid side chain to antibody binding.

37.3.3.1 Mutant Library Construction

Random mutations are introduced into the antigen gene using error prone PCR.

1. Make up 50 μL PCR reaction mixes containing the following: Heat to 94°C for 5 min in a PCR thermo-cycler. The number of reactions should be set up to yield 20 μg of antigen DNA after gel purification.

Water	34.0 μL
20× dNTPs (5 mM each)	2.0 μL
10× Taq Buffer	5.0 μL
pYDFor (10 pm/μL)	2.0 μL
pYDRev (10 pm/μL)	2.0 μL
Antigen gene in pYD2 vector (10 ng)	2.0 μL
Taq DNA polymerase (NEB, 2.5 units)	0.5 μL
$MnCl_2$ (10 mM)	2.5 μL

2. Cycle 20 times to amplify the antigen gene at 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min. Finally, cycle at 72°C for 10 min.
3. Purify the PCR amplified DNA (20 μg) on a 0.8% agarose gel. Extract the antigen fragment from the gel using GeneClean Turbo Kit.
4. Gel purify double digested (NcoI and NotI) pYD2 plasmid, as described in Sect. 37.3.1, above; 20 μg of purified vector is required.
5. Transform 1×10^9 (10 × transformation reactions) LiAc-treated EBY 100 cells using 20 μg of antigen DNA and 20 μg of vector DNA as described in Sect. 37.3.1.3, above.
6. After transformation, dilute transformation mixtures to 0.25 OD_{600} /mL of SD-CAA media. Plate 1:10, 1:100, and 1:1000 dilutions of the culture on SD-CAA plates to estimate library size and characterize individual clones for insert size and DNA sequence.
7. Grow the remaining yeast in SD-CAA media at 30°C with shaking at 250 rpm for approximately 36 h until the $O.D._{600}$ is approximately 8.
8. Subculture the library by diluting in SD-CAA media to an OD_{600} of 0.5 at the same volune as the original culture.

9. Grow the subculture at 30°C for 24 h or until an OD_{600} of approximately 8.
10. Centrifuge the subculture for 5 min at 3,000 rpm and aspirate the supernatant. The library can be stored for long term at −80°C by resuspending the yeast in 1 mL aliquots in SD-CAA media containing 15% glycerol at an OD_{600} of 30. An aliquot of the centrifuged yeast library can be induced for use in subsequent sorting for epitope identification.
11. To induce yeast surface display of antigen, take an aliquot of yeast at least ten times larger than the library size (see below) and resuspend in SG-CAA media to an $O.D._{600}$ of 1 OD/mL. Induce antigen surface display by growing the culture at 24–48 h with shaking at 250 rpm at 18°C.
12. Before preceding, estimate the library size by counting the number of colonies on the SG-CAA plates. Determine the frequency and diversity of the antigen inserts by PCR amplifying and sequencing the antigen gene from 10–20 individual colonies on the SD-CAA plates (see Sect. 37.3.1.4).

37.3.3.2 Selection of Mutated Antigen Library for Loss of Antibody Binding

Inorder to identify the antibody fine epitope, the randomly mutated antigen library is selected for loss of antibody binding by flow cytometry. A number of approaches can be used (Chao et al. 2004; Oliphant et al. 2005; Levy et al. 2007). Like Oliphant et al., we use an approach where yeast cells are stained with two antibodies binding non-overlapping conformational epitopes, one of which is being mapped and one of which is not. Yeast-displayed antigen mutants are selected for loss of binding to the first antibody, but retention of binding to the second antibody. Retention of binding of the second mAb ensured that mutants were selected on the basis of mutations in the epitope, rather than selection of unfolded or poorly displayed mutants. This is the method that is described below. This method can be further refined by also staining for SV5 epitope tag expression, as well as the binding of two mAbs. This allows separation of well expressed mutants from mutants with *C*-terminal truncations due to the random insertion of stop codons. Analysis of such mutants helped identify the minimal binding fragment and allowed simplification of mutant analysis. This modification is decribed in Levy et al. (Levy et al. 2007), but is not detailed below.

1. After induction wash and resuspend cells in ice-cold FACS buffer, as described before. In order to ensure that the entire library diversity is present, the number of cells stained should be at least ten times greater than the library size, or ten times greater than the sort output from the previous round of sorting.
2. For staining, follow protocol 37.3.2.2. For the first round of sorting, yeast cells are co-stained with two Alexa-647 labeled monoclonal IgG antibodies binding non-overlapping epitopes on the antigen. Antibodies are labeled using kits from Molecular probes and used at a final concentration of 1 μg/ml. Yeast cells are also stained with SV5 monoclonal antibody labeled with Alexa-488, at a 1:200 dilution (v/v). Incubate yeast at 4°C, with shaking, 2 h. Wash and

resuspend cells in 100 μL to 1 mL of FACS buffer to achieve a final concentration of aproximately 2×10^8 cells/mL.

3. Sort the stained yeast library by gating the Alexa-647 positive cells (APC gate) (bound by either Alexa-647-antigen binding mAbs). All cells should be collected, regardless of expression level (SV5 staining).
4. Grow and induce the sort output yeast by suspending the yeast in 5 mL of SD-CAA media and following protocol 37.3.2.1.
5. For the second round of sorting, wash yeast and stain with the two antigen binding non-overlapping IgGs labeled with different flourophores, one labeled with Alexa-647 and the other labeled with Alexa-488, both at 1:200 dilutions. Incubate for 2 h at 4°C. Wash with FACS buffer and resuspend cells in 100 μL to 1 mL of FACS buffer. Set sort gates to select yeast bound by one of the IgG (either APC positive or FITC positive) but not by the other IgG (APC negative or FITC negative). Each of these populations can be sorted simultaneously, allowing the simulataneous mapping of the fine epitopes of two antibodies.
6. Grow and induce the sort output yeast by suspending the yeast from sort output in 5 mL of SD-CAA media and following protocol 37.3.2.1.
7. Perform a third round of selection exactly like the second round.
8. Plate the third round sort output on 150 mm SD-CAA plates and incubate at 30°C for 36–72 h to obtain well separated colonies.
9. Streak 50 well separated clones, form each plate of the two outputs, and grow plates at 30°C, for 48–72 h.
10. Grow and induce each of the streaked out colonies as described in protocol 37.3.2.1 and analyze for binding by each of the two antigen binding mAbs as described in protocol 37.3.2.2 to verify that the clone no longer binds the antibody being mapped but is still bound by the other antibody.
11. Sequence the antigen gene from each of the 50 clones as described in protocol 37.3.1.4.
12. Analyze the location of mutations by modeling onto the three dimensional structure of the antigen. Putative epitopes will be those locations on the antigen where there are mutations in more than one of the sequenced clones. In the absence of a three dimensional structure, this analysis can be challenging.

37.3.3.3 Alanine Scanning

To verify the fine epitope of the antibody, single alanine mutations are made at each amino acid hypothesized to be in the epitope based on analysis of clones from the random mutant library.

1. For each position to be mutated, design a complementary set of primers, approximately 45 nucleotides long, (forward and reverse primers) which generate a single alanine mutant in the middle of the primer at each desired position.
2. Amplify the 5′portion of the antigen gene, by using PCR and protocol 37.3.1.1, using the primers pYDFor and the specific designed reverse primer which

introduces the alanine mutation. Similarly, amplify the 3′portion of the antigen gene and use the primers pYDRev and the specific designed forward primer which introduces the alanine mutation.
3. Gel purify both products as described in protocol 37.3.1.1.
4. Prepare NcoI-NotI digested pYD2 as described in protocol 37.3.1.2.
5. Co-transform yeast with the two PCR products and the digested pYD2 vector as deseribed in protocol 37.3.1.3. This results in a three fragment gap repair where the two PCR products are combined into the vector to recreate the antigen gene with a single alanine mutation at the desired location.
6. Confirm the location of the mutation by DNA sequencing by following protocol 37.3.1.4.
7. Grow and induce each yeast displayed alanine mutant by following protocol 37.3.2.1, steps 1 and 2.

37.3.3.4 Measurement of the Impact of Individual Antigen Amino Acids on Antibody Binding

The energetic impact of individual antigen amino acid side chains contained within the epitope on antibody binding can be determined by commparing the affinity of antibody for wild-type antigen and for an alanine mutant of that antigen. Once the two affinties are known, they can be used to calculate the change of free energy between the Ala mutants relative to the wild type (wt). The following standard formula is used for each mutated residue:

$$\Delta\Delta G_{mut-wt} = RT \ln(K_{Dmut}/K_{Dwt})$$

These calculations provide a measure of the energetic contribution of each one of the alanine substituted amino acid residues, for the mAb, therefore indicating the position of its functional epitope. For measuring the dissociation equilibrium constants, we use the Fab prepared from the IgG. We use Fab as it is monovalent, compared to the bivalent IgG. This eliminates the avidity effect that can occur with bivalent binding which can lead to falsely high (and inaccurate) affinities (Levy et al. 2007).

1. Prepare Fab from IgG by enzymatic digestion with immobilized papain using the Pierce Fab preparation kit.
2. Grow and induce EBY-100 yeast harboring the PYD2-wt or the alanine mutant as previously described in protocl 37.3.2.1, steps 1 and 2.
3. Transfer 10^6 yeast cells to a 1.5 mL microfuge tube, and pellet cells by centrifugation at top speed for 15 s Discard the supernatant.
4. Wash yeast once by resuspending in 1 mL of FACS buffer, followed by centrifugation, and resuspend in 1 mL of FACS buffer.
5. Aliquot 100 μL of the cell suspension containing 1×10^5 cells into 96-well V-bottom plates.

6. Add 50 μL of Fab to each well at decreasing concentration. Fab concentrations should be such that the K_D would be spanned by at least tenfold. Incubation volumes are chosen to ensure that a tenfold molar excess of the ligand over the displayed domain (wt or Ala mutant) would be maintained. Consider 10^5 as the number of copies of displayed protein on the cell surface of yeast. This may require adjustment of the incubation volumes upward, especailly at very low Fab concentrations.
7. Incubate at room temperature at 4 °C, with rocking for 2 h.
8. Wash cells, pellet cells by centrifugation at top speed for 15 s Discard the supernatant and resuspend in a solution of FACS buffer with the secondary antibodies; APC conjugated Fab-specific goat-anti-human $(Fab)_2$ plus SV5-Alexa-488, at 1:200 dilution. Incubate at 4°C, for 30 min.
9. Wash and resuspend in 200 μL FACS buffer. Measure mean cell fluorescent intensity (MFI) in a FACS LSRII flow cytometer or other comparable cytometer using the APC and FITC channels. Measure the mean fluorescence intensity of the antigen displaying yeast population (SV5 positive).
10. K_D is calculated from the different MFI as a function of Fab concentration (Boder and Wittrup 2000).

References

Benichou S, Inchauspe G (1996) Random fragment libraries using yeast expression plasmid. Methods Mol Biol 66:241–255

Boder ET, Wittrup KD (1997) Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol 15(6):553–557

Boder ET, Wittrup KD (2000) Yeast surface display for directed evolution of protein expression, affinity, and stability. Methods Enzymol 328:430–444

Chao G, Cochran JR, Wittrup KD (2004) Fine epitope mapping of anti-epidermal growth factor receptor antibodies through random mutagenesis and yeast surface display. J Mol Biol 342 (2):539–550

Christmann A, Wentzel A, Meyer C, Meyers G, Kolmar H (2001) Epitope mapping and affinity purification of monospecific antibodies by Escherichia coli cell surface display of gene-derived random peptide libraries. J Immunol Methods 257(1–2):163–173

Cochran JR, Kim YS, Olsen MJ, Bhandari R, Wittrup KD (2004) Domain-level antibody epitope mapping through yeast surface display of epidermal growth factor receptor fragments. J Immunol Methods 287(1–2):147–158

Cunningham BC, Wells JA (1989) High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. Science 244(4908):1081–1085

Frank R, Overwin H (1996) SPOT synthesis. Epitope analysis with arrays of synthetic peptides prepared on cellulose membranes. Methods Mol Biol 66:149–169

Gietz RD, Schiestl RH (1991) Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. Yeast 7(3):253–263

Levy R, Forsyth CM, LaPorte SL, Geren IN, Smith LA, Marks JD (2007) Fine and domain-level epitope mapping of botulinum neurotoxin type A neutralizing antibodies by yeast surface display. J Mol Biol 365(1):196–210

Mehra V, Sweetser D, Young RA (1986) Efficient mapping of protein antigenic determinants. Proc Natl Acad Sci USA 83(18):7013–7017

Mullaney BP, Pallavicini MG, Marks JD (2001) Epitope mapping of neutralizing botulinum neurotoxin A antibodies by phage display. Infect Immun 69(10):6511–6514

Oliphant T, Engle M, Nybakken GE, Doane C, Johnson S, Huang L, Gorlatov S, Mehlhop E, Marri A, Chung KM, Ebel GD, Kramer LD, Fremont DH, Diamond MS (2005) Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nat Med 11(5):522–530

Orr-Weaver TL, Szostak JW (1983) Yeast recombination: the association between double-strand gap repair and crossing-over. Proc Natl Acad Sci USA 80(14):4417–4421

Razai A, Garcia-Rodriguez C, Lou J, Geren IN, Forsyth CM, Robles Y, Tsai R, Smith TJ, Smith LA, Siegel RW, Feldhaus M, Marks JD (2005) Molecular evolution of antibody affinity for sensitive detection of botulinum neurotoxin type A. J Mol Biol 351(1):158–169

Vajdos FF, Adams CW, Breece TN, Presta LG, de Vos AM, Sidhu SS (2002) Comprehensive functional maps of the antigen-binding site of an anti-ErbB2 antibody obtained with shotgun scanning mutagenesis. J Mol Biol 320(2):415–428

Weiss GA, Watanabe CK, Zhong A, Goddard A, Sidhu SS (2000) Rapid mapping of protein functional epitopes by combinatorial alanine scanning. Proc Natl Acad Sci USA 97 (16):8950–8954

Chapter 38
Size Exclusion Chromatography

Bernd Voedisch and Holger Thie

38.1 Introduction

Biomolecules can be separated by different chromatographic techniques, based on various molecular specific properties, such as biorecognition, charge, hydrophobicity, and size.

The size exclusion chromatography (SEC) (Lathe and Ruthven, 1955), also known as gel filtration (Porath and Flodin, 1959) or gel permeation chromatography (Moore, 1964), separates molecules on the basis of differences in hydrodynamic volume. The separation depends on different permeation properties of the molecules through a porous carrier matrix (i.e., spherical gel particles) with distinct pore sizes. While carried through the matrix bed by the buffer flow, small molecules move in and out of the pores of the matrix by diffusion. This results in more retention when the molecules are smaller. SEC is a mild and robust technique that is able to handle biomolecules in physiological conditions in the presence of cofactors, ions, or detergents or at different temperatures. It is used to separate noncovalent multimeric protein complexes from monomers or to separate low molecular substances, e.g., for desalting or buffer exchange. Another possible application of SEC is the estimation of the molecular mass of protein samples. Here, a calibration of the column with proteins of known molecular mass is necessary. In contrast to SDS-PAGE or mass spectrometry, it is preferred, despite its lower resolution, if noncovalent complexes have to be kept intact.

H. Thie (✉)
Technische Universität Braunschweig, Institut für Biochemie und Biotechnologie, Abteilung Biotechnologie, Spielmannstr. 7, 38106 Braunschweig, Germany
e-mail: h.thie@tu-bs.de

B. Voedisch
Technische Universität Braunschweig, Institut für Biochemie und Biotechnologie, Abteilung Biotechnologie, Spielmannstr. 7, 38106 Braunschweig, Germany
Present address: Novartis Institutes for Biomedical Research, NBC-PA, Novartis Pharma AG, Basel, Switzerland

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_38,

SEC is a powerful tool for preparative separation of multimeric protein complexes or structures under biological conditions. A multitude of SEC columns are commercially available on the market, allowing separations in a range from 500 to several hundred thousand Da.

SEC is utilized in the field of antibodies to determine, for example, the correct assembly of IgG antibodies. Another example is the analysis of antibody fragments, especially scFv fragments, which sometimes tend to form multimeric structures by themselves. The formation of either dimers or multimers or aggregates depends on the specific antibody fragment, the linker design (Schmiedl et al. 2006), and the concentration of the antibody fragments (Arndt et al. 1998). The tendency of scFv antibody fragments to form aggregates is also an indicator for their stability, because high aggregation levels lead to lower long-term stability of the scFvs. By using SEC, antibody fragments can be quickly validated for their aggregation tendency, so it became a standard technique to determine the properties of antibody fragments or improved engineered antibody fragments.

38.2 Outline

The following method describes the analysis of scFv antibody fragments after purification concerning their aggregation properties. A representative exemplary chromatogram will be shown in the results section.

38.3 Materials

- Filtration device
- Filter membrane (pore size, 0.2 μm)
- Syringe filters (pore size 0.2 μm)
- HPLC or FPLC device with pump, tubing, sample loops, valves, monitors (UV, conductivity), and recorder (e.g., ÄKTA Purifier)
- Gel filtration column (e.g., Superdex matrix series provided by GE Healthcare)
- Protein molecular weight standards (e.g., "Gel Filtration Calibration Kit," GE Healthcare)
- Running buffer (e.g., PBS)
- Ethanol (20% v/v)
- Deionized water

38.4 Procedure

1. Choose a gel filtration column that is suitable for your application (e.g., Superdex200 for scFv or Fab fragments). Different matrices have different

properties in respect of resolution, pH and pressure stability, and unspecific adsorption of protein. Refer to the column manufacturer's manual for details.

2. Prepare and degas all necessary buffers (e.g., PBS as running buffer, EtOH (20 % v/v) for storage).
   Note: Filter all buffers and samples through a 0.2 μm membrane. For aqueous solutions, this is sufficient to degas the buffers. Equilibrate all buffers and samples to the temperature used for the chromatography run.
   The amount of buffer needed depends on the column volume:
   (a) For each run at least one column volume of running buffer is needed. To equilibrate the gel column calculate three column volumes of buffer.
   (b) For storing the column after the final run another three column volumes of deionized water and three column volumes of ethanol are used.
   Keep in mind that the tubing of the chromatography system has to be immersed in suitable buffer as well, so prepare some surplus volume for each buffer and solution.
3. For analytical gel filtration runs, sample buffer and running buffer should be identical. The sample protein has to be applied to the column in a minimal volume. Refer to the manufacturer's recommendation of what is the optimal and the maximal sample volume to be applied to a column.
   Note: In order to get a reliable readout, the amount of protein loaded onto the column should be in the low microgram range at least. However, test several sample concentrations to avoid precipitation.
4. If the column is stored in 20% ethanol, first wash out the ethanol with 1.5–3 column volumes degassed, deionized water before equilibrating the column with running buffer.
5. Immerse the FPLC system tubing and equilibrate the column with degassed running buffer using 1.5–3 times the column volume. Equilibration is complete when the conductivity signal (near zero during washout of ethanol and water) stabilizes at an increased value. Depending on the column volume and the flow rate, this process might take several hours and can be done over night in an automated system (e.g., ÄKTA Purifier).
   Note: The equilibration step can be done with the fastest flow rate possible without exceeding the maximum backpressure.
6. Before starting analytical runs, calibrate the column using standard proteins (e.g., from "Gel Filtration Calibration Kit," GE Healthcare). Plot the elution volume vs. the logarithm of the molecular mass of the protein which should result in a linear relation (see Fig. 38.2).
   Note: Do a separate run for each standard protein, do not mix standard proteins.
7. Apply the sample to the column using a sample loop. Elute the sample using a constant flow rate (isocratic flow conditions).
   Note: Whenever possible during analytical runs, use the flow rate that was used when calibrating the column. Refer to the column manufacturer's recommendation on optimal and maximal flow rates.
8. If required, elute the sample in fractions.

Note: By a rule of thumb, no protein will elute from the column before reaching an elution volume of one third the column volume.

9. After the last run, the system column should be washed with 1.5–3 column volumes of water and the same amount of 20% ethanol for storage.

38.5 Results

The peak position gives the size of the proteins in the elution fraction by comparing the elution volume with that from the standard proteins (Fig. 38.1).

The peak area is proportional to the amount of protein. Compare the peak area for the peak at the expected elution volume for the scFv antibody fragment with the area of peaks at lower elution volumes to determine the ratio of monomeric, dimeric, and multimeric protein (Fig. 38.2).

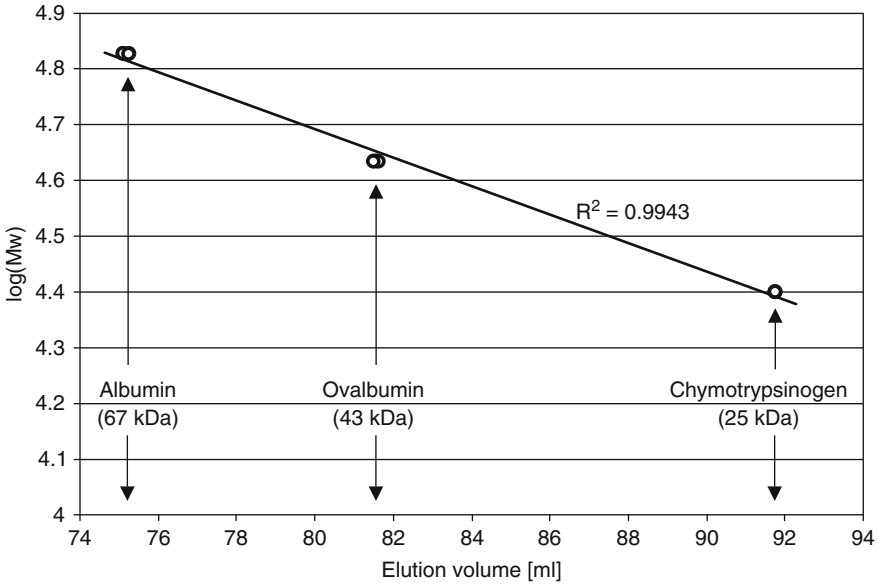


Fig. 38.1 Calibration of a gel filtration column (HiLoad16/60 column, Superdex200 matrix, GE Healthcare) with a separation range of 10,000–500,000 Da and a column volume of 123 ml. Three standard proteins (albumin, ovalbumin and chymotrypsinogen) were separated in independent runs two times each. The logarithm of their molecular weight was plotted against the elution volume. The data points should lie on a straight best fit line. If not, the column either has a separation range not suited or the gel-bed is not correctly packed and the column should be repacked or discarded

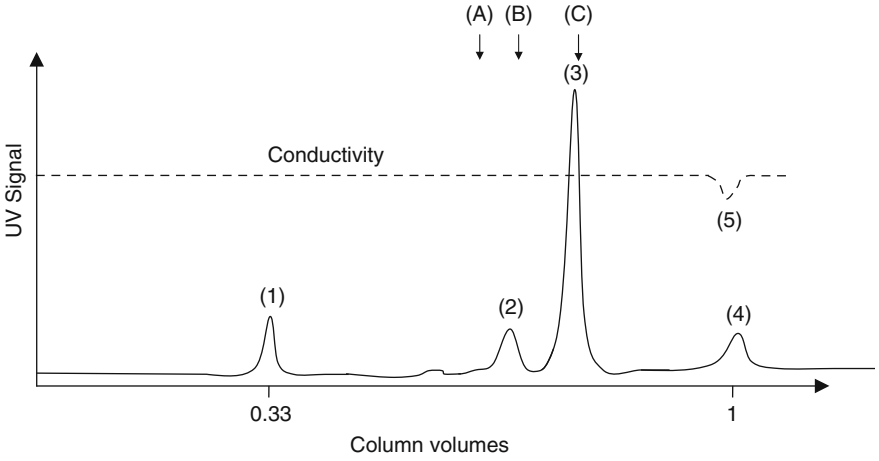


Fig. 38.2 Exemplified chromatogram of a separation of a purified scFv antibody fragment sample on a gel filtration column (HiLoad16/60 column, Superdex200 matrix, GE Healthcare) with a separation range of 10,000–500,000 Da, a column volume of 123 ml and a flow rate of 0.5 mL/min. Large aggregates elute from the column at about 0.33 column volumes (1). Dimers (2) and monomers (3) are well resolved. After one column volume, small fragments, UV active substances from the sample buffer and peptides elute from the column (4), indicating the end of analysis. If sample buffer and running buffer are not of the same composition the conductivity signal may show a change at one column volume (5). Peak locations of calibration proteins are indicated by arrows (A: Albumin, 67 kDa, B: Ovalbumin, 43 kDa, C: Chymotrypsinogen, 25 kDa)

38.6 Troubleshooting

1. The retention of molecules does not only depend on the size but also on the shape of the molecule and the amount of water bound to it (hydrodynamic volume). Long, fibrous proteins can show a different retention (appear larger) than globular proteins of the same mass. A direct correlation between the hydrodynamic volume, molecular mass, and retention is found only for optimal globular proteins. This has to be kept in mind when using protein standards for calibration. Test the column and chromatography system with BSA in the buffer and under the conditions (e.g., temperature, flow rate) you want to use for your analytical runs. If no problems occur, continue with the calibration runs. After that do your analytical runs.
2. Carefully check the conditions (e.g., pH, pressure) allowed for every particular column material. Never let the column run dry because repacking of the gel bed is difficult – the performance of the column will suffer most likely. Air bubbles detected upstream of the column material have to be removed by washes with degassed buffer before any run (if necessary, change the flow direction).
3. The column should be cleaned in place regularly and especially if you suspect precipitation of proteins on the column. Refer to the column manufacturer's

recommendations on what cleaning solutions are suitable for the column (e.g., 0.5 M NaOH, detergents).
4. The calibration should be tested for validity regularly.
5. Slower flow rates will result in a sharper elution profile. However, this might lengthen a run considerably.
6. Short and thin tubing used in the chromatography system gives sharper elution profiles. As soon as any tubing, valves or pumps in the FPLC device are changed, a new calibration curve must be recorded.
7. Especially when working at low temperatures (e.g., 4°C in a cooling cabinet), the flow rate probably has to be reduced to prevent problems with backpressure.
8. Ideally, running buffer and sample buffer are identical. Exchange the sample buffer prior to the chromatography run (e.g., by dialysis). However, that is not always necessary if sample buffer and running buffer are similar or the protein is not affected by the buffer exchange (i.e., no precipitation occurs). During the SEC, running buffer exchange is facilitated anyway as the buffer of the elution fractions will be running buffer. Elution fractions from immobilized metal ion afinity chromatography (IMAC) where a phosphate buffer containing imidazole or EDTA was used for elution usually can directly be used in a SEC run where PBS serves as running buffer.
9. Non-symmetrical elution peaks may result from protein overload (reduce sample volume or sample concentration) or too high pressure on the column (backpressure too high, gel matrix particles are deformed; reduce flow rate).

References

Arndt KM, Müller KM, Plückthun A (1998) Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. Biochemistry 15(37):12918–12926

Lathe GH, Ruthven CR (1955) The separation of substances on the basis of their molecular weights, using columns of starch and water. Biochem J 60(4):xxxiv

Moore JC (1964) Gel permeation chromatography. I. A new method for molecular weight distribution of high polymers, by J. C. Moore. J Polym Sci A 2:835

Porath J, Flodin P (1959) Gel filtration: a method for desalting and group separation. Nature 183:1657–1659

Schmiedl A, Zimmermann J, Scherberich JE, Fischer P, Dübel S (2006) Recombinant variants of antibody 138H11 against human gamma-glutamyltransferase for targeting renal cell carcinoma. Human antibodies 15:81–94

Gel Filtration Principles and Methods (GE Healthcare) 18-1022-18 Edition AI

Chapter 39
Structural Characterization of Antibodies by Mass Spectrometry

Marie-Claire Janin-Bussat, Jean-Marc Strub, Elsa Wagner-Rousset, Olivier Colas, Christine Klinguer-Hamour, Nathalie Corvaia, Alain van Dorsselaer, and Alain Beck

39.1 Introduction

G-type Immunoglobulins (IgGs) are large tetrameric glycoproteins (150 kDa range) composed of two light and two heavy chains. These H2L2 heterodimers are connected by multiple interchain disulfide bonds, their number varying and depending on the isotype. Among all analytical methods used to characterize monoclonal antibodies (mAbs), mass spectrometry (MS) plays an increasingly important role for both global and fine structural characterization of therapeutic candidates, and many micro-variants have been described; Fig. 39.1 (Srebalus Barnes and Lim 2007, Liu et al. 2008a, Zhang et al. 2009). Recent developments in MS, including liquid chromatography – electrospray ionization – time-of-flight (LC-ESI-TOF) (Dillon et al. 2004, Dillon et al. 2006, Gadgil et al. 2006b), matrix-assisted-laser desorption/ionization time-of-flight (MALDI-TOF), tandem MALDIs (MALDI-TOF/TOF), and liquid chromatography ion trap mass spectrometry (LC-IT-MS) (Le and Bondarenko 2005), show that each technique is complementary for primary structure assessment, glycosylation and production-system fingerprinting, IgG structural isotyping by disulfide pairing determination, and hot-spot mapping (Beck et al. 2005, Beck et al. 2008a).

M.C. Janin-Bussat, E. Wagner-Rousset, O. Colas, C. Klinguer-Hamour, N. Corvaia, and A. Beck (✉)
Centre d'Immunologie Pierre Fabre (CIPF), 5 Avenue Napoléon III, F74160 Saint-Julien-en-Genevois, France
e-mail: alain.beck@pierre-fabre.com

A. van Dorsselaer and J.-M. Strub
Laboratoire de Spectrométrie de Masse Biologique (LSMBO), Université de Strasbourg, 67000 Strasbourg, France

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_39,

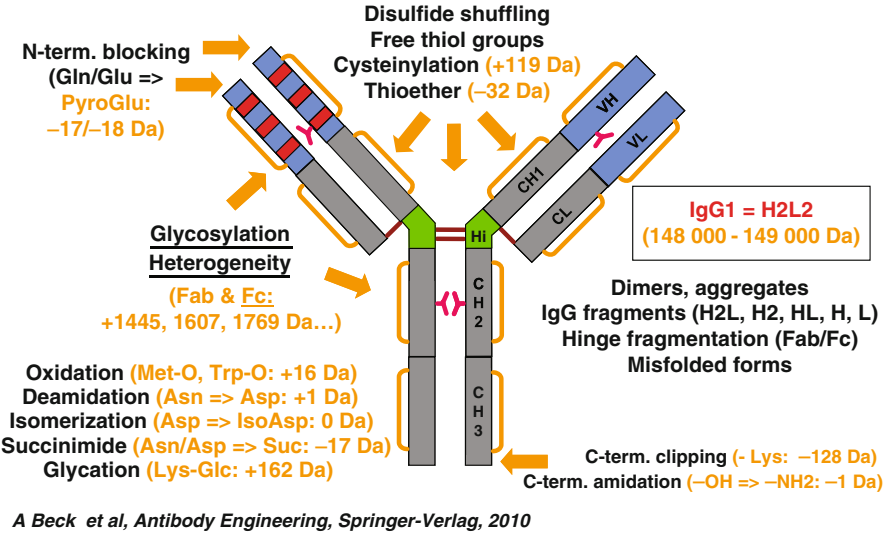


Fig. 39.1 Reported huIgG structure heterogeneity

39.1.1 Intact Antibody and Light and Heavy Chain Primary Structure Characterization by MS

All IgGs show common posttranslational modifications (Yan et al. 2007): light and heavy chains pyroglutamic acid cyclization for mAbs containing *N*-terminal glutamine (Yu et al. 2006, Dick et al. 2007) or glutamic acid residues (Chelius et al. 2006b) heavy chains CH_2 domain N-glycosylation, and *C*-terminal lysine processing; Fig. 39.1. All these microvariants can be characterized by liquid chromatography, electrophoretic, and mass-spectrometry based methods (Beck et al. 2005, Rehder et al. 2006, Wang et al. 2005, Wagner-Rousset et al. 2008). Interestingly, with the last generation of LC-ES-TOF mass spectrometers, the mass of intact antibodies can be measured with a precision reaching 40 ppm (+/− 6 Da of total mass), which speeds-up considerably the screening and routine mAbs identification. Moreover, the current resolution of mass spectra allows also investigating the non-symmetry of N-linked biantennary oligosaccharides between the two heavy chains. This analysis was not feasible up to now with classical carbohydrate analytical methods used after enzymatic glycans release. These new types of information may contribute to the better knowledge of Fc gamma receptor binding structure relationships. On the other hand, MALDI-TOF mass measurements are less accurate for the intact H2L2 antibody but give direct information on the light chain mass and on the H2L fragment for example. After disulfide bridge reduction, very acute data can be recorded for both light (25 kDa range) and heavy chains (50 kDa range). In addition, the efficacy of mass spectrometry over classical

electrophoresis and liquid-chromatography methods was also recently demonstrated for posttranslational modifications that were not previously reported for mAbs (Beck et al. 2007, Ying and Liu 2007).

39.1.2 Glycosylation and Production-System Fingerprinting by MS

Glycans represent only an average of 2% of the total mass of IgGs and are located generally on Asn^{297} in the Fc domain. Despite this low percentage, particular glycoforms are involved in important immune effector functions (e.g., ADCC, CDC) (Beck et al. 2008a). Glycosylation plays also an essential role in the long plasmatic half-life via binding to neonatal FcR and resistance to proteolytic cleavage (Raju and Scallon 2006). A second N-glycosylation site may also be present in the variable domain of IgGs and extensively characterized by mass spectrometry (Huang et al. 2006, Qian et al. 2007). Glycoforms that are not commonly synthesized in humans may be immunogeneic and have to be identified, controlled, and limited for therapeutic use of mAbs by systemic routes of administration. Glycosylation depends on the production system (i.e., cell line), the selected monoclonal population, and the process (i.e., feeding strategy). Mass spectrometry is a powerful technique to differentiate glycoform fingerprints of mAbs produced for instance in CHO and NS0 cells, the two predominant mammalian production systems (Beck et al. 2005, Siemiatkoski et al. 2006). Murine NS0 cells produce mAbs exhibiting small amounts of glycoforms with additional α-1,3-galactose and different sialic acids (NGNA vs NANA). Characteristic glycoform patterns can also be observed by MS for PER.C6 human retina cells, chicken, yeast, insect cells, and plant-derived antibodies (e.g., xylose or fucose-1,3). Recently, glyco-engineered antibody variants were also produced in several of the above-mentioned production systems and MS-characterized. For instance, yeast strains were genetically transformed to produce mAbs with "humanized" glycoforms or with enhanced effectors functions (e.g., third bisecting arm and/or lack of fucose) (Cox et al. 2006, Beck et al. 2008c).

39.1.3 Structural Isotyping and Disulfide Connection Determination by MS

All therapeutics currently approved or in late clinical stages, which are either humanized or human immunoglobulins, belong to IgG1, IgG2, or IgG4 isotypes (Beck et al. 2008b). Only a couple of IgMs and IgAs have been investigated in early clinical trials and for particular indications and applications. From a structural point of view, IgGs share more than 90% sequence homologies but are characterized by

different interchain disulfide bridges in the flexible hinge region and between the heavy and light chains. Direct high-accuracy LC-MS maps of trypsin or endoprotease Lys-C digested non-reduced mAbs result in specific fingerprints for each isotype, that allows a fast identification and can be associated with correct protein folding.

39.1.4 Non-Stable "Hot Spots" Mapping by MS

During the pharmaceutical development, non-stable "hot spots" in the primary structure can be monitored by MS (Chelius et al. 2005) used as fine stability-indicating method (Liu et al. 2006). For instance, deamidation of asparagines in aspartic/isoaspartic acids yielded negative charges, which can be globally highlighted by separation methods based on differences of charges (IEF, CE-IEF, and C-IEX) (Lyubarskaya et al. 2006) or by enzymatic methods (isoaspartate dosage). Analysis of peptide maps containing this unstable amino acid by MALDI-TOF or LC-ES-TOF shows a 1 Da increase in mass. Additional LC-MS/MS sequence data recorded on an LC-ES-Ion-Trap mass spectrometer or a MALDI-TOF/TOF allow a fine and unambiguous localization of the deamidation sites. In some cases, such deamidation events may result in dramatic effects on the antibody production yields or on the antigen binding affinity, when located in the CDRs (Liu et al. 2008a, Vlasak and Ionescu 2008). Accumulation of succinimide intermediate products may also be identified by MS (Chelius et al. 2006a, Terashima et al. 2007, Xiao et al. 2007, Chu et al. 2007, Chelius et al. 2006a), as well as additional cysteinylation (Banks et al. 2008, Gadgil et al. 2006b,a), tryptophan (Wei et al. 2007, Yang et al. 2007) or methionine oxidation (Liu et al. 2008b), hinge non-enzymatic cleavage (Cordoba et al. 2005, Cohen et al. 2007), thioether cross-link of heavy and light chains (Tous et al. 2005), glycation (Gadgil et al. 2007), and *C*-terminal amidation (Johnson et al. 2007).

39.2 Materials

39.2.1 LC-ESI-TOF Characterization of an Intact, and PNGase F Treated and Reduced Humanized IgG4

- High performance liquid chromatography (HPLC) system Alliance™ 2695 separation module equipped with a column-heating compartment and a 996™ photodiode array detector (Waters, Saint-Quentin-en-Yvelines, France).
- Zorbax™ 300SB-C8 Rapid Resolution Cartridge™, 2.1 mm × 30 mm, 3.5 m (Agilent Technologies, Massy, France)

– Mass spectrometer LCT Premier^{TM} equipped with an ESI source (Waters, Saint-Quentin-en-Yvelines, France).

39.2.2 LC-ESI-TOF Characterization of Trypsin Digested Light and Heavy Chains

– RP-HPLC system (Waters, Saint- Quentin-en-Yvelines, France) consisting of two 510 pumps, a 717 auto-injector, and a 490 UV detector.
– G3000SW size-exclusion column 7.8 mm × 300 mm (Tosoh Bioscience, VWR, Fontenay-sous-Bois, France)
– Amicon ultra centrifugal filter units, nominal molecular weight cut-off of 10 and 30 kDa (Millipore, Saint-Quentin-en-Yvelines, France)
– See 2.1 for LC-ESI-MS spectrometer.
– Buffer: 0.1 M Tris/Tris–HCl; 0.02 M CaCl2; pH 8.1.
– Sequencing grade trypsin (EC 3.4.21.4 from bovine pancreas, Roche Diagnostics, Meylan, France).
– Speed-Vac.
– Acetonitrile.
– Trifluoacetic acid (TFA).

39.2.3 Humanized IgG1 and IgG4 Disulfide Bridges Mapping and Isotyping by LC-ES-TOF (Endoprotease Lys-C Maps)

– Autoflex MALDI-TOF or Ultraflex MALDI TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany)
– See 2.1 for LC-ESI-MS spectrometer
– Buffer: 0.1 M Tris/Tris–HCl; 0.02 M CaCl2; CaCl2; pH 8.1
– Sequencing grade endoprotease Lys-C (EC 3.4.21.50 from *Lysobacter enzymogenes*, Roche Diagnostics, Meylan, France).

39.2.4 Deamidation Site Identification by MALDI-TOF and LC-IT MS/MS

– Ultraflex MALDI TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany)
– HCT ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany)
– Agilent 1100 series HLPC-Chip/MS systems (Agilent Technologies, Palo Alto, USA).

39.3 Protocols

39.3.1 LC-ESI-TOF Characterization of an Intact, and PNGase F Treated and Reduced Humanized IgG4

39.3.1.1 LC-ESI-TOF Experiments

1. Run LC–MS experiments at 0.25 ml/min (no flow rate split for MS detection).
2. MS analysis in W positive ion mode on a Waters LCT Premier™ equipped with an ESI source (Waters, Saint-Quentin-en-Yvelines, France).
3. Source and desolvation temperatures were set at 120 and 300°C, respectively.
4. Nitrogen gas flow rates were set at 50 l/h for the cone and 350 l/h for desolvation.
5. Calibration carried out using a 2 g/l solution of cesium iodide (Sigma–Aldrich, Saint-Quentin-Fallavier, France) for the protein mass measurements.
6. MS data acquisition and processing using MassLynx™ 4.1 (Waters, Saint-Quentin-en-Yvelines, France).
7. Deconvolution of the protein mass spectra performed with Transform™ (Waters, Saint-Quentin-en-Yvelines, France).
8. Standard deviations for mass measurements were given by treatment of the multiply charged ion spectrum using Mass Lynx™ (Waters, Saint-Quentin-en-Yvelines, France).

39.3.1.2 LC-ESI-TOF of Intact IgG4

1. Load 10 μg intact or deglycosylated antibody onto a Zorbax™ 300SB-C8 Rapid Resolution Cartridge™, 2.1 mm × 30 mm, 3.5 m (Agilent Technologies, Massy, France) maintained at 60°C.
2. The protein was first rinsed on the cartridge for 20 min with 90% solvent A (water + 0.02% TFA and 1% HCOOH), 10% solvent B (water:*n*-propanol, 10:90 + 0.02% TFA and 1% HCOOH). Elution was then performed using an isocratic step consisting of 50% of B for 10 min, followed by a 10 min step washing at 80% B, and by a final equilibration period of 10 min at 10% B. Capillary and cone voltages were optimized for maximum sensitivity at each experiment. Ion guide one and Aperture 1 V were both set at 40 V.

39.3.1.3 Reduction and Alkylation of IgG4

1. Lyophilized and solubilized in 60 μl Tris–HCl 100 mM, 2 mM EDTA, guanidine HCl 6 M, pH 8.0 buffer.
2. Flush sample with N2.

3. Add 20 mM DTT (Sigma–Aldrich, Saint-Quentin Fallavier, France) for 2 h at 37°C.
4. Add 25 mM iodoacetamide (Sigma–Aldrich, Saint-Quentin Fallavier, France) in the dark at room temperature within 1 h.
5. Quench by addition of acetic acid and *n*-propanol (final concentration 10 and 20%, respectively).

39.3.1.4 LC-ESI-TOF of Reduced IgG4

1. Load 5 μg of reduced antibody onto the RP-HPLC column maintained at 60°C.
2. Elute proteins from the column using a linear gradient from 20 to 35% of B in 30 min (solvent A = water + 0.02% TFA and 1% HCOOH; solvent B = water: *n*-propanol, 10:90 + 0.02% TFA and 1% HCOOH).
3. Flush the column with 80% B for 5 min followed by an equilibration step of 10 min at 20% B.
4. Optimize capillary and cone voltages for maximum sensitivity at each experiment.
5. Set ion guide one and aperture 1 V at 20 and 30 V, respectively.

39.3.2 LC-ESI-TOF Characterization of Trypsin Digested Light and Heavy Chains

39.3.2.1 Desalting of Mixture of Light and Heavy Chain Prior to Digestion

1. Desalt reduced and alkylated LC and HC using a Zorbax 300SB-C8 Rapid Resolution Cartridge™, 2.1 mm × 30 mm, 3.5 μm (Agilent Technologies, Massy, France) maintained at 60°C.
2. Mobile phase A = acidified water (0.05% trifluoroacetic acid (TFA)); solvent B = *n* propanol:water, 90:10 containing 0.1% formic acid (HCOOH).
3. Set the flow rate at 0.25 ml/min.
4. After a 5 min desalting step at 100% A, perform an isocratic elution at 70% B for 5 min.
5. Monitor the elution of the fraction of interest at 280 nm.
6. The LC and HC mixture was collected and lyophilized.

39.3.2.2 Separation of Light and heavy Chain by Size-Exclusion Chromatography (SEC)

1. Load 10 μl of mixture of IgG4 heavy and light chains on the SEC column.
2. Mobile phase: 3.0 M guanidine hydrochloride in 50 mM sodium phosphate, pH 6.2.

3. Flow rate: 1 ml/min and isocratic elution.
4. Column temperature ambient
5. Monitor the elution at 280 nm.
6. Concentrate fractions containing light and heavy chains on Amicon ultra centrifugal filter units (10 kDa nominal molecular weight cut-off for light chains and 30 kDa for heavy chains, respectively)
7. Wash three times with 10% acetic acid.

39.3.2.3 Light and Heavy Chain Trypsin Digestion

1. Prepare 0.1 M Tris/Tris–HCl; 0.02 M CaCl2; pH 8.1 buffer.
2. Solution A: dry sample to digest with Speed- Vac and then add 10 μl of acetonitrile and 40 μl of buffer.
3. Solution B: solubilize 25 μg of trypsin in 50 μl of de-ionised water (0.5 g/l).
4. Add solution B to solution A (enzyme/substrate: 1/10 to 1/100 ratio, depending on the protein amount to digest).
5. Incubate the sample at 37°C for 7 h.
6. Add 1 μl of TFA to stop the reaction.

39.3.3 Humanized IgG1 and IgG4 Disulfide Bridges Mapping and Isotyping by LC-ES-TOF

39.3.3.1 Endoproteinase Lys-C Digestion

1. Prepare buffer with 0.1 M Tris/Tris–HCl; 0.02 M CaCl2; pH 8.1.
2. Solution A: dry the sample to digest with Speed-Vac; add 10 μl of acetonitrile and 40 μl of buffer.
3. Solution B: solubilize 5 μl endoproteinase Lys-C in 50 μl of water Direct-QTM (0.1 μg/μl).
4. Add solution B to solution-A (enzyme/substrate: 1/10 to 1/100 ratio, depending on protein amount to digest). Incubation was performed at 37°C overnight
5. Add 1 μl of TFA to stop the reaction.

39.3.3.2 LC-ESI-TOF Analyzes of Endoproteinase Digested Humanized IgG1 and IgG4

1. Load 5 μg of reduced antibody onto the RP-HPLC column maintained at 60°C.
2. Elute peptides from the column using a linear gradient from 20 to 35% of B in 30 min (solvent A + water + 0.02% TFA and 1% HCOOH; solvent B = water: *n*-propanol, 10:90 + 0.02% TFA and 1% HCOOH).

3. Flush the column with 80% B for 5 min followed by an equilibration step of 10 min at 20% B.
4. Optimize capillary and cone voltages for maximum sensitivity at each experiment.
5. Set ion guide one and aperture 1 V at 20 and 30 V, respectively.

39.3.4 Deamidation Site Identification by MALDI-TOF and LC-IT MS/MS

39.3.4.1 Trypsin Digestion of Reduced and Alkylated Humanized IgG

1. Prepare a 1 mg/ml IgG solution in guanidine-HCl 6 M, Tris Base 0.1 M, EDTA 2 mM at pH8.
2. Add a DTT solution at a final concentration of 5 mM and incubate 3 h at 37°C.
3. Add an iodoacetamide solution at a final concentration of 12.5 mM and incubate -40 min in the dark.
4. Wash and exchange buffer exchange with 10% acetic acid (Vivaspin 10 kDa).
5. Add Trypsin 1:50 w:w.
6. Increase incubations periods at 37°C to 4 h, 21 h, 96 h, and 168 h to generate positive controls.
7. Analyze the digests by MALDI-MS and LC-MS/MS.

39.3.4.2 Deamidation Site Identification by MALDI-TOF

1. Prepare samples with standard dried droplet on stainless steel MALDI target and cyano-4-hydroxycinnamicacid as matrix.
2. MS and MS–MS spectra were acquired with a Bruker Ultraflex TOF-TOF with gridless ion optics under control of Flexcontrol 2.0. This instrument, equipped with the Scout (Bruker Daltonics, Bremen, Germany) high-resolution optics with X-Y multisample probe and gridless reflector, was used at a maximum accelerating potential of 25 kV and was operated in the reflector mode.
3. Ionization was accomplished with a 337 nm beam from a nitrogen laser with a repetition rate of 20 Hz.
4. The output signal from the detector was digitized at a sampling rate of 2 GHz.
5. External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of human angiotensin II (m/z 1046.542), substance P (m/z 1349.643), bombesin (m/z 1619.823), and ACTH 18–39 (m/z 2465.199). Monoisotopic peptide masses were automatically annotated using FlexAnalysis 2.4 (Build11) software package.
6. Collision cell and the Ultraflex device providing a second ion source were accelerated to 7 kV and the post-acceleration after Ultraflex was 19 kV. In the

standard mode, the Ultraflex TOF/TOF uses laser-induced decomposition (LID) for the generation of MS–MS fragments.
7. Their masses were analyzed after the ion reflector passage.
8. Ultraflex data were processed using the de novo analysis module of Biotools protein sequence analysis package (Version 2.2) and DataAnalysis 3.4.

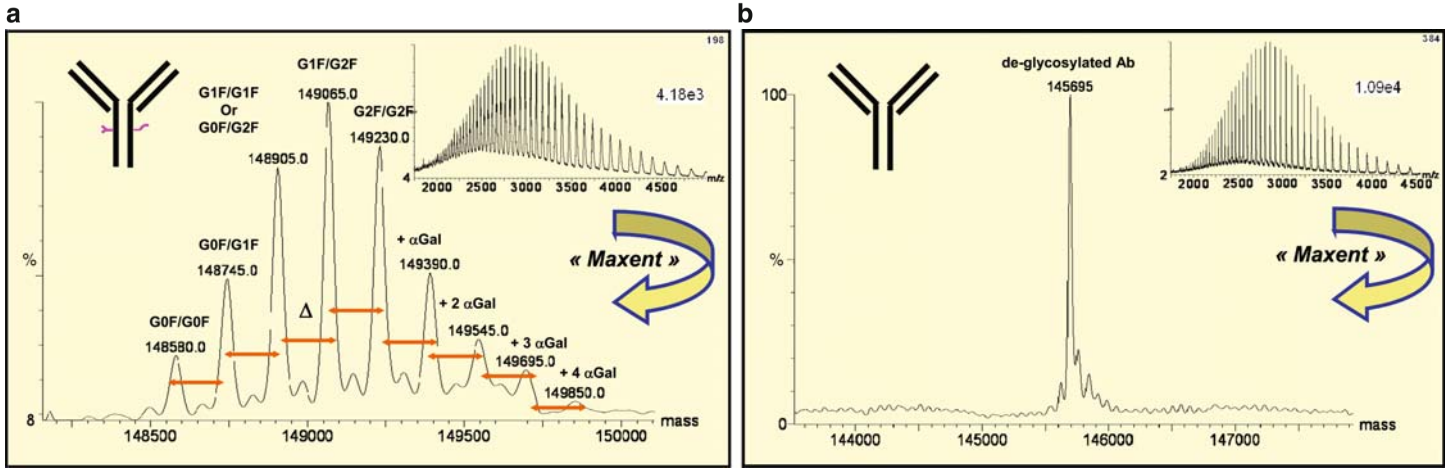
39.3.4.3 Deamidation Site Confirmation by LC-IT MS/MS

1. The sample (3 µl) analysis was performed using an Agilent 1100 series HPLC-Chip/MS system coupled to an HCT Ultra ion trap.
2. The chip contained a Zorbax 300SB-C18 43 mm × 75 µm, with a 5 µm particle size column and a Zorbax 300SB-C18 (40 nL, 5 µm) enrichment column.
3. The solvent system consisted of 2% acetonitrile, 0.1% formic acid in water (solvent A), and 2% water, 0.1% formic acid in acetonitrile (solvent B).
4. The sample was loaded into the enrichment column at a flow rate set to 3.75 µL/min with a H2O/2%ACN/0.1 HCOOH solution during 3 min.
5. Elution was performed at a flow rate of 300 nl/min with a 8–40% linear gradient (solvent B) over the first 20 min and followed by a 70% stage (solvent B) over 5 min before the reconditioning of the column with 92% of solvent A.
6. The MS/MS data were analyzed by DataAnalysis 3.4. The MS and MS/MS tolerance were set at 0.2 Da.

39.4 Results

39.4.1 LC-ESI-TOF Characterization of an Intact, and PNGase F Treated and Reduced IgG4

Figure 39.2 shows ESI mass spectra of a humanized IgG4 produced in NS0, either intact (A) or treated with PNGase F (B) as well as the corresponding light (A) and heavy chains (B) of the same antibody, analyzed after reduction by dithiothreitol (DTT); Fig. 39.3. The spectra show typical series of multiply charged ions with m/z values ranging from 1,500 to about 4,000 m/z. The associated deconvoluted spectra are shown in each figure. Experimental masses were compared to calculated masses deduced from the cDNA-derived amino acid sequence, assuming 16 disulfide bridges, heavy chain *N*-terminal pyroglutamic acids formation, and *C*-terminal lysine clipping (145 685 Da). The experimental mass of the PNGase F treated mAb is in good agreement with the calculated one (145 695 Da; + 10 Da mass difference). The spectrum of the non-deglycosylated mAb is characterized by 9 equidistant peaks (162 Da increment), which correspond to the mass of additional hexoses (e.g., galactose). Very close experimental masses are also measured for the light chain (exp. 24 063 Da/calc. 24 063 Da) and for the heavy chain

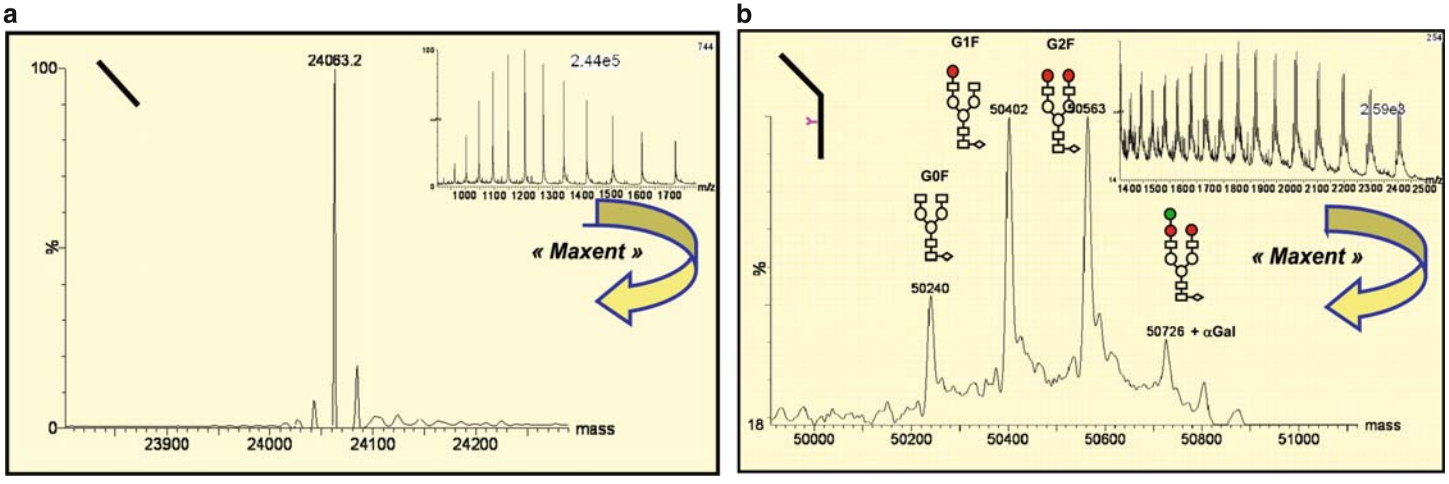


IgG4 Glycoforms	Calc. Mass (Da)	Exp. Mass (Da)	Difference (Da)
G0F/G0F	148576.4	148580.0	+3.6
G0F/G1F	148738.5	148745.0	+6.5
G0F/G2F, G1F/G1F	148900.7	148905.0	+5.7
G1F/G2F	149062.8	149065.0	+2.2
G2F/G2F	149224.9	149230.0	+3.1

IgG4 + PNGase F	Calc. Mass (Da)	Exp. Mass (Da)	Difference (Da)
H2L2	145 685.0	145695.0	+10

A Beck et al, Antibody Engineering, Springer-Verlag, 2010

Fig. 39.2 Humanized IgG4 LC-ESI-TOF mass spectra (A) without and (B) after PNGase F digestion



Light Chain	Calc. Mass (Da)	Exp. Mass (Da)	Difference (Da)
	24063.0	24063.2	+0.2 Da

Heavy Chain	Calc. Mass (Da)	Exp. Mass (Da)	Difference (Da)
G0F	50241.4	50240	–1.4
G1F	50403.5	50402	–1.5
G2F	50565.6	50563	–2.6
G2FαGal	50727.7	50726	–1.7

A Beck et al, Antibody Engineering, Springer-Verlag, 2010

Fig. 39.3 Humanized IgG4 LC-ESI-TOF mass spectra (A) Light Chain (B) Heavy Chain

(calc. 48 796 Da) corrected by the masses of the main reported glycoforms (G0F: 50 240 Da/ + 1444 Da; G1F: 50 402 Da/ + 1606 Da; G2F: 50 563 Da/ + 1767 Da; G2FG: 50 726 Da/ + 1930 Da).

39.4.2 LC-ESI-TOF Characterization of Trypsin Digested Light and Heavy Chains

The light chain of the IgG4 contains 219 amino-acids and five Cys, with four Cys involved in two intra-molecular bridges (Cys23–Cys93, Cys139–Cys199) for both variable and constant domains and one (Cys219-(HC)), involved in an intermolecular disulfide bond with a heavy chain. Trypsin cleaves polypeptides after Lys or Arg residues, except when they are followed by a Pro (Beck et al. 2005). LC–MS tryptic maps of reduced and alkylated isolated light and heavy chains were detected by UV (280 nm) and by mass spectrometry (TIC: total ion current). All 20 expected tryptic fragments are identified confirming the primary structure of the light chain; Fig. 39.3 and Table 39.1.

For the heavy chains, glycopeptides containing the glycosylated Asn297 are identified by LC-TOF linked to a nonameric peptide (- R/EEQYN297STYR/V-; 9-mer). The three observed main peaks are interpreted as the so-called "G0F" (HexNAc4Hex3DeoxyHex1), "G1F" (HexNAc4Hex4DeoxyHex1), and "G2F" forms (HexNAc4Hex5DeoxyHex1) in a ratio (15/52/25). One additional galactose α(1–3) linked glycoform was also identified (HexNAc4Hex6DeoxyHex1) as previously reported for other antibodies produced in NS0 cells (Beck et al. 2007). Such a glycopeptide LC-MS maps are characteristic of each protein expression system (mouse or hamster) and can be used as fingerprint to assess the NS0 or CHO origin of the humanized mAbs.

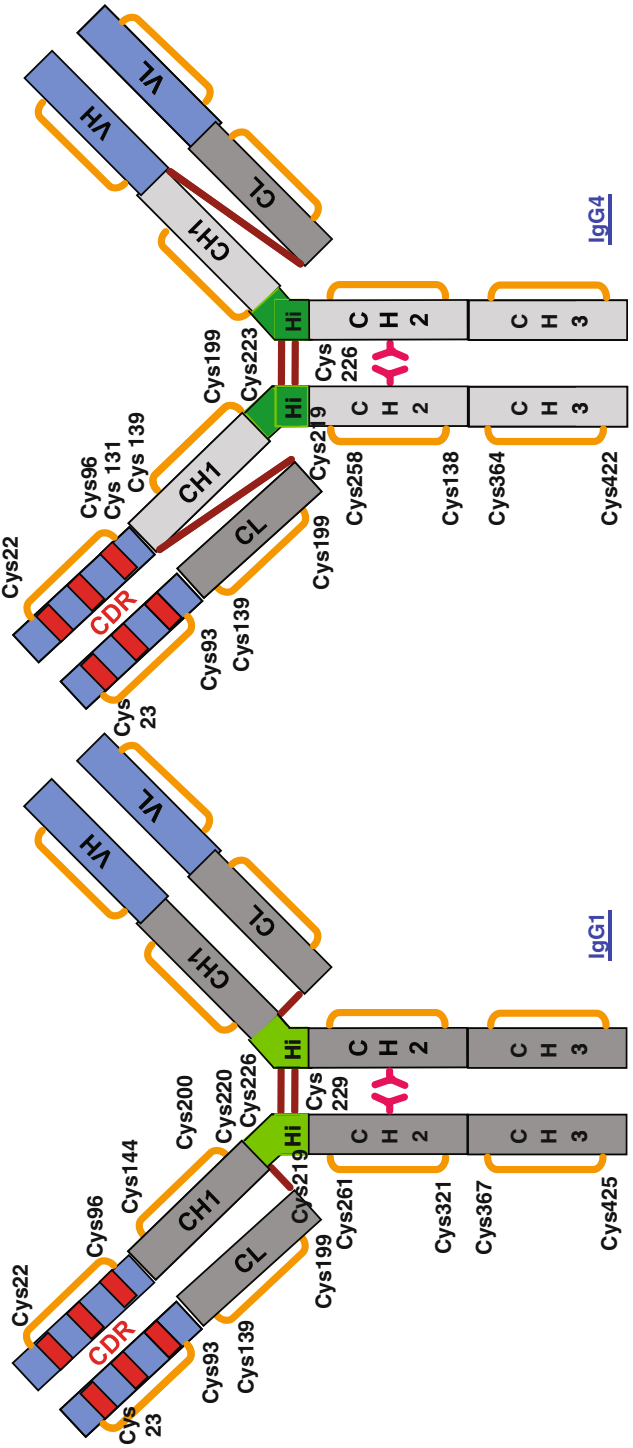
39.4.3 Humanized IgG1 and IgG4 Disulfide Bridges Mapping and Isotyping by LC-ES-TOF

Differential humanized IgG1 and IgG4 disulfide bridges pairing (Fig. 39.4) can be investigated by LC–ESI-TOF after endoprotease Lys-C or other enzymes. Because of the symmetry of IgG, nine of the 16 disulfide bonds are unique for both isotypes (Zhang et al. 2002, Beck et al. 2005). As shown in Table 39.2, specific peptide paring is found on the LC-MS maps, particularly in the hinge region (IgG4: H-H Cys^{223}-Cys^{223} H/H Cys^{226}-Cys^{226}; IgG1: H-H Cys^{226}-Cys^{226} H/H Cys^{229}-Cys^{229}) and for the light-heavy chain connections (IgG4: L-H Cys^{219}-Cys^{131}/H-H Cys^{139}-Cys^{199}); IgG1: L-H Cys^{219}-Cys^{220}).

Direct high-accuracy LC-MS maps of all digested, nonreduced mAbs result in specific fingerprints for each isotypes, which allow a fast identification and can be

Table 39.1 (A) IgG Light Chain trypsin LC-MS peptide map (B) IgG Heavy Chain trypsin LC-MS posttranslational-modified peptides

Trypsin	Peptide	Sequence	Calculated	LC-ESI-TOF	
(A) IgG Light Chain trypsin LC-MS: full peptide map					
LT1	1–24	DIVM4TQSPLSLPVTPGEPASISC^{23}R	2,555.3	2,554.4	
LT2	25–55	SSQSIVHSNGNTYLQWYLQKPGQSPQLLIYK	3,579.0	3,579.0	
LT3	56–59	VSNR	474.3	474.3	
LT4	60–66	LYGVPDR	818.4	818.5	
LT5	67–69	FSGSGSGTDFTLK	1,302.6	1,302.6	
LT6	80–82	ISR	374.2	374.2	
LT7	83–108	VEAEDVGVYYC^{93}FQGSHVPWTFGQGTK	2,960.3	2,962.2	
LT8	109–112	VEIK	487.3	487.3	
LT8+9	109–113	VEIK-R	643.4	643.4	
LT10	114–131	TVAAPSVFIFPPSDEQLK	1,945.0	1,945.0	
LT11	132–147	SGTASVVC^{139}LLNNFYPR	1,798.0	1,796.9	
LT12	148–150	EAK	346.2	346.2	
LT13	151–154	VQWK	559.3	559.3	
LT14	155–174	VDNALQSGNSQESVTEQDSK	2,135.0	2,135.9	
LT15	175–188	DSTYSLSSTLTLSK	1,501.8	1,502.8	
LT16	189–193	ADYEK	624.3	624.3	
LT17	194–195	HK	283.2	283.2	
LT18	196–212	VYAC^{199}EVTHQGLSSPVTK	1,874.9	1,875.0	
LT19	213–216	SFNR	522.3	522.3	
LT20	217–219	GEC^{219}	364.1	364.1	
(B) IgG Heavy Chain trypsin LC-MS: posttranslational-modified peptides					
Trypsin	peptide	Sequence	Calculated	LC-ESI-TOF	Interpretation
HT1.2	1–44	Q^{1}VQLQ...	4,835.5	not found	Nt-PyroGlu
HT1.2	1–44	PyroE^{1}VQLQ...	4,835.5	4,818.4	−17 Da (-NH_3)
HT23	293–301	EEQYN^{297}STYR	1,189.5	not found	
			2,430.2	2,431.1	+1241.6 (+ G0F-NAcGlc)
			2,592.2	2,593.3	+1403.8 (+ G1F-NAcGlc)
			2,633.2	2,634.7	+1445.2 (+ G0F)
			2,795.2	2,796.5	+1607.0 (+ G1F)
			2,957.2	2,958.5	+1769.0 (+ G2F)
			3,119.2	3,121.7	+1932.2 (+ G2F + Gal)
HT40	440–447	SLSLSPG	787.9	786.9	No Ct-Lys



A Beck et al, Antibody Engineering, Springer-Verlag, 2010

Fig. 39.4 Humanized IgG1 vs IgG4 disulfide bridge maps

Table 39.2 (A) Whole humanized IgG1 and (B) IgG4 endoprotease- LysC LC-MS disulfide peptide maps

Disulfide containg-peptides	Fragments	Calculated	LC-ESI-TOF
(A) Whole humanized IgG1 endoprotease- LysC LC-MS: disulfide peptide maps			
L-L Cys23-Cys93 (2×)	L1-L3	9,319.5	9,319.8
H-H Cys22-Cys96 (2×)	L1-L5	9,018.2	–
L-L Cys139-Cys199 (2×)	L6-L12	3,886.4	3,886.3
L-H Cys219-Cys220 (2×)	L13-L11	1,261.4	1,260.9
H-H Cys144-Cys200 (2×)	L7-L8	7,921.9	7,921.7
H-H Cys226-Cys226 H/H Cys229-Cys229	L12-L12	5,458.5	5,458.5
H-H Cys261-Cys321 (2×)	L13-L17	3,146.6	3,146.3
H-H Cys367-Cys425 (2×)	L23-L27	4,090.6	4,090.5
(B) Whole humanized IgG4 endoprotease- LysC LC-MS: disulfide peptide maps			
Disulfide containg-peptides	Fragments	Calculated	LC-ESI-TOF
L-L Cys23-Cys93 (2×)	L1-L3	9,319.5	–
	L1-L3 (KP)	8,094.1	8,093.2
H-H Cys22-Cys96 (2×)	L1-L5	9,018.2	–
L-L Cys139-Cys199 (2×)	L6-L12	3,886.4	3,886.3
L-H Cys219-Cys131/H-H Cys139-Cys199 (2×)	L13-L6-L8	4,994.6	4,994.3
H-H Cys223-Cys223 H/H Cys226-Cys226	L11-L11	5,708.7	5,708.7
H-H Cys258-Cys318 (2×)	L12-L15	4,797.4	4,797.3
H-H Cys364-Cys422 (2×)	L21-L24	4,091.6	4,091.5

associated with correct protein folding. IgG4 half-antibodies or bispecific antibodies can also be assessed by MS as well as IgG2 structural isoforms related to hinge different disulfide bridge pairing (Chelius et al. 2006a).

39.4.4 Deamidation Site Identification by MALDI-TOF and LC-IT MS/MS

Deamidation of asparagine (Asn) residues are common degradation of proteins. They can significantly impact on protein structure and function (Chelius et al. 2005, Vlasak and Ionescu 2008). At neutral and basic pH, deamidation proceeds via formation of a five-member ring intermediate–succinimide. The reaction is initiated by deprotonation of the peptide bond nitrogen, followed by a nucleophilic attack on the side chain carbonyl, loss of ammonia, and formation of succinimide. Succinimide is unstable and hydrolyzes into a mixture of two products – isoaspartate (isoAsp) and Asp. At acidic pH, deamidation occurs as well, but predominantly by direct hydrolysis of the side chain amide, yielding only Asp. The rate of deamidation depends on protein sequence and conformation, as wells as on external factors such as temperature and pH. The rate of deamidation mainly depends on the amino acid residue following Asp, Asn-Gly being the most susceptible to deamidation.

In the case of Asn deamidation, a mass increase of 1 Da may be observed both for Asp and IsoAsp deamidation products. For full-length antibodies (150 kDa), a difference of 1 Da cannot be accurately measured with most current mass

spectrometers. For derived Fab and Fc fragments (50 kDa), a 1 Da mass difference is at the limit of resolution (20 ppm). For smaller fragments like peptide on trypsin maps for example, a 1 Da mass increase can be clearly measured and compared to the control material, providing a first line of evidence for deamidation as illustrated for a Fc peptide containing a NG motive and common to human IgG1, 2 and 4; Fig. 39.5. Deamidation is a slow process as long as the mAb is intact at pH 7.5 and 37°C, because 3D-globular structure of the mAb protect from degradation (Chelius et al. 2005). The phenomenon is greatly enhanced after tryptic digestion as shown in the time-course presented in Fig. 39.5. After 7 Days of trypsin treatment at 37°C, deamidation is observed on peptide 302–317 by MALDI-TOF and confirmed by MS/MS (Fig. 39.6), which demonstrates the suitability of both assays. No deamidation was observed for this peptide at T0.

39.5 Conclusion

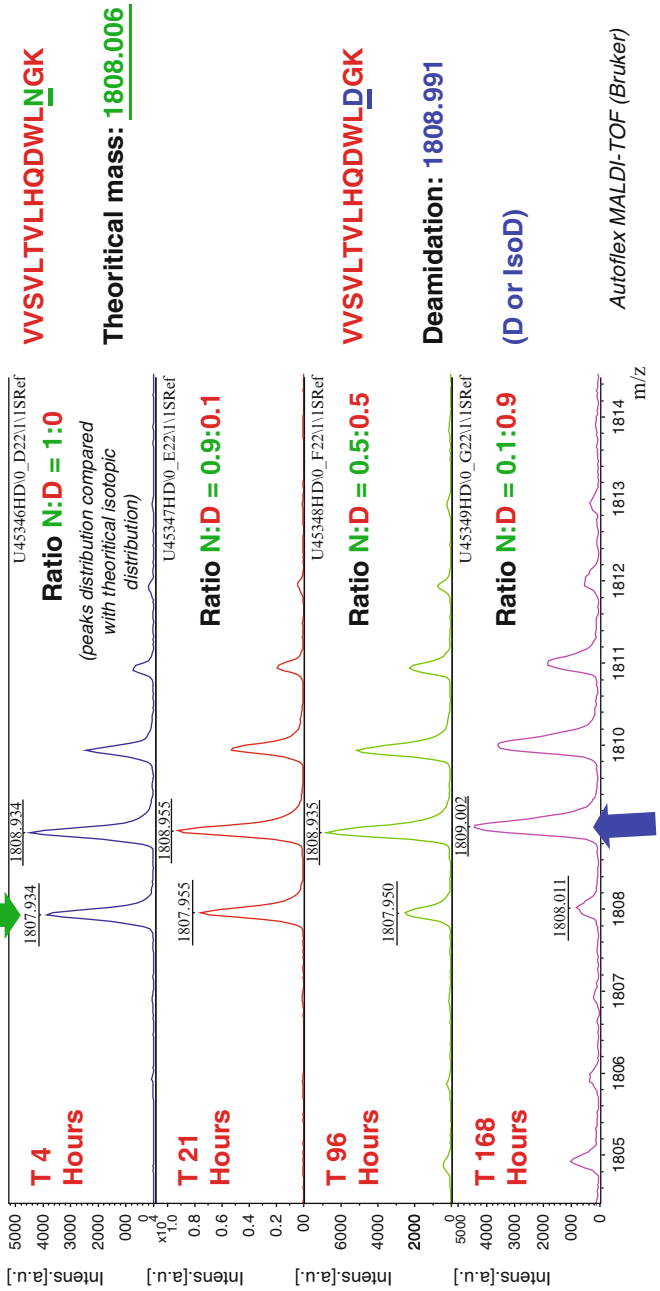
Multiple and complementary mass spectrometry methods and measures are now applied at multiple stage of therapeutic antibody discovery and development: (1) selection of the best antibody-producing clone from a structural point of view, (2) optimization of a suitable formulation for long term stability and conservation, (3) full structural characterization of clinically-developed candidates, (4) comparability assays for process transfer and bridging and for scaling-up, and (5) routinely as QC-batch release assays. In most cases, the higher mass accuracy and resolution of the new LC-ES-TOFs have real advantages over the former techniques and tandem MS tends to replace traditional protein micro sequencing by Edman degradation. This is particular true for new generations of mass spectrometers like Orbitrap (Zhang and Shah 2007) or Ion-Mobility mass spectrometers (Olivova et al. 2008, Atmanène et al. 2009).

References

Atmanène C, Wagner-Rousset E, Malissard M, Chol B, Robert A, Corvaïa N, Van Dorsselaer A, Beck A, Sanglier-Cianferani S. (2009) Extending Mass Spectrometry contribution to therapeutic monoclonal antibody lead selection and development: characterization of antigen/antibody immune complexes using non-covalent ESI-MS. Analytical Chemistry. *In Press*

Banks DD, Gadgil HS, Pipes GD, Bondarenko PV, Hobbs V, Scavezze JL, Kim J, Jiang XR, Mukku V, Dillon TM (2008) Removal of cysteinylation from an unpaired sulfhydryl in the variable region of a recombinant monoclonal IgG1 antibody improves homogeneity, stability, and biological activity. J Pharm Sci 97:775–790

Beck A, Bussat MC, Zorn N, Robillard V, Klinguer-Hamour C, Chenu S, Goetsch L, Corvaia N, Van Dorsselaer A, Haeuw JF (2005) Characterization by liquid chromatography combined with mass spectrometry of monoclonal anti-IGF-1 receptor antibodies produced in CHO and NS0 cells. J Chromatogr B Analyt Technol Biomed Life Sci 819:203–218



A Beck et al, Antibody Engineering, Springer-Verlag, 2010

Fig. 39.5 Time course analysis by MALDI-TOF of an IgG-Fc peptide prone to de-amidation

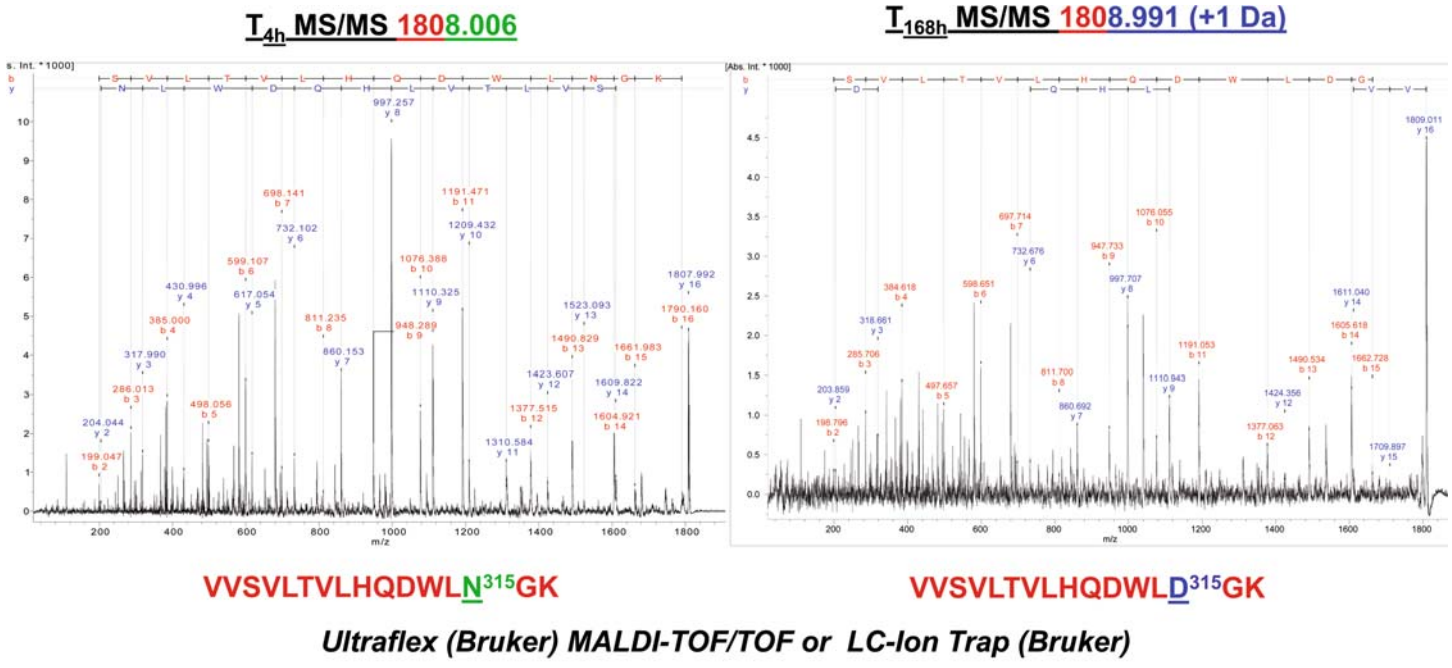


Fig. 39.6 LC-MS/MS characterization of an IgG-Fc peptide prone to de-amidation

Beck A, Klinguer-Hamour C, Bussat MC, Champion T, Haeuw JF, Goetsch L, Wurch T, Sugawara M, Milon A, Van Dorsselaer A, Nguyen T, Corvaia N (2007) Peptides as tools and drugs for immunotherapies. J Pept Sci 13:588–602

Beck A, Wagner-Rousset E, Bussat MC, Lokteff M, Klinguer-Hamour C, Haeuw JF, Goetsch L, Wurch T, Van Dorsselaer A, Corvaia N (2008a) Trends in Glycosylation, Glycoanalysis and Glycoengineering of Therapeutic Antibodies and Fc-Fusion Proteins. Curr Pharm Biotechnol 9:482–501

Beck A, Wagner-Rousset E, Goetsch L, Corvaïa N (2008b) Therapeutic antibodies: structure assessment by mass spectrometry from screening to clinical batches. Screen Trends Drug Discov 9:18–20

Beck A, Wurch T, Corvaïa N (2008c) Editorial: therapeutic antibodies and derivatives: from the bench to the clinic. Curr Pharma Biotechnol 9:421–422

Chelius D, Rehder DS, Bondarenko PV (2005) Identification and characterization of deamidation sites in the conserved regions of human immunoglobulin gamma antibodies. Anal Chem 77:6004–6011

Chelius D, Huff Wimer ME, Bondarenko PV (2006a) Reversed-phase liquid chromatography in-line with negative ionization electrospray mass spectrometry for the characterization of the disulfide-linkages of an immunoglobulin gamma antibody. J Am Soc Mass Spectrom 17:1590–1598

Chelius D, Jing K, Lueras A, Rehder DS, Dillon TM, Vizel A, Rajan RS, Li T, Treuheit MJ, Bondarenko PV (2006b) Formation of pyroglutamic acid from N-terminal glutamic acid in immunoglobulin gamma antibodies. Anal Chem 78:2370–2376

Chu GC, Chelius D, Xiao G, Khor HK, Coulibaly S, Bondarenko PV (2007) Accumulation of succinimide in a recombinant monoclonal antibody in mildly acidic buffers under elevated temperatures. Pharm Res 24:1145–1156

Cohen SL, Price C, Vlasak J (2007) Beta-elimination and peptide bond hydrolysis: two distinct mechanisms of human IgG1 hinge fragmentation upon storage. J Am Chem Soc 129:6976–6977

Cordoba AJ, Shyong BJ, Breen D, Harris RJ (2005) Non-enzymatic hinge region fragmentation of antibodies in solution. J Chromatogr B Analyt Technol Biomed Life Sci 818:115–121

Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, Peele CG, Black A, Passmore D, Moldovan-Loomis C, Srinivasan M, Cuison S, Cardarelli PM, Dickey LF (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant Lemna minor. Nat Biotechnol 24:1591–1597

Dick LW Jr, Kim C, Qiu D, Cheng KC (2007) Determination of the origin of the N-terminal pyroglutamate variation in monoclonal antibodies using model peptides. Biotechnol Bioeng 97:544–553

Dillon TM, Bondarenko PV, Speed RM (2004) Development of an analytical reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry method for characterization of recombinant antibodies. J Chromatogr A 1053:299–305

Dillon TM, Bondarenko PV, Rehder DS, Pipes GD, Kleemann GR, Ricci MS (2006) Optimization of a reversed-phase high-performance liquid chromatography/mass spectrometry method for characterizing recombinant antibody heterogeneity and stability. J Chromatogr A 1120:112–120

Gadgil HS, Bondarenko PV, Pipes GD, Dillon TM, Banks D, Abel J, Kleemann GR, Treuheit MJ (2006a) Identification of cysteinylation of a free cysteine in the Fab region of a recombinant monoclonal IgG1 antibody using Lys-C limited proteolysis coupled with LC/MS analysis. Anal Biochem 355:165–174

Gadgil HS, Pipes GD, Dillon TM, Treuheit MJ, Bondarenko PV (2006b) Improving mass accuracy of high performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry of intact antibodies. J Am Soc Mass Spectrom 17:867–872

Gadgil HS, Bondarenko PV, Treuheit MJ, Ren D (2007) Screening and sequencing of glycated proteins by neutral loss scan LC/MS/MS method. Anal Chem 79:5991–5999

Huang LH, Biolsi S, Bales KR, Kuchibhotla U (2006) Impact of variable domain glycosylation on antibody clearance: An LC/MS characterization. Anal Biochem 349:197–207

Johnson KA, Paisley-Flango K, Tangarone BS, Porter TJ, Rouse JC (2007) Cation exchange-HPLC and mass spectrometry reveal C-terminal amidation of an IgG1 heavy chain. Anal Biochem 360:75–83

Le JC, Bondarenko PV (2005) Trap for MAbs: characterization of intact monoclonal antibodies using reversed-phase HPLC on-line with ion-trap mass spectrometry. J Am Soc Mass Spectrom 16:307–311

Liu H, Gaza-Bulseco G, Sun J (2006) Characterization of the stability of a fully human monoclonal IgG after prolonged incubation at elevated temperature. J Chromatogr B Analyt Technol Biomed Life Sci 837:35–43

Liu H, Gaza-Bulseco G, Faldu D, Chumsae C, Sun J (2008a) Heterogeneity of monoclonal antibodies. J Pharm Sci 97:2426–2447

Liu H, Gaza-Bulseco G, Xiang T, Chumsae C (2008b) Structural effect of deglycosylation and methionine oxidation on a recombinant monoclonal antibody. Mol Immunol 45: 701–708

Lyubarskaya Y, Houde D, Woodard J, Murphy D, Mhatre R (2006) Analysis of recombinant monoclonal antibody isoforms by electrospray ionization mass spectrometry as a strategy for streamlining characterization of recombinant monoclonal antibody charge heterogeneity. Anal Biochem 348:24–39

Olivova P, Chen W, Chakraborty AB, Gebler JC (2008) Determination of N-glycosylation sites and site heterogeneity in a monoclonal antibody by electrospray quadrupole ion-mobility time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 22:29–40

Qian J, Liu T, Yang L, Daus A, Crowley R, Zhou Q (2007) Structural characterization of N-linked oligosaccharides on monoclonal antibody cetuximab by the combination of orthogonal matrix-assisted laser desorption/ionization hybrid quadrupole-quadrupole time-of-flight tandem mass spectrometry and sequential enzymatic digestion. Anal Biochem 364:8–18

Raju TS, Scallon BJ (2006) Glycosylation in the Fc domain of IgG increases resistance to proteolytic cleavage by papain. Biochem Biophys Res Commun 341:797–803

Rehder DS, Dillon TM, Pipes GD, Bondarenko PV (2006) Reversed-phase liquid chromatography/mass spectrometry analysis of reduced monoclonal antibodies in pharmaceutics. J Chromatogr A 1102:164–175

Siemiatkoski J, Lyubarskaya Y, Houde D, Tep S, Mhatre R (2006) A comparison of three techniques for quantitative carbohydrate analysis used in characterization of therapeutic antibodies. Carbohydr Res 341:410–419

Srebalus Barnes CA, Lim A (2007) Applications of mass spectrometry for the structural characterization of recombinant protein pharmaceuticals. Mass Spectrom Rev 26:370–388

Terashima I, Koga A, Nagai H (2007) Identification of deamidation and isomerization sites on pharmaceutical recombinant antibody using H(2)(18)O. Anal Biochem 368:49–60

Tous GI, Wei ZP, Feng JH, Bilbulian S, Bowen S, Smith J, Strouse R, McGeehan P, Casas-Finet J, Schenerman MA (2005) Characterization of a novel modification to monoclonal antibodies: Thioether cross-link of heavy and light chains. Anal Chem 77:2675–2682

Vlasak J, Ionescu R (2008) Heterogeneity of Monoclonal Antibodies relvealed by charge-sensitive methods. Curr Pharm Biotechnol 9:468–481

Wagner-Rousset E, Bednarczyk A, Bussat MC, Colas O, Corvaia N, Schaeffer C, Van Dorsselaer A, Beck A (2008) The way forward, enhanced characterization of therapeutic antibody glycosylation: comparison of three level mass spectrometry-based strategies. J Chromatogr B Analyt Technol Biomed Life Sci 872:23–37

Wang L, Amphlett G, Lambert JM, Blattler W, Zhang W (2005) Structural characterization of a recombinant monoclonal antibody by electrospray time-of-flight mass spectrometry. Pharm Res 22:1338–1349

Wei Z, Feng J, Lin HY, Mullapudi S, Bishop E, Tous GI, Casas-Finet J, Hakki F, Strouse R, Schenerman MA (2007) Identification of a single tryptophan residue as critical for binding activity in a humanized monoclonal antibody against respiratory syncytial virus. Anal Chem 79:2797–2805

Xiao G, Bondarenko PV, Jacob J, Chu GC, Chelius D (2007) 18O labeling method for identification and quantification of succinimide in proteins. Anal Chem 79:2714–2721

Yan B, Valliere-Douglass J, Brady L, Steen S, Han M, Pace D, Elliott S, Yates Z, Han Y, Balland A, Wang W, Pettit D (2007) Analysis of posttranslational modifications in recombinant monoclonal antibody IgG1 by reversed-phase liquid chromatography/mass spectrometry. J Chromatogr A 1164:153–161

Yang J, Wang S, Liu J, Raghani A (2007) Determination of tryptophan oxidation of monoclonal antibody by reversed phase high performance liquid chromatography. J Chromatogr A 1156:174–182

Ying H, Liu H (2007) Identification of an alternative signal peptide cleavage site of mouse monoclonal antibodies by mass spectrometry. Immunol Lett 111:66–68

Yu L, Vizel A, Huff MB, Young M, Remmele RL Jr, He B (2006) Investigation of N-terminal glutamate cyclization of recombinant monoclonal antibody in formulation development. J Pharm Biomed Anal 42:455–463

Zhang Z, Shah B (2007) Characterization of variable regions of monoclonal antibodies by top-down mass spectrometry. Anal Chem 79:5723–5729

Zhang W, Marzilli LA, Rouse JC, Czupryn MJ (2002) Complete disulfide bond assignment of a recombinant immunoglobulin G4 monoclonal antibody. Anal Biochem 311:1–9

Zhang Z, Pan H, Chen X (2009) Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrom Rev 28:147–176

Chapter 40
Antibody Glycans Characterization

Marie-Claire Janin-Bussat, Elsa Wagner-Rousset, Christine Klinguer-Hamour, Nathalie Corvaia, Alain van Dorsselaer, and Alain Beck

40.1 Introduction

Glycosylation is one of the most important posttranslational protein modifications and has essential roles in antibody effectors functions, immunogenicity, plasmatic clearance (Jefferis 2005; Beck et al. 2008a), and resistance toward proteases (Raju and Scallon 2006).

Like for natural IgGs, all recombinant antibodies contain an Asn-Xxx-Ser/Thr (Xxx is not Pro) consensus sequence for N-glycosylation in their C_H2 constant domain. IgG glycans represent only an average of 2% of the total antibody mass and are located generally on residue Asn^{297}. Despite this low percentage, particular glycoforms can be involved in important immune cytotoxic functions. Glycosylation plays also a role in the plasmatic half-life *via* binding to neonatal Fcgamma Receptor (FcγRn). Glycoforms that are not commonly biosynthesized in humans may potentially be immunogenic and must be identified, quantified, and limited for therapeutic use. Glycans of normal polyclonal human IgGs belong to the bi-antennary complex type. A conserved heptasaccharide core is composed of 2 N-acetylglucosamine (GlcNAc), three mannose (Man), and two GlcNAc residues that are β-1,2 linked to α-6 Man and α-3 Man, forming 2 arms. Additional fucose (Fuc), galactose (Gal), and N-acetylneuraminic acid (NANA) residues may or may not be present; Fig. 40.1. Antibody glycans present considerable heterogeneity, with potentially more than 400 glycoforms due to the random pairing of heavy

M-C. Janin-Bussat, E. Wagner-Rousset, C. Klinguer-Hamour, N. Corvaia, and A. Beck (✉)
Centre d'Immunologie Pierre Fabre (CIPF), 5 Avenue Napoléon III, F74160, Saint-Julien-en-Genevois, France
e-mail: alain.beck@pierre-fabre.com

A. van Dorsselaer
Laboratoire de Spectrométrie de Masse Biologique (LSMBO), Université de Strasbourg, 67000 Strasbourg, France

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_40,

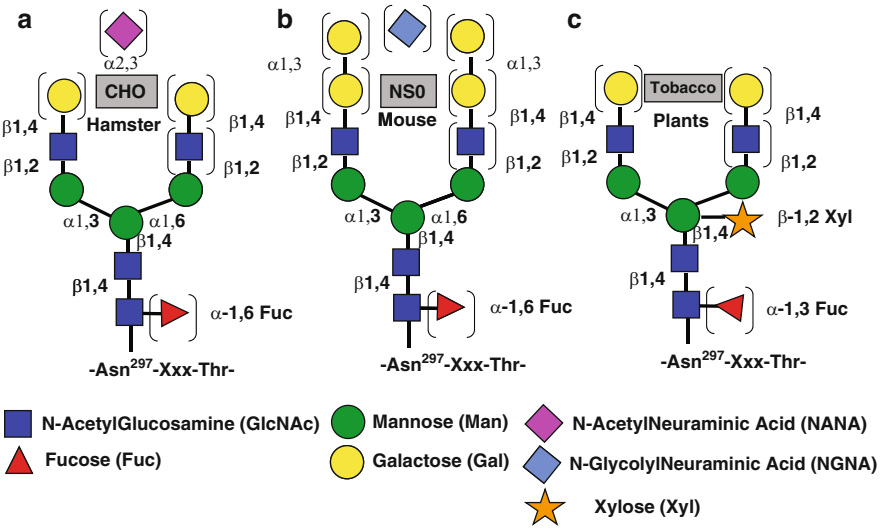


Fig. 40.1 Recombinant antibody glycans produced in (**a**) hamster (CHO cell line), (**b**) murine (NS0 or SP2/0 cell lines as well as mice hybridoma), or (**c**) plant cells (e.g., tobacco leaves). These schematic structures are used to calculate theoretical masses for MS analysis (GlcNac = 203.19 Da; Man, Gal = 162.14 Da; Fuc = 146.14 Da; NGNA = 291.09 Da; NANA = 275.00 Da; 2AB = 136.15 Da). The structures are represented according the nomenclature outlined by the Consortium for Functional Glycomics (http://www.functionalglycomics.org/)

chains, each one containing glycans of different structures (Jefferis 2005; Walsh and Jefferis 2006).

IgG homogeneity and determination of level of glycosylation may be achieved by capillary electrophoresis-sodium dodecyl sulfate (CE-SDS). Orthogonal glycoprofiling of IgG released glycans can be performed by capillary electrophoresis with a laser-induced fluorescent detector (CE-LIF) and by normal-phase high performance liquid chromatography (NP-HPLC). Glycans fine structure assessment can be determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and by nano-liquid chromatography tandem mass spectrometry fragmentation (nano-LC-MS/MS).

40.1.1 Glycosylation of Current Marketed Therapeutic Antibodies

40.1.1.1 Immunoglobulin Isotypes and Glycosylation

Since the first registration of a murine monoclonal antibody 20 years ago (Orthoclone/muromomab), 28 further antibodies and related-products (Fab fragments

naked or PEGylated, Fc-fusion protein or peptides, antibody-drug conjugates, or radio-immunoconjugates) have been approved in various therapeutic indications such as oncology, organ transplantation, rheumatoid and autoimmune diseases, cardiology, infectious diseases, allergy, as well as tissue growth and repair (Beck et al. 2008b). Most of the chimeric, humanized, and human IgGs belong to the IgG1 isotype. Nevertheless, two IgG4 (Mylotarg/gemtuzumab and Tysabri/natalizumab) and one IgG2 (Vectibix/panitumumab) also reached the market (Jefferis 2006). Despite their higher micro-heterogeneity compared to IgG1 (half-antibodies in IgG4, Forrer et al. 2004; 2 vs. 4 disulfide bridges linking the heavy chains in IgG2, Chelius et al. 2006), both isotypes were chosen and developed to avoid effectors functions, which are directly linked to Fc structure and to their glycosylation. It has also been demonstrated that in human plasma around 20% of IgG have an N-glycosylation site in their variable domains (Huang et al. 2006). This was observed for the recombinant Erbitux/cetuximab, which was shown to be hypogalactosylated in its constant domain but fully galactosylated in its variable domain (Holland et al. 2006). Generally, when possible, glycosylation sites are removed from CDRs by genetical engineering to get more homogeneous mAbs, which are easier to develop as biopharmaceuticals. Affinity maturation is then often necessary to reconstruct framework portions to accommodate the CDR mutations. A few IgMs and IgAs were also investigated in clinical trials for particular indications. They show several glycosylation sites, and batch consistency is more difficult to achieve than for IgGs (O-glycosylation in the hinge domain of IgA1s; multiple N-glycosylation in the pentameric IgMs; Arnold et al. 2005).

40.1.1.2 Cell Production Systems and Glycosylation

Recombinant protein glycosylation is highly dependent on the production system, the selected clonal cell population, and the culture process (i.e., feeding strategy). Chinese hamster ovary cells (CHO) and mouse myeloma cells (NS0, SP2) have become the gold-standard eucaryotic host cells used for the production of therapeutic antibodies. These cell lines have been adapted to grow in suspension culture and are well suited for reactor culture, scaling-up, and large volume production, features which are essential for blockbuster antibodies (Butler 2005; Birch and Racher 2006; Chartrain and Chu 2008); Fig. 40.2. Murine NS0 cells produce mAbs exhibiting small amounts of glycoforms with additional α-1,3-galactose and different sialic acids (N-Glycolyl Neuraminic acid vs. N-Acetyl Neuraminic Acid) (Beck et al. 2007). NGNA is the predominant sialic acid in mice and was described as immunogenic in humans (Sheeley et al. 1997). But from a practical point of view, the NGNA amounts present in NS0-produced mAbs are generally very low (around 1–2%). No adverse event linked to these glycoforms was reported for the current nine marketed NS0/SP2-produced mAbs, including Synagis/palivizumab anti-RSV mAb used in neonates since 1998. The same stands for the mouse α-1,3-galactose residue (2–4%) (Beck et al. 2008b).

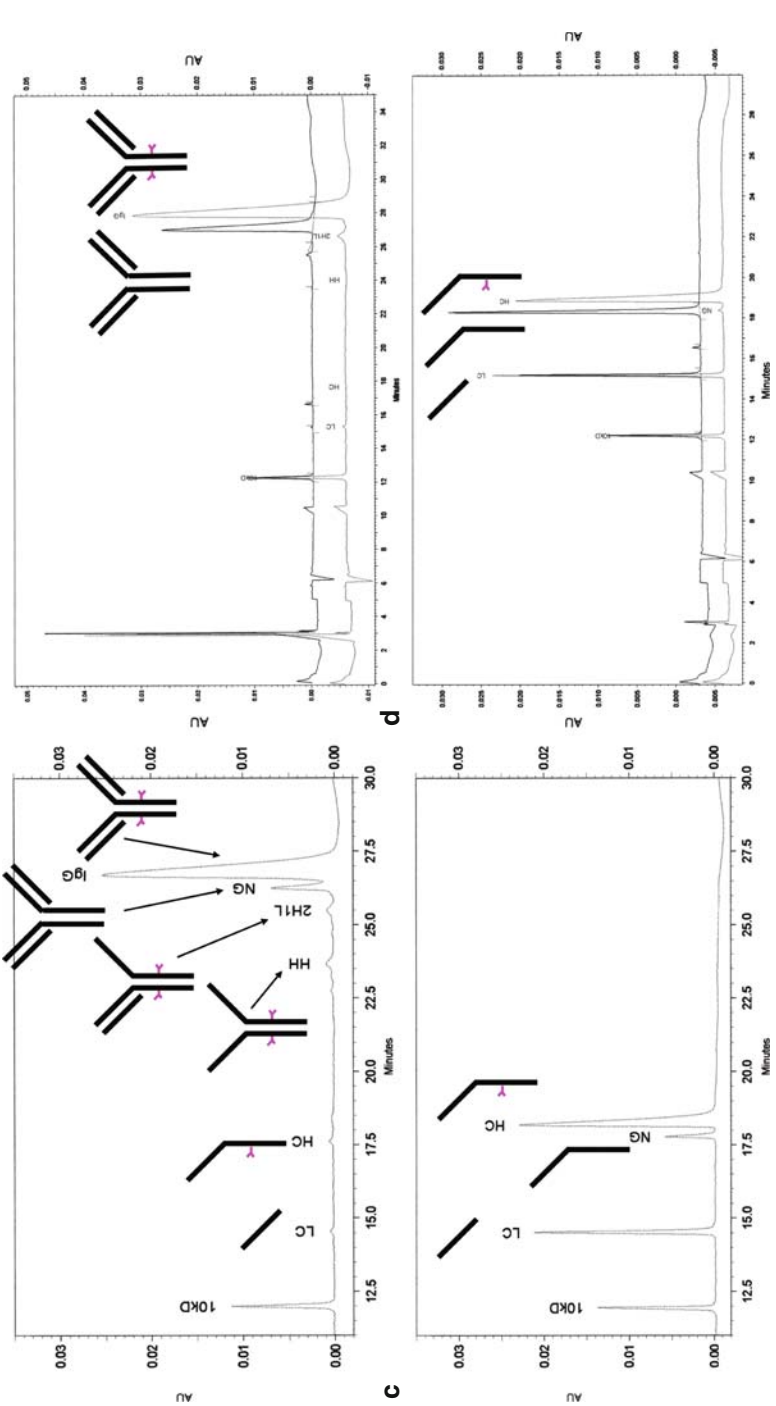


Fig. 40.2 Capillary Electrophoresis of (**a**) an intact recombinant IgG; (**b**) an intact and a PNGase F treated recombinant IgG; (**c**) a reduced IgG and (**d**) a reduced and PNGase F treated recombinant IgG

40.1.2 Benefits of Manipulating the Carbohydrate Components of Antibodies

40.1.2.1 Alternative Cell Production Systems and Glycosylation

Antibodies produced in alternative systems to the current mammalian cells will certainly reach the market in the future. PER.C6 human retina cells were recently proposed as a new system to produce mAbs carrying a human glycosylation profile and a G0F/G1F/G2F ratio close to 1/2/1 (Jones et al. 2003). Actually, this ratio can also be observed for NS0-produced mAbs (Beck et al. 2005) and other glycoforms are essentially a minority.

40.1.2.2 Humanization of Glycosylation in Heterologous Expression Systems

Tremendous research efforts are ongoing to produce proteins with humanized engineered glycoforms. These systems include transgenic animals (Echelard et al. 2006), chicken (Suzuki and Lee 2004), insect cells (Kost et al. 2005; Hodoniczky et al. 2005), and plant-derived antibodies, for example, that featured high level of galactose, undetectable levels of xylose, and traces of fucose (Gomord et al. 2005; Bakker et al. 2006). Yeast strains were also genetically transformed to produce mAbs with "humanized" glycoforms or with enhanced effectors functions (e.g., third bisecting arm, lack of fucose) (Wildt and Gerngross 2005; Hamilton et al. 2006; Li et al. 2006) and the perspective to achieve higher proteins titers than with the current mammalian cells systems, and with lower production costs.

40.1.2.3 Plasmatic Half-Life Improvement

The half-life and therapeutic potency of most glycoproteins (e.g., EPO) are clearly dependent on the presence of terminal sialic acid, with the exception of antibodies (Hamilton et al. 2006).

The role of antibody glycosylation in the long plasmatic half-life via binding to neonatal FcgammaRn remains controversial (Hinton et al. 2006). Serum half-life of human IgGs is remarkably long (21–23 days) when compared to IgAs (6 days), IgMs (5 days), or antibodies fragments (a couple of hours). The half-life of recombinant, current marketed, humanized, and human antibodies is around 20 days, close to natural human IgGs and independent of the CHO or NS0 production system (Lobo et al. 2004). Engineered antibodies with longer half-life (e.g., 6 weeks) may not only reduce the frequency of administration in patients undergoing long-term therapy but may also allow reducing the doses in the future.

40.1.2.4 ADCC Enhancement

Antibody-Dependent Cellular Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC) are important effector functions especially for mAbs developed in Oncology, where the major goal is to kill tumor cells. The presence of a bisecting N-acetylglucosamine (Ferrara et al. 2006) associated with the depletion in fucose residues from oligosaccharides in the conserved attachment region to Fc receptors clearly results in an increase of ADCC (Kanda et al. 2006). This specific glycosylation profile can be favored by glyco-engineering of the production systems, thereby improving significantly in vitro and in vivo ADCC functions.

40.2 Materials

40.2.1 IgG Homogeneity and Level of N-Glycosylation by Capillary Electrophoresis-Sodium Dodecyl Sulfate (CE-SDS)

- Capillary electrophoresis system equipped with a UV detector (PA800, Beckman Coulter, Villepinte, France) or equivalent); UV detection was conducted at 220 nm.
- IgG Purity/Heterogeneity Assay Kit (Beckman Coulter, Villepinte, France):
  - IgG control standard stored at −20°C
  - 10 kDa Internal Standard stored at +4°C
  - SDS gel buffer stored at room temperature
  - SDS sample buffer stored at +4°C
  - Bare Fused-Silica Capillary (Beckman Coulter, Villepinte, France) of 50 μm I.D. and 375 μm O.D. with a total length of 30.2 cm and a effective length of 20.2 cm.
  - Acidic wash solution (0.1 N HCl)
  - Basic wash Solution (0.1 N NaOH)
- 2-Mercaptoethanol (Sigma, Saint-Quentin-Fallavier, France)
- Water Bath (37°C to 100°C) or Heat Block
- Microcon YM-30 Centrifugal Unit (Millipore, Saint-Quentin-en-Yvelines, France)
- 250 mM Iodoacetamide (Sigma, Saint-Quentin-Fallavier, France) solution in water
- Sonicator
- Vortex
- Pipettes of various sizes
- Microfuge Centrifuge

- 0.5 ml Micro-centrifuge Capped Vials
- 2 ml Glass Vials with Caps (Beckman Coulter, Villepinte, France)
- 200 µl PCR Vials
- Micro Vial Springs (Beckman Coulter, Villepinte, France)
- PCR Vial Holders with caps (Beckman Coulter, Villepinte France)

40.2.2 Oligosaccharides Profiling by Capillary Electrophoresis with a Laser-Induced Fluorescent Detector (CE-LIF)

40.2.2.1 Sample Preparation and Purification of Oligosaccharides

- PNGase F (New England Biolabs).
- Graphitized carbon black SPE columns, 3 ml (Supelclean™ ENVI™, Supelco).
- Columns washing solvent: 20% (v/v) water, 0.1% (v/v) trifluoroacetic acid in acetonitrile (HPLC grade).
- Solvant for oligosaccharides elution: 30% (v/v) water, 0.02% (v/v) trifluoroacetic acid in acetonitrile (HPLC grade).
- Heating block, oven, or similar dry heater set at 100°C.
- Reaction vials 1.5 ml (e.g., polypropylene microcentrifuge vials).
- Pipettes of various sizes.

40.2.2.2 Derivatization and CE Analysis

- Capillary electrophoresis system [PA800 (Beckman Coulter, Villepinte France) or equivalent] equipped with a LIF detector (Beckman Coulter, Villepinte France). The LIF detector uses a 3.5 mW argon ion laser with $\lambda_{ex} = 488$ nm and $\lambda_{em} = 560$ nm.
- Carbohydrate labeling and analysis Kit (Beckman Coulter, Villepinte France)
  - N-CHO Capillary (Beckman Coulter, Villepinte France) of 50 µm I.D. and 375 µm O.D. with a total length of 50.2 cm and an effective length of 40 cm.
  - Carbohydrate Separation Gel Buffer.
  - Labeling Dye (APTS)
  - Labeling Dye Solvent
  - Glucose Ladder Standard (G20)
  - Maltose Mobility Marker (G22)
  - APTS-Labeling Reagent (monosaccharide grade)
- 0.5 ml Micro-centrifuge Capped Vials.
- 2 ml Glass Vials with Caps (Beckman Coulter, Villepinte, France).
- 200 µl PCR Vials.
- Micro Vial Springs (Beckman Coulter, Villepinte, France).

- PCR Vial Holders with caps (Beckman Coulter, Villepinte, France).
- Pipettes of various sizes.
- Vortex.
- Water Bath.
- Centrifugal vacuum evaporator.
- Sodium Cyanoborohydride 1 M/THF (Aldrich, Saint-Quentin-Fallavier, France).

40.2.3 Oligosaccharides Profiling by (NP-HPLC)

40.2.3.1 Sample Preparation and Deglycosylation

- Ultra centrifugal filter units, nominal molecular weight limit = 30 kDa (Millipore, Saint Quentin en Yvelines, France)
- Centrifuge up to 10,000 g
- 100 mM sodium bicarbonate (Sigma, Saint-Quentin-Fallavier, France), pH 8.6
- 2% SDS in 100 mM sodium bicarbonate (Sigma, Saint-Quentin-Fallavier, France), pH 8.6
- 1% Igepal (Sigma, Saint-Quentin-Fallavier, France) in water
- PNGase F (New England Biolabs)
- Reaction vials
- Heating block, oven or similar dry heater set at 100°C
- Reaction vials 1.5 ml (e.g., polypropylene microcentrifuge vials)
- Pipettes of various sizes.

40.2.3.2 Desalting of PNGase Released Oligosaccharides

- Graphitized carbon black SPE columns, 1 ml, (Carbograph, Altech or equivalent)
- Columns washing solvent: 20% (v/v) water, 0.1% (v/v) trifluoroacetic acid in acetonitrile HPLC grade
- Solvent for oligosaccharides elution: 30% (v/v) water, 0.02% (v/v) trifluoroacetic acid in acetonitrile HPLC grade
- Centrifugal evaporator.

40.2.3.3 Oligosaccharides Fluorescent Derivation by 2-Aminobenzamide (2-AB)

- Signal™ 2-AB labeling kit (Prozyme, San Leandro, USA)
  - 2-aminobenzamide (2 –AB) Dye 2 × 5 mg
  - DMSO 2 × 400 µl

- Glacial acetic acid 2 × 200 µl
- Reductant (Sodium cyanoborohydride) 2 × 6 mg

– GlycoClean™ S Cartridges (Promozyme, San Leandro, USA)
– Trifluoroacetic acid, analysis grade
– Acetonitrile, HPLC grade
– Heating block, oven, or similar dry heater set at 65°C
– Centrifugal evaporator
– Reaction vials (e.g., polypropylene microcentrifuge vials)
– Oligomannose standards (Promozyme, San Leandro, USA)

40.2.3.4 Normal Phase HPLC

– Chromatography System with fluorescence detector (Waters, Saint-Quentin-en-Yvelines, France)
– Column: Glyco-Sep N (Promozyme, San Leandro, USA)
– Mobile phase A: 50 mM ammonium formate, pH 4.4
– Mobile phase B: acetonitrile, HPLC grade.

40.2.4 Oligosaccharides Analysis by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)

– Ultraflex™ TOF/TOF Mass Spectrometer (Bruker Daltonics, Bremen, Germany) with gridless ion optics under the control of Flexcontrol 2.0, instrument equipped with a SCOUT™ high-resolution optics with X-Y multisample probe and a grid-less reflector
– 2,5-Dihydroxybenzoic acid (DHB)

40.2.5 Oligosaccharides Fragmentation by Nano-Liquid Chromtogarphy Tandem Mass Spectrometry Fragmentation (Nano-LC-MS/MS)

– Esquire 3000+™ Ion Trap (Brukey Daltonics, Bremen, Germany) equipped with an electrospray ion source working in positive mode.

40.3 Protocols

40.3.1 IgG homogeneity and Level of N-Glycosylation by CE-SDS

40.3.1.1 Reduced IgG Control Standard and Samples Preparation

1. Take
   (a) for IgG control standard: 1 micro-centrifuge vial of 95 µl aliquots of IgG control standard.
   (b) for sample analysis: Place 100 µg of IgG sample in a volume less than 45 µl, in a 0.5 ml micro-centrifuge vial and add 50–95 µl of sample buffer to give a final volume of 95 µl.
   Note: If the sample concentration is less than 10 mg/ml and the buffer concentration is more than 50 mM, the buffer (Volume = V) of the IgG sample must be exchanged with 4 V SDS-MW sample buffer, by using the Microcon YM30 centrifuge filter unit.
2. Add 2 µl of 10 KDa
3. Add 5 µl 2-mercaptoethanol
4. Cap the vial and mix thoroughly
5. Centrifuge at 1,000 *g* for 1 min
6. Heat the mixture at 70°C for 10 min
7. Centrifuge at 12,000 *g* for 6 min.
8. Transfer 100 µl of the prepared sample into a 200 µl PCR vial.

40.3.1.2 Non-Reduced IgG Control Standard and Samples Preparation

1. Apply the protocol of reduced IgG control (3.1.1) except for step 3: add 5 µl of 250 mM iodoacetamine solution instead of 2-mercaptoethanol.

40.3.1.3 CE IgG Profiling

The CE program for mapping is as follows:

1. Prior injection, rinse capillary at 70 psi with 0.1 N NaOH, 0.1 N HCl, water, and SDS running buffer for 3, 1, 1, and 10 min, respectively.
2. Dip capillary in water two times.
3. Inject sample electrokinetically by applying voltage at −5 kV for 20 s.
4. Dip capillary in water.
5. Conduct CE analysis in negative polarity mode (−15 kV, −497 V/cm) 35 min. The current generated is approximately 27 µAmps. Maintain capillary and samples at 25°C and apply 20 psi to both inlet and outlet.

40.3.1.4 Notes

1. Use PNGase F for oligosaccharides release of mammalian-cell produced mAbs (CHO, NS0, SP2/0, hybrodoma...) and PNGase A for vegetal-cell produced mAbs.
2. Capillary Preconditioning method: Precondition a new capillary or a re-used capillary that has been stored for a long period as follows:
   (a) Rinse capillary at 20 psi with 0.1 N NaOH, 0.1 N HCl, and water for 10, 5, and 2 min, respectively.
   (b) Rinse capillary at 70 psi with SDS running buffer.
   (c) Equilibrate capillary at 15KV for 10 min, 5 min ramping.
3. Capillary cleaning method for long term storage of capillary, perform a cleaning method to clean the capillary and fill the capillary with DDI H_2O.

40.3.2 Oligosaccharides Profiling by CE-LIF

40.3.2.1 Sample and Deglycosylation

1. Take 1 mg of IgG solution (IgG concentration between 2 and 10 mg/ml).
2. Add 3 μl PNGase F and incubate overnight with at 37°C

40.3.2.2 Purification of Oligosaccharides

1. Prepare graphitized carbon black SPE columns: Wash three times each cartridge with 1 ml water/CH_3CN 20/80 with TFA 0.1% and with 3 × 1 mL H_2O.
2. Apply the sample to a graphitized carbon black SPE column.
3. Elute the column with 2 ml of water to remove salts.
4. Elute oligosaccharides with 2 ml of 30% (v/v) water, and 0.02% (v/v) trifluoacetic acid in acetonitrile into a 1.5 ml Eppendorf tube.
5. Prepare a tube with 3 nmoles glucose ladder standard.
6. Add 5 nmoles of maltose mobility marker to each sample.
7. Dry samples overnight on centrifugal evaporator.

40.3.2.3 Derivatization of Oligosaccharides

1. Add 2 μl of sodium cyanoborohydride 1 M/THF to each sample.
2. Add 2 μl APTS-labeling reagent to each sample; APTS-labeling reagent is prepared by addition of 48 μl of labeling dye solvent to a vial of ATPS labeling dye.
3. Leave reaction mixture at 55°C for 2 h with stirring.
4. Add to sample and glucose ladder standard 20 μl and 96 μl of water, respectively.

40.3.2.4 CE-LIF Oligosaccharides Profiling

The CE program for mapping is as follows:

1. Prior to injection, rinse capillary at 30 psi with carbohydrate separation gel buffer for 10 min.
2. Inject sample by pressure at 0.5 psi for 4.0 s.
3. Dip capillary in water.
4. Conduct CE-LIF analysis in reverse polarity mode (−30 kV, −598 V/cm) 20 min with 0.17 increment ramp. The current generated is approximately 13 μAmps. Maintain Capillary at 25°C and samples at 10°C.

40.3.2.5 Notes

1. Derivatization of oligosaccharides: work above a fume hood
2. Capillary long term storage:
   (a) Rinse capillary at 30 psi with water.
   (b) Rinse capillary at 30 psi with carbohydrate separation gel buffer

40.3.3 Oligosaccharides Analysis by NP-HPLC

40.3.3.1 Sample Preparation and Deglycosylation

1. Concentrate native antibody (600 μg) on ultra centrifugal filter units by centrifugation.
2. Wash three times with 400 μl of 100 mM sodium bicarbonate, pH 8.6 on Amicon ultra centrifugal filter unit by centrifugation.
3. Concentrate to have a final volume of 150 μl (3 × 50 μl aliquots) on Amicon ultra centrifugal filter unit by centrifugation.
4. Add to each aliquot 50 μl 2% SDS in 100 mM sodium bicarbonate and denature for 10 min at 100°C.
5. Add 900 μl of 1% Igepal and incubate overnight with 1 μl PNGase F at 37°C.

40.3.3.2 Desalting of Oligosaccharides

1. Condition SPE columns with 3 column volumes 80% (v/v) acetonitrile/water in 0.1% (v/v) TFA and with 3 column volumes water.
2. Apply the sample to a carbon column.
3. Elute the column with 2 ml of water to remove salts and detergent.
4. Elute neutral and sialylated oligosaccharides with 2 ml of 30% (v/v) water, 0.02% (v/v) trifluoacetic acid in acetonitrile.
5. Dry samples overnight on centrifugal evaporator.

40.3.3.3 2-AB Oligosaccharide Labeling and Standards

1. Prepare fresh labeling reagent: with components supplied in the SignalTM 2-AB labeling kit:
   (a) Add 150 μl of acetic acid (from Vial C) to a vial of DMSO (Vial B) and mix by pipetting action
   (b) Add 100 μl of acetic acid/DMSO mixture to a vial of 2-AB Dye (Vial A) and mix until the dye is dissolved
   (c) Add all of the acetic acid/DMSO/2-AB dye mixture to a vial of reductant (Vial D) and mix by pipetting action until the reductant is completely dissolved. This is the Labeling Reagent; protect from exposure to moisture and use within 1 h.
2. Add 5 μl of Labeling Reagent to each dried glycan sample, cap the microtube, mix thoroughly, and centrifuge at low speed.
3. Place the reaction vials in a heating block at 65°C and incubate for 3 h.
4. After incubation, centrifuge each reaction tube.
5. Allow to cool completely to room temperature.

40.3.3.4 Clean-up of Glycan Samples

1. Prepare GlycoClean S cartridges:
   (a) Wash each cartridge with 1 ml water.
   (b) Wash with 5 ml acetic acid solution (allow to drain completely).
   (c) Wash with 3 ml acetonitrile (allow to drain completely).
   (d) Finally wash with an additional 1 ml acetonitrile and allow to drain completely.
2. Spot each sample onto a freshly washed cartridge membrane.
3. Leave for 15 min to allow the glycans to adsorb onto membrane.
4. Wash each cartridge with 1 ml of acetonitrile, followed by 5 × 1 ml of 96% acetonitrile solution.
5. Elute the glycans with three washes of 0.5 ml water into a 2 ml Eppendorf tube.
6. Prepare standards as follows: into 2 ml Eppendorf tubes, add 5 μl of 1 mg/ml oligomannose solution in water.
7. Dry samples and standards overnight on centrifugal evaporator.

40.3.3.5 NP- HPLC of 2-AB Derived Oligosaccharides

1. The gradient program for mapping is as follows (Glyco-Sep N column, detection at $\lambda_{ex} = 330$ nm and $\lambda_{em} = 420$ nm): initial conditions 20% mobile phase A at a flow rate of 0.4 ml/min, linear gradient of 20–53% mobile phase A over 132 min at a flow rate of 0.4 ml/min, linear gradient of 53–100% mobile phase A over

3 min at a flow rate of 0.4 ml/min, wash with 100% mobile phase A for 5 min at a flow rate of 1.0 ml/min, and equilibrate for 35 min at 0.4 ml/min.

2. Standards and samples preparation:
   (a) Add to samples 200 µl water/acetonitrile 30/70 (v/v) solution and 400 µl to oligomannose standards.
   (b) Mix samples.
3. Inject 5 µl to standards and 20 µl to samples.

40.3.4 Oligosaccharides Mass Determination by MALDI-TOF

1. Dried droplet preparation was performed for MALDI analyzes:
   (a) Place 0.5 µl 2.5-dihydroxybenzoic acid (DHB, Bruker), 20 mg/mL in 50/50 (v/v) water/acetonitrile on stainless steel MALDI target.
   (b) Depose 0.5 µl of sample on matrix.
   (c) Dry the target under atmospheric conditions
2. Perform externally calibration spectra with a mixture of mono-isotopic peaks of peptide calibration mixture [Bradykinin 1–7 (m/z 757.400), Angiotensin II (m/z 1046.542), Angiotensin I (m/z 1296.685), Substance P (m/z 1347.735), bombesin (m/z 1619.822), rennin (m:z 1758.933), ACTH clip 1–17 (m/z = 2093.087), ACTH 1–17 (m/z = 2093.087), and ACTH 18–39 (m/z = 2465.199).
3. MALDI-TOF analyzes: use the instrument at a maximum accelerating potential of 25 kV and operate in reflector mode.

40.3.5 Oligosaccharides Fragmentation by Nano-LC-MS/MS

1. The sample solutions were infused at 4 µl/min with a syringe pump. The voltage applied to the counter electrode was set at 4,000 V.
2. The voltage on the metal glass capillary interface was optimized at 225 V and the voltage applied to the skimmer was set at 40 V.
3. For tandem mass experiments, the precursor ions were fragmented applying a resonance frequency on the end cap electrodes (peak to- peak amplitude 0.8 V) matching to the frequency of the selected ions.
4. Fragmentation of the precursor ions occurred in the ion trap due to collisions with helium gas buffer (pressure 5 mPa).
5. Scanning was performed in standard resolution mode at a scan rate of 13,000 *m*/*z* per second.
6. A total of 50 scans were averaged to obtain a mass spectrum.
7. Calibration of the ion trap was performed using ions from a Tuning Mix™ solution (Bruker Daltonics, Bremen, Germany) over the mass range.

40.4 Results

40.4.1 IgG Purity and Level of N-Glycosylation by CE-SDS

Capillary Electrophoresis-Sodium Dodecyl Sulfate (CE-SDS) is a resolutive and quantitative method (peak area surface integration), which tends to replace slab-gels (Rustandi et al. 2008). Figure 40.2 shows electropherograms of an intact IgG (glyco-H2L2) treated or not with PNGase F under reducing and non-reducing conditions. This method allows relative quantifications of the main peak (glyco-H2L2), of a non-glycosylated variant (NG-H2L2) as well as of antibody fragments incompletely processed during the culture step (H2L, H2, H and L) (Kamoda and Kakehi 2008).

40.4.2 Oligosaccharides Profilling by CE

Capillary electrophoresis with laser induction fluorescence detection (LIF) is a powerful tool for oligosaccharide analysis due to its high resolving ability to separate oligosaccharides having similar structures and automatic operating systems. For peak identification in CE analysis, comparison of the migration time of the unknown oligosaccharide with that of the standard oligosaccharide is useful.

It is also possible to discriminate between the isomers of G1F and G1F' in antibody oligosaccharides by digestion with combinations of glycosidases.

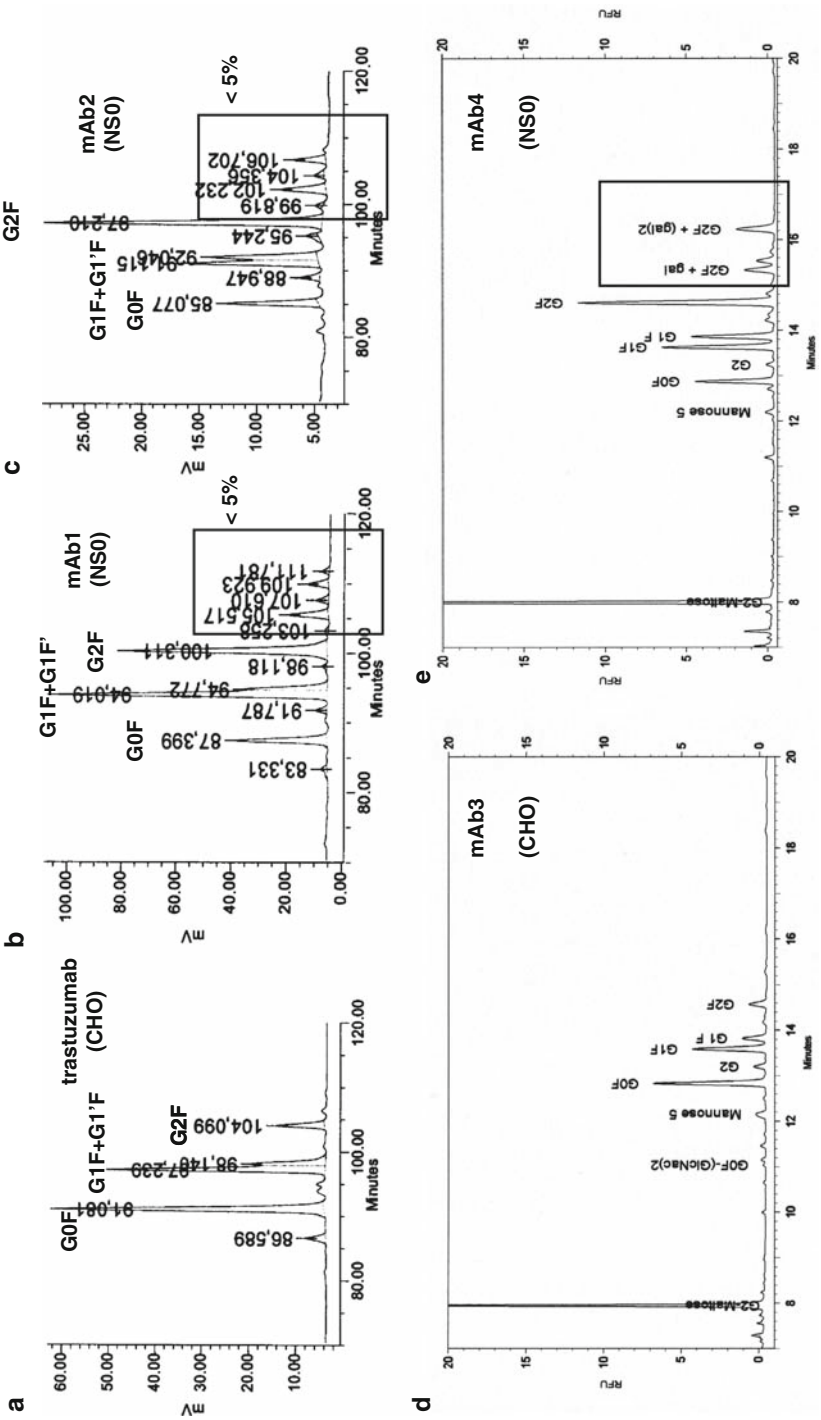
Structural analysis using capillary electrophoresis combined to mass-spectrometry (CE-MS) is a very effective method (Kamoda and Kakehi 2006), but not currently available in most of the laboratories.

40.4.3 Oligosaccharides Profilling by NP-HPLC

NP-HPLC is a well-established analytical method to separate mixtures of oligosaccharides after enzymatic release (e.g., PNGase F or A) and fluorescent derivation (e.g., 2-aminobenzamide). Sub-picomolar levels can be detected and accurately quantified (Guile et al. 1996). Alternative methods like zwitterionic type of hydrophilic-interaction chromatography (ZIC-HILIC) were recently proposed for isomeric N-glycan separations (Takegawa et al. 2006).

Figures 40.3 and 40.4 show representative examples of NP-HPLC chromatograms obtained for oligosacharides, which were released after PNGAse F digestion of IgGs. The different peaks can be isolated and submitted to off-line analysis by MALDI-TOF (see above).

NP-HPLC can routinely be used in most of the laboratories, for IgGs glycan fingerprinting, as illustrated in Fig. 40.3 for mAbs produced in different cell lines



Janin-Bussat MC_Beck A, Chp 37, Glycoanalysis, Kontermann & Dübel, Springer, 2009

(e.g., CHO or NS0), which clearly exhibit different patterns. NS0 cell produced mAbs exhibit small amounts of glycoforms with additional α-1,3-galactose and different sialic acids (NGNA vs. NANA). The same stands for plant vs. CHO produced mAbs (Beck et al. 2005, 2008c), using PNGase A for the release of the glycans.

40.4.4 Oligosaccharides Mass Determination by MALDI-TOF

The different peaks, NP-HPLC isolated peaks, can be isolated and submitted to off-line analysis by MALDI-TOF mass spectrometry. For seven of them, the experimental masses were in excellent agreement with the calculated masse, as shown in Fig. 40.4.

40.4.5 Oligosaccharides Fragmentation by Nano-LC-MS/MS

Tandem mass spectrometry is a method of choice to determine linear sequences, branching, and linkages of glycans. Full structural information can be obtained by fragmentation analysis of isolated N-glycans by ESI–MS/MS with high or low collision-induced dissociation (CID) (Wagner-Rousset et al. 2008). For that purpose, an ion trap mass spectrometer (IT-MS) with its ability to perform multiple stages of fragmentation (MS^n), can be used. Figure 40.5 displays a representative MS/MS spectrum for the G1F glycoform. By collision with helium, the singly charged parent $[M+H]^+$ ion (*m*/*z* 1625.6) produces a large variety of ions resulting exclusively from glycosidic cleavages. The pattern of ions yields structural information consistent with the expected N-linked oligosaccharide structure. The parent ion and some fragments show primary loss of a molecule of water (−18 Da). The prominent ion at *m*/*z* 1260.6 is due to the loss of a single GlcNAc residue from the non-reducing terminus. The spectrum reflects two main fragmentation pathways. Within the first pathway, the Y ion series at *m*/*z* 1260.6, 1057.5, and 895.4 conserves the core fucosyl region intact and results in progressive loss of mannose from the non-reducing terminus. The second pathway, which generates smaller

Fig. 40.3 NP-HPLC fingerprinting of three recombinant IgGs produced in (**a**) CHO cells and in (**b** and **c**) NS0 cells, respectively. For all three IgGs, four mains glycoforms (so called G0F, G1F/G1'F, G2F) are observed in different ratios. For the mAbs produced in the NS0 cell line additional glycoforms are present in low amounts and are characteristic of mice (Mono and Di alpha-1, 3 GalGal are present as well as traces of mono and di-NGNA). CE-LIF fingerprinting of two recombinant IgGs produced in (**d**) CHO cells and (**e**) NS0. For both mAbs, four mains glycoforms (G0F, G1F/G1'F, G2F) are observed in different ratios. Capillary Electrophoresis (CE) compared to NP-HPLC allows easier and faster sample preparation, shorter running time (20 min vs. 115 min), and better resolution (e.g., G1F/G1'F resolution, G2. . .)

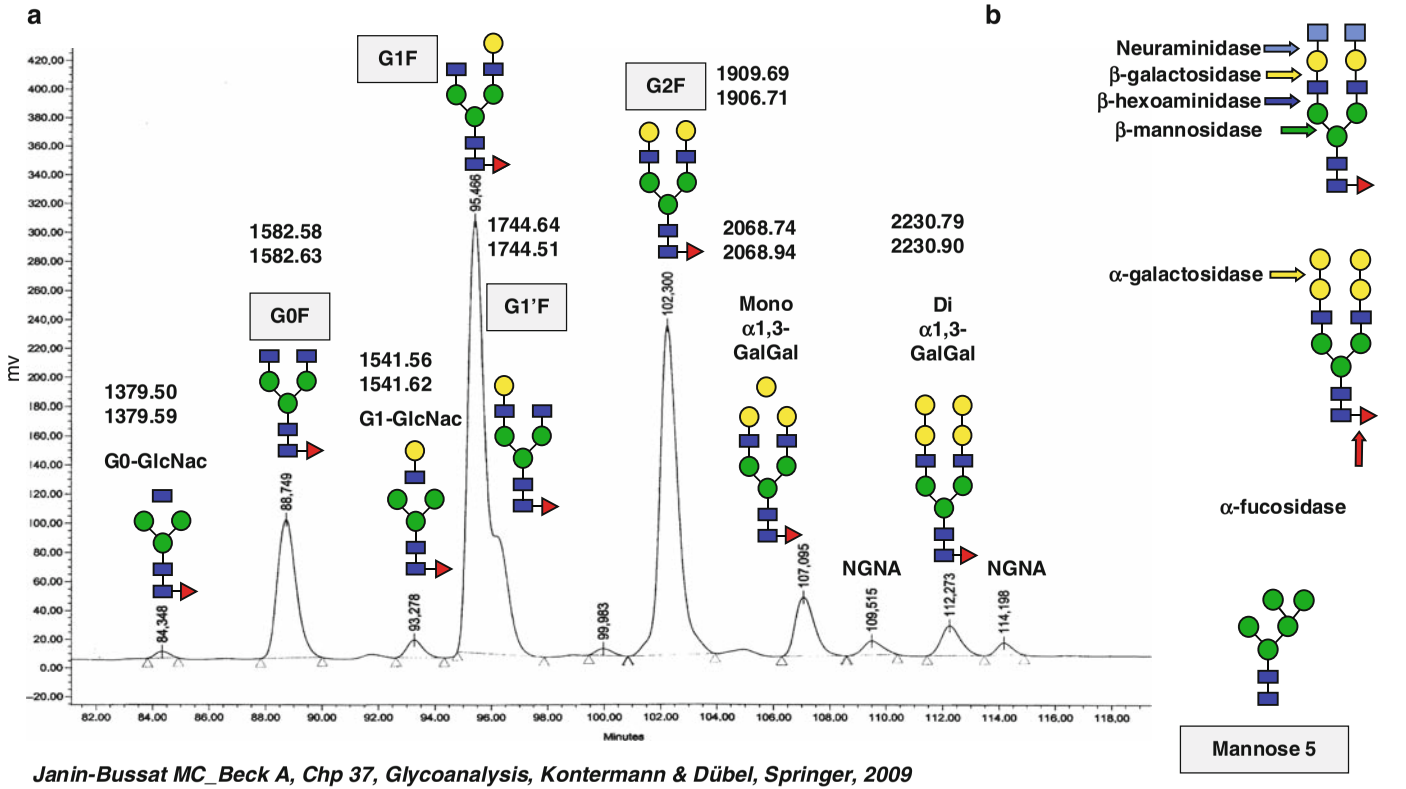


Fig. 40.4 NP-HPLC of oligosaccharides released by a PNGase treatment of a recombinant IgG and derivatized with a fluorescent dye (2-AB). The structure of each isolated peak was submitted to MALDI-TOF mass spectrometry analyzes (calculated mass/experimental mass) and/or to sequential digestion with exoglycosidases for interpretation

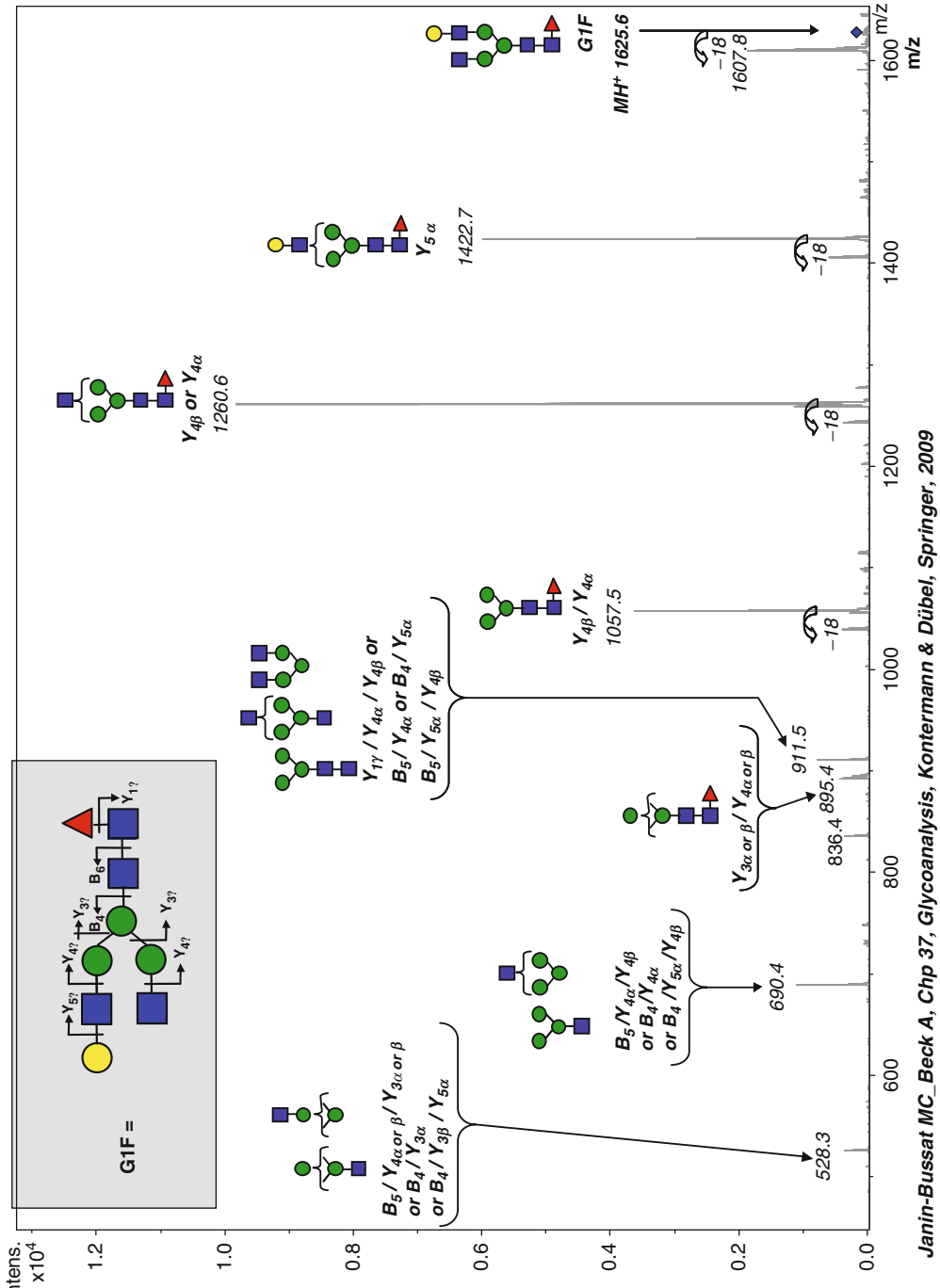


Fig. 40.5 Tandem mass spectra of glycan G1F isolated with *m/z* 1625.6 by ES-Ion Trap and structural interpretation of the fragmentation pattern (see the text)

peaks, first triggers the loss of the fucosyl residue together with a GlcNAc (*m*/*z* 1114.5). Several ions (*m*/*z* 690.4 and 528.3) are coincident in mass with multiple glycosidic cleavages and may be the result of both pathways. Such ions correspond to the internal fragments (Man)2(GlcNAc)2, (Man)3(GlcNAc)1, and (Man)1 (GlcNAc)1, respectively. We were not able to see smaller fragments due the low mass cut-off of the ion trap.

40.5 Conclusion

Capillary Electrophoresis with Laser-induced Fluorescence detection (CE-LIF) is a powerful technique for oligosaccharide analysis due to its high resolution and compatibility with automatic operating systems (Chen et al. 2008). CE-LIF can also be used with IgG released oligosaccharides with 2-AB derivatization (Kamoda et al. 2006). As illustrated in Fig. 40.4, CE-LIF offers advantages over the classical method of NP-HPLC: easier and faster sample preparation, shorter analytical runs (20 vs. 120 min), and better resolution (e.g., the G1F/G1'F glycoforms are fully resolved in the case of CE analysis and may be better quantified for structure-activity relationship studies). In complement, structural analysis using capillary electrophoresis combined to mass-spectrometry (CE-MS) looks also promising for the future (Gennaro and Salas-Solano 2008).

Beyond classical electrophoretic and chromatographic methods, mass spectrometry (MS) coupled to liquid chromatography plays an increasing role both during mAb screening and for fine carbohydrate structural characterization (Gadgil et al. 2006; Rehder et al. 2006; Prater et al. 2009). New-high throughput methods of glycan analysis are in development based on MS. All these high-performance new analytical methods are also used to analyze the glycan patterns of the targeted antigens in structure-activity relationship studies.

References

Arnold JN, Royle L, Dwek RA, Rudd PM, Sim RB (2005) Human immunoglobulin glycosylation and the lectin pathway of complement activation. Adv Exp Med Biol 564:27–43

Bakker H, Rouwendal GJA, Karnoup AS, Florack DEA, Stoopen GM, Helsper JPFG, Van Ree R, Van Die I, Bosch D (2006) An antibody produced in tobacco expressing a hybrid β-1, 4-galactosyltransferase is essentially devoid of plant carbohydrate epitopes. Proc Natl Acad Sci USA 103:7577–7582

Beck A, Bussat MC, Zorn N, Robillard V, Klinguer-Hamour C, Chenu S, Goetsch L, Corvaia N, Van Dorsselaer A, Haeuw JF (2005) Characterization by liquid chromatography combined with mass spectrometry of monoclonal anti-IGF-1 receptor antibodies produced in CHO and NS0 cells. J Chromatogr B Analyt Technol Biomed Life Sci 819:203–218

Beck A, Klinguer-Hamour C, Bussat MC, Champion T, Haeuw JF, Goetsch L, Wurch T, Sugawara M, Milon A, Van Dorsselaer A, Nguyen T, Corvaia N (2007) Peptides as tools and drugs for immunotherapies. J Pept Sci 13:588–602

Beck A, Wagner-Rousset E, Bussat MC, Lokteff M, Klinguer-Hamour C, Haeuw JF, Goetsch L, Wurch T, Van Dorsselaer A, Corvaia N (2008a) Trends in Glycosylation, Glycoanalysis and Glycoengineering of Therapeutic Antibodies and Fc-Fusion Proteins. Curr Pharm Biotechnol 9:482–501

Beck A, Wurch T, Corvaïa N (2008b) Editorial: therapeutic antibodies and derivatives: from the bench to the clinic. Curr Pharm Biotechnol 9:421–422

Beck A, Wagner-Rousset E, Goetsch L, Corvaïa N (2008c) Therapeutic antibodies: structure assessment by mass spectrometry from screening to clinical batches. Screen Trends Drug Discov 9:18–20

Birch JR, Racher AJ (2006) Antibody production. Adv Drug Deliv Rev 58:671–685

Butler M (2005) Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. Appl Microbiol Biotechnol 68:283–291

Chartrain M, Chu L (2008) Development and production of commercial therapeutic monoclonal antibodies in mammalian cell expression system: an overview of the current upstream technologies. Curr Pharm Biotechnol 9:447–467

Chelius D, Huff Wimer ME, Bondarenko PV (2006) Reversed-phase liquid chromatography in-line with negative ionization electrospray mass spectrometry for the characterization of the disulfide-linkages of an immunoglobulin gamma antibody. J Am Soc Mass Spectrom 17:1590–1598

Chen X, Tang K, Lee M, Flynn GC (2008) Microchip assays for screening monoclonal antibody product quality. Electrophoresis 29:4993–5002

Echelard Y, Ziomek CA, Meade HM (2006) Production of recombinant therapeutic proteins in the milk of transgenic animals. BioPharm Int 19:36–46

Ferrara C, Brunker P, Suter T, Moser S, Puntener U, Umana P (2006) Modulation of therapeutic antibody effector functions by glycosylation engineering: influence of Golgi enzyme localization domain and co-expression of heterologous beta1, 4-N-acetylglucosaminyltransferase III and Golgi alpha-mannosidase II. Biotechnol Bioeng 93:851–861

Forrer K, Hammer S, Helk B (2004) Chip-based gel electrophoresis method for the quantification of half-antibody species in IgG4 and their by- and degradation products. Anal Biochem 334:81–88

Gadgil HS, Pipes GD, Dillon TM, Treuheit MJ, Bondarenko PV (2006) Improving mass accuracy of high performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry of intact antibodies. J Am Soc Mass Spectrom 17:867–872

Gennaro LA, Salas-Solano O. (2008) On-Line CE-LIF-MS Technology for the Direct Characterization of N-Linked Glycans from Therapeutic Antibodies. *Analytical Chemistry* (in press)

Gomord V, Chamberlain P, Jefferis R, Faye L (2005) Biopharmaceutical production in plants: problems, solutions and opportunities. Trends Biotechnol 23:559–565

Guile GR, Rudd PM, Wing DR, Prime SB, Dwek RA (1996) A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. Anal Biochem 240:210–226

Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi BK, Hopkins D, Wischnewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S, Gerngross TU (2006) Humanization of yeast to produce complex terminally sialylated glycoproteins. Science 313:1441–1443

Hinton PR, Xiong JM, Johlfs MG, Tang MT, Keller S, Tsurushita N (2006) An engineered human IgG1 antibody with longer serum half-life. J Immunol 176:346–356

Hodoniczky J, Zheng YZ, James DC (2005) Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling in vitro. Biotechnol Prog 21:1644–1652

Holland M, Yagi H, Takahashi N, Kato K, Savage COS, Goodall DM, Jefferis R (2006) Differential glycosylation of polyclonal IgG, IgG-Fc and IgG-Fab isolated from the sera of patients with ANCA-associated systemic vasculitis. Biochim Biophys Acta Gen Subj 1760:669–677

Huang LH, Biolsi S, Bales KR, Kuchibhotla U (2006) Impact of variable domain glycosylation on antibody clearance: an LC/MS characterization. Anal Biochem 349:197–207

Jefferis R (2005) Glycosylation of recombinant antibody therapeutics. Biotechnol Prog 21:11–16
Jefferis R (2006) Criteria for selection of IgG isotype and glycoform of antibody therapeutics. BioProcess Int 4:40–43
Jones D, Kroos N, Anema R, van Montfort B, Vooys A, van Der KS, van Der HE, Smits S, Schouten J, Brouwer K, Lagerwerf F, van Berkel P, Opstelten DJ, Logtenberg T, Bout A (2003) High-level expression of recombinant IgG in the human cell line per.c6. Biotechnol Prog 19:163–168
Kamoda S, Kakehi K (2006) Capillary electrophoresis for the analysis of glycoprotein pharmaceuticals. Electrophoresis 27:2495–2504
Kamoda S, Kakehi K (2008) Evaluation of glycosylation for quality assurance of antibody pharmaceuticals by capillary electrophoresis. Electrophoresis 29:3595–3604
Kamoda S, Ishikawa R, Kakehi K (2006) Capillary electrophoresis with laser-induced fluorescence detection for detailed studies on N-linked oligosaccharide profile of therapeutic recombinant monoclonal antibodies. J Chromatogr A 1133:332–339
Kanda Y, Yamane-Ohnuki N, Sakai N, Yamano K, Nakano R, Inoue M, Misaka H, Iida S, Wakitani M, Konno Y, Yano K, Shitara K, Hosoi S, Satoh M (2006) Comparison of cell lines for stable production of fucose-negative antibodies with enhanced ADCC. Biotechnol Bioeng 94:680–688
Kost TA, Condreay JP, Jarvis DL (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat Biotechnol 23:567–575
Li H, Sethuraman N, Stadheim TA, Zha D, Prinz B, Ballew N, Bobrowicz P, Choi BK, Cook WJ, Cukan M, Houston-Cummings NR, Davidson R, Gong B, Hamilton SR, Hoopes JP, Jiang Y, Kim N, Mansfield R, Nett JH, Rios S, Strawbridge R, Wildt S, Gerngross TU (2006) Optimization of humanized IgGs in glycoengineered Pichia pastoris. Nat Biotechnol 24:210–215
Lobo ED, Hansen RJ, Balthasar JP (2004) Antibody pharmacokinetics and pharmacodynamics. J Pharm Sci 93:2645–2668
Prater BD, Connelly HM, Qin Q, Cockrill SL (2009) High-throughput immunoglobulin G N-glycan characterization using rapid resolution reverse-phase chromatography tandem mass spectrometry. Anal Biochem 385:69–79
Raju TS, Scallon BJ (2006) Glycosylation in the Fc domain of IgG increases resistance to proteolytic cleavage by papain. Biochem Biophys Res Commun 341:797–803
Rehder DS, Dillon TM, Pipes GD, Bondarenko PV (2006) Reversed-phase liquid chromatography/mass spectrometry analysis of reduced monoclonal antibodies in pharmaceutics. J Chromatogr A 1102:164–175
Rustandi RR, Washabaugh MW, Wang Y (2008) Applications of CE SDS gel in development of biopharmaceutical antibody-based products. Electrophoresis 29:3612–3620
Sheeley DM, Merrill BM, Taylor LC (1997) Characterization of monoclonal antibody glycosylation: comparison of expression systems and identification of terminal alpha-linked galactose. Anal Biochem 247:102–110
Suzuki N, Lee YC (2004) Site-specific *N*-glycosylation of chicken serum IgG. Glycobiology 14:275–292
Takegawa Y, Deguchi K, Keira T, Ito H, Nakagawa H, Nishimura S (2006) Separation of isomeric 2-aminopyridine derivatized *N*-glycans and *N*-glycopeptides of human serum immunoglobulin G by using a zwitterionic type of hydrophilic-interaction chromatography. J Chromatogr A 1113:177–181
Wagner-Rousset E, Bednarczyk A, Bussat MC, Colas O, Corvaia N, Schaeffer C, Van Dorsselaer A, Beck A (2008) The way forward, enhanced characterization of therapeutic antibody glycosylation: comparison of three level mass spectrometry-based strategies. J Chromatogr B Analyt Technol Biomed Life Sci 872:23–37
Walsh G, Jefferis R (2006) Post-translational modifications in the context of therapeutic proteins. Nat Biotechnol 24:1241–1252
Wildt S, Gerngross TU (2005) The humanization of N-glycosylation pathways in yeast. Nat Rev Microbiol 3:119–128

Chapter 41
Affinity Measurements by Competition ELISA

Pierre Martineau

41.1 Introduction

Antibodies and their fragments are widely used tools for research, diagnostics, and therapy. Among the different parameters that characterize antibody molecules, the affinity for the antigen is of premium importance. There are several available approaches to measure the affinity constant of an antibody molecule, but most of them either rely on expensive equipment (Surface Plasmon resonance), or are not generally applicable (Fluorescence modifications, dialysis) or require a modified antigen (precipitation of radio-labeled antigen). This is not the case of the ELISA-based procedure developed by M. Goldberg and collaborators (Friguet et al. 1985, 1995, 1997), which only relies on the availability of small amounts of purified antigen and antibody.

To determine the true affinity constant in solution, proper experimental conditions must be chosen to avoid introducing a bias that could lead to a tenfold underestimate of the affinity value. First, it is best to use a monovalent antibody fragment (Fab or scFv). It is possible to adapt the mathematics for bivalent fragments but only if the antigen contains a single binding site per molecule (Stevens 1987). However, with the advent of antibody engineering, it is now easy to produce such fragments in *Escherichia coli*. Second, to measure accurately the free antibody concentration, the ELISA should not modify significantly the liquid phase equilibrium (Friguet et al. 1995). This requires to perform preliminary

P. Martineau
IRCM, Institut de Recherche en Cancérologie de Montpellier, Montpellier 34298, France
INSERM, U896, Montpellier 34298, France
Université Montpellier1, Montpellier 34298, France
CRLC Val d'Aurelle Paul Lamarque, Montpellier 34298, France
e-mail: pierre.martineau@valdorel.fnclcc.fr

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_41,

experiments to determine the correct experimental conditions, as described in Sect. 41.3.1. Finally, it is better to use a curve-fitting program to avoid the bias introduced by equation linearization, such as the Scatchard transformation (Leatherbarrow 1990).

When an antibody fragment (scFv) is mixed with the antigen in solution, we can write

$$a = [\text{scFv}] = [\text{scFV}]_{\text{f}} + [\text{scFv}]_{\text{b}} \tag{41.1}$$

$$[\text{BS}] = [\text{BS}]_{\text{f}} + [\text{BS}]_{\text{b}} \tag{41.2}$$

where a, $[\text{scFv}]_{\text{f}}$, $[\text{scFv}]_{\text{b}}$ are, respectively, the total, free, and bound scFv concentrations, and $[\text{BS}]_{\text{f}}$ and $[\text{BS}]_{\text{b}}$ are, respectively, the free and bound "Binding Site" concentrations. If the antigen is monomeric, [BS] is equal to the antigen concentration. But if the antigen is multimeric,

$$[\text{BS}] = \text{n}[\text{Ag}] = \text{nx} \tag{41.3}$$

where n is the number of sites per antigen (e.g., 2 for a homodimer), and x the antigen concentration.

Since there is one scFv bound per binding site,

$$[\text{BS}]_{\text{b}} = [\text{scFv}]_{\text{b}} \tag{41.4}$$

and the Mass action law is written

$$K_{\text{d}} = \frac{[\text{scFv}]_{\text{f}} \cdot [\text{BS}]_{\text{f}}}{[\text{BS}]_{\text{b}}} \tag{41.5}$$

Since the method will measure the free scFv concentration, we will rename $[\text{scFv}]_{\text{f}}$ to y.

By combining (41.1), (41.2), and (41.4)

$$[\text{BS}]_{\text{f}} = \text{nx} - [\text{BS}]_{\text{b}} = \text{nx} - [\text{scFv}]_{\text{b}} = \text{nx} - \text{a} + \text{y} \tag{41.6}$$

$$[\text{BS}]_{\text{b}} = \text{a} - \text{y} \tag{41.7}$$

and (41.5), (41.6), and (41.7)

$$K_{\text{d}} = \frac{y(nx - a + y)}{a - y} \tag{41.8}$$

This quadratic equation can be solved to obtain y as a function of x:

$$y = \frac{-(nx - a + K_d) + \sqrt{(nx - a + K_d)^2 + 4aK_d}}{2} \quad (41.9)$$

Finally, in the ELISA described in Sect. 41.3.1, we will measure an absorbance, proportional to the free scFv concentration, i.e., $y = a(A - A_0)/(A_{max} - A_0)$. We thus obtain the final equation:

$$A = (A_{max} - A_0) \times \frac{-(nx - a + K_d) + \sqrt{(nx - a + K_d)^2 + 4aK_d}}{2a} + A_0 \quad (41.10)$$

where A is the absorbance, A_{max} and A_0 are, respectively, the maximal and minimal ELISA signal obtained (when $x = 0$ and $x = \infty$), K_d is the dissociation constant, a is the total scFv concentration, n is the number of binding sites per antigen, and x is the total antigen concentration.

41.2 Materials

41.2.1 Setting up the ELISA Conditions

- Plate washer (*see Note 1*).
- PBSx10: 6.1 g Na_2HPO_4 (43 mM), 2 g KH_2PO_4 (15 mM), NaCl 80 g (1.37 M), 2 g KCl (27 mM), make up to 1 L with H_2O, autoclave.
- PBST: PBS with 0.1% (v/v) Tween-20.
- PBSM: PBS with 3% (w/v) non-fat dried milk.
- Carbonate buffer (if required): 50 mM sodium carbonate, pH 9.6. Mix 16 ml of 0.2 M sodium carbonate, 34 ml of 0.2 M sodium bicarbonate, and 150 ml of H_2O.
- Flat-bottom ELISA plates or strips (Nunc Maxisorp).
- 9E10 monoclonal antibody (*see Note 2*).
- Alkaline Phosphatase-conjugated anti-mouse antibody (*see Note 3*).
- PNPP: 1 mg/ml solution of *p*-Nitrophenyl Phosphate, Disodium Salt in 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$ pH 9.5.

41.2.2 Measuring Free scFv as a Function of the Antigen Concentration

- Curve-fitting software (*see Note 4*).

41.3 Methods

In the step-by-step procedures described below, we will use a scFv produced in *E. coli* and tagged with the *c*-myc peptide recognized by the 9E10 monoclonal antibody. You will have to adapt the detection method (Steps 10 and 13 in Sect. 41.3.1) if your scFv is linked to a different tag.

41.3.1 Setting up the ELISA Conditions

This ELISA will measure the scFv concentration. However, to determine accurately the free scFv concentration in the presence of the antigen in Sect. 41.3.2, we must be sure that the fraction of scFv captured in the ELISA does not modify significantly the liquid phase equilibrium (Friguet et al. 1995). The maximum allowed displacement is usually fixed to 10%, but we will see in Sect. 41.4 that a higher value can be acceptable. To estimate the amount of bound scFv in the solid-phase ELISA, a first ELISA is performed, the unbound scFv is then recovered and used in a second ELISA. By comparing the ELISA signal obtained in the two ELISAs, we can measure the decrease in scFv concentration and thus the fraction of bound scFv (Fig. 41.1). In order to be comparable, the two ELISAs must be performed in the same conditions (*see Note 5*).

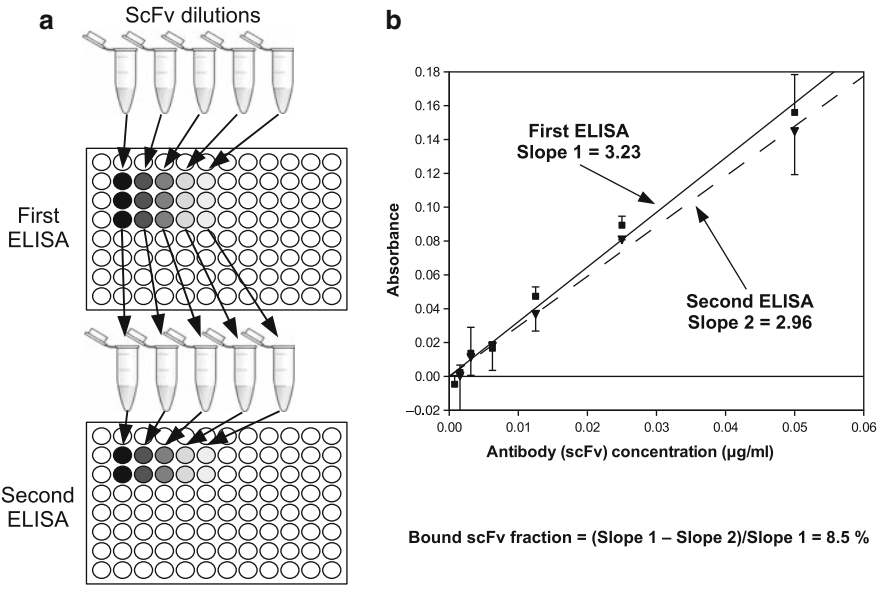


Fig. 41.1 Setting up of the ELISA. (**a**) Schematic outline of the procedure described in Sect. 41.3.1. (**b**) Example of a result obtained using correct conditions

1. Coat two identical ELISA plates with 100 µL of a 1 µg/ml solution of purified antigen prepared in either PBS or Carbonate buffer (*see Note 6*).
2. Wash the *two* plates three times with PBST.
3. Add 200 µL of PBSM to each well and incubate for at least 2 h at RT.
4. Wash the *first* plate three times with PBST.
5. Prepare a series of 2-step dilutions, in PBS, of the purified scFv (*see Note 7*). Add 100 µL of each dilution, in triplicate, to the *first* plate.
6. Incubate for 1 h at RT.
7. Wash the *second* plate three times with PBST.
8. Carefully pipette out the scFv dilutions of each well of the *first* plate using a micropipette, pool the three triplicates in a single tube, and add them in duplicate to the *second* plate as in Step 5.
9. Wash the *first* plate three times with PBST.
10. Add 100 µL of 9E10 culture supernatant diluted 1/10th in PBST to the *first* plate (*see Note 2*).
11. Incubate the *two* plates for 1 h at RT.
12. Wash the *two* plates three times with PBST.
13. *First* plate: Add 100 µL of AP-conjugated anti-mouse antibody diluted 1/200 (*see Note 3*). *Second* plate: Add 9E10 monoclonal antibody as in Step 10 above.
14. Incubate the *two* plates for 1 h at RT.
15. Wash the *two* plates three times with PBST.
16. *First* plate: Add 100 µL of PNPP and incubate for 1 h at RT (*see Note 8*). *Second* plate: Add AP-conjugated anti-mouse antibody as in Step 13.
17. Wash the *second* plate three times with PBST, add PNPP as in Step 16 and incubate for 1 h at RT.
18. Read the two plates at 405 nm after the same revelation time (*see Note 8*).

Compare the signals obtained with the two plates. If the conditions are correct, the signal should decrease by less than 10% (Fig. 41.1). If the decrease in signal intensity is too strong, decrease the coating concentration tenfold and re-perform the procedure. Also, determine the highest concentration that still gives a signal proportional to the scFv concentration , and use it as the maximal concentration in the next ELISA (Step 5) and in Sect. 41.3.2 below.

41.3.2 Measuring Free scFv as a Function of the Antigen Concentration

The ELISA in this section must be performed in the same conditions than those determined in Sect. 41.3.1.

1. Prepare a coated and saturated plate as in Sect. 41.3.1.
2. During the saturation, prepare dilutions of the antigen in PBS, in a final volume of 150 µL (*see Note 9*).

3. Prepare a scFv dilution at twice the maximal concentration determined in Sect. 41.3.1. Prepare at least a volume of 150 µL × (number of antigen dilutions +1).
4. Add 150 µL of scFv to each antigen dilution. Mix gently and incubate for 1 h at RT (*see Note 10*).
5. Use these mixes to perform the ELISA in duplicate, using the conditions determined in Sect. 41.3.1 (Steps 5–18).
6. Plot the absorbance at 405 nm as a function of the antigen concentration. Typical results are shown in Fig. 41.2.
7. Calculate the Kd using (41.10) and a curve-fitting program (*see* Sect. 41.4 for an example).

41.4 Results

Until recently, because computers were not widely available, binding data were fitted to a linearized form of (41.10) using a Klotz or Scatchard plot (Friguet et al. 1985). It is, however, neither necessary nor advisable since these transformations result in distribution of the error in both x and y directions, which does not fulfil some of the assumptions made by the curve-fitting programs (Leatherbarrow 1990). There is no reason today not to use non-linear fitting programs which are widely available and will give more correct results. Once the fitted parameters are

a

	Coating	
Tubulin (nM)	0.1 ug/ml	10 ug/ml
100.00	0.246	0.593
25.00	0.292	0.845
12.50	0.318	1.006
6.25	0.458	1.279
3.13	0.586	1.784
1.56	0.671	1.815
0.78	0.837	2.273
0.39	1.043	2.430
0.00	1.036	2.263

b

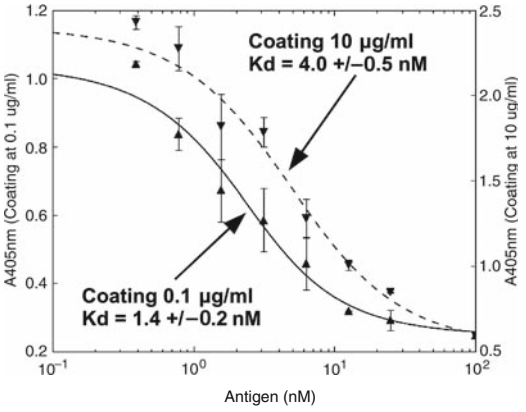


Fig. 41.2 K_d determination. (**a**) Absorbance measured at 405 nm in the ELISA described in Sect. 41.3.2. The antigen (αβ-tubulin) was coated at two concentrations: 0.1 µg/ml is the correct concentration, as determined in Sect. 41.3.1 (Fig. 41.1) and 10 µg/ml results in a value of 40% for the fraction of bound scFv. (**b**) Data from Fig. 41.2a were fitted to 41.10 as explained in Sect. 41.4. Comparison of the two coating concentrations shows the effect of a too high antigen coating concentration on the determined K_d value (*see* Sect. 41.4)

determined, data and the linear equation can be represented using a Scatchard plot, if required. An added benefit of this approach is that it could be used even with non-purified scFv since the scFv concentration can also be considered as a parameter in the equation. We will exemplify the procedure in this section.

As an example, we used the 2F12 anti-α-tubulin scFv (Philibert et al. 2007). The scFv was purified from *E. coli* cytoplasm using immobilized metal chelate affinity, and dialyzed against PBS, as described (Guglielmi and Martineau 2009; Philibert et al. 2007). Purified αβ-tubulin dimer was coated in PBS, at the concentrations of 10, 1 and 0.1 μg/ml, overnight at 4°C. The proportion of bound scFv in the ELISA described in Sect. 41.3.1 was, respectively, 40, 25, and 8.5% (Fig. 41.1). We thus selected the last coating conditions (0.1 μg/ml) for the binding experiment described in Sect. 41.3.2.

Binding experiment was performed using a scFv concentration of 0.05 μg/ml (1.79 nM) and αβ-tubulin concentrations of 100, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0 nM. It is always necessary to use a concentration high enough to completely inhibit the scFv (here, we used 100 nM) and to make a point in the absence of antigen. The ELISA was performed in duplicate and the average of the two values plotted in Fig. 41.2. The principle of non-linear regression is to minimize, by adjusting a set of unknown parameters (here, the K_d), the sum of the squares of the deviations between the experimental values and the curve described by 41.10 (*see Note 4*). You will have to give the software a first estimate of the unknown parameters and the software will refine the parameters by successive iterations. In 41.10 we have five parameters: K_d, A_{max}, A_0, a and n. The last two parameters are normally known (in our case it is, respectively, 1.79 nM and 1), but they could also be fitted (see below). A good estimate of A_{max} and A_0 are the values of the absorbance when there is, respectively, no or a saturating amount (100 nM) of antigen in the assay: In Fig. 41.2, we used $A_{max} = 1.036$ and $A_0 = 0.246$. The only really unknown parameter is K_d. A sensible starting value is the IC_{50}, that is, the antigen concentration giving a 50% signal inhibition, that is about 1.6 nM ($A = 0.671 \sim (A_{max}+A_0)/2$). The best fitted curve is shown in Fig. 41.2b and was obtained for $K_d = 1.4 \pm 0.2$ nM (*see Note 11*).

In this example, we have used fixed values for A_{max}, A_0, a, and n. These parameters could also be fitted together with the K_d. However, this will increase the error on the estimates resulting in a lower precision in the K_d determination. An interesting application is when the scFv is not purified. With the same data, such a fitting will give $K_d = 1.5 \pm 0.6$ nM and $a = 1.5 \pm 1.2$ nM. Even if the precision on the scFv concentration is low in this case, the real experimental value (1.79 nM) is indeed within the interval of the fitted parameter. In addition, when the real scFv concentration is known, performing this fitting demonstrates that, within experimental errors, the whole scFv preparation is active.

The most difficult step of this approach is to successfully obtain a displacement of less than 10% in Sect. 41.3.1. This requires to use a low coated antigen concentration, resulting in a weak signal in the ELISA. Sometimes, the signal is too weak to be usable, and you must tolerate a value higher than 10%. To evaluate the effect of breaking this requirement of the method, we have analyzed the same

scFv but with a 100-fold higher αβ-tubulin coating concentration (10 μg/ml). In this case, the displacement, as measured in Sect. 41.3.1, is 40%. The result is shown in Fig. 41.2. We obtained 4.0 ± 0.5 nM for the Kd instead of the value of 1.4 ± 0.2 nM obtained using the correct coating conditions. In this case, the affinity is underestimated by a factor of 3. The error will always result in an underestimation of the affinity and you can thus say that the affinity of this scFv for its antigen is at least of 2.5×10^8 M^{-1} (correct value = 7.1×10^8 M^{-1}).

41.5 Notes

1. If you don't have access to an automatic plate washer, wash the plate by soaking it in a large container filled with PBST and turning it upside down over a sink. Pay attention not to have bubbles in the wells during soaking. At the end, dry the wells by hitting the plate strongly on a pile of paper towels.
2. 9E10 hybridoma is available from ATCC (#CRL-1729) and ECACC (#85102202). If using culture supernatant, the suitable dilution must be first determined by testing 1:10, 1:20 and 1:50 dilutions. Purified 9E10 can also be purchased from several suppliers (Santa-Cruz, Abcam, Sigma) and is generally used at a 1 μg/ml concentration.
3. Never use HRP-coupled antibodies for quantitative measurements. Because we have to use a low coating concentration, the signal obtained is usually low. In order to get a good signal, you must use the AP-coupled anti-mouse antibody at a much higher concentration than the supplier-recommended one.
4. Several curve-fitting softwares are available, either commercial or free. A good quality software should give you an estimate of the error. A simple, free software based on the scipy scientific package (http://www.scipy.org) is available on our web site (http://www.ircm.fr) or on request.
5. Since we will compare the results of the two ELISAs, we must carefully perform them in the same conditions. It is important to use the same temperature, incubation time and dilution of the secondary antibodies for both plates.
6. You don't have to coat a whole ELISA plate. We usually use 6 scFv dilutions (*see Note 7*). You thus have to coat 18 wells for the first plate and 12 for the second.
7. We usually use, as a starting point, about 35 nM (1 μg/ml) for the highest concentration and we prepare 2-step dilutions down to 2 nM. Add a tube without antibody as blank.
8. Incubation time can be increased to get a strong signal. We routinely use up to 3 h if the signal is too low. You can stop the reaction by adding 50 μL of NaOH 2 N if you want to wait for the second plate before reading.
9. The concentration range will depend on the affinity. For most antibodies, use 100–500 nM for the first point and prepare 2-step dilutions. Add a tube without antigen to obtain the A_{max}.

10. You must incubate long enough to reach equilibrium. Use the temperature at which you want to determine your K_d (usually 4–37°C). Keep the same temperature during the whole experiment.
11. It is also possible to pool the results of several experiments. First, transform the absorbance into fraction of free scFv using the formula $f = (A–A_0)/(A_{max}–A_0)$. Then, fit the data to 41.10 with $A_{max} = 1$ and $A_0 = 0$.

References

Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME (1985) Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J Immunol Methods 77:305–319

Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME (1995) Under proper experimental conditions the solid-phase antigen does not disrupt the liquid phase equilibrium when measuring dissociation constants by competition ELISA. J Immunol Methods 182:145–150

Friguet B, Djavadi-Ohaniance L, Goldberg ME (1997) Immunochemical analysis of protein conformation. In: Creighton T (ed) Protein structure: a practical approach. Oxford University Press, Oxford, pp 323–348

Guglielmi L, Martineau P (2009) Expression of single-chain Fv fragments in E. coli cytoplasm. Methods Mol Biol 562:215–224

Leatherbarrow RJ (1990) Using linear and non-linear regression to fit biochemical data. Trends Biochem Sci 15:455–458

Philibert P, Stoessel A, Wang W, Sibler A, Bec N, Larroque C, Saven JG, Courtete J, Weiss E, Martineau P (2007) A focused antibody library for selecting scFvs expressed at high levels in the cytoplasm. BMC Biotechnol 7:81

Stevens FJ (1987) Modification of an ELISA-based procedure for affinity determination: correction necessary for use with bivalent antibody. Mol Immunol 24:1055–1060

Chapter 42
Anti-Histidine Antibodies as Tools for Reversible Capturing of His-Tagged Fusion Proteins for Subsequent Binding Analysis

H.-M. Zenn, S. Hutschenreiter, and F.W. Herberg

42.1 Introduction

Protein-binding reagents, most commonly polyclonal or monoclonal antibodies, are essential tools for protein characterization in basic research, biotechnology, medical therapy, and diagnostics. Antibody-based therapeutics has become available as a means to efficiently target several human diseases such as cancer treatment where antibody-based immunotherapy complements chemotherapy (Adams and Weiner 2005). Two prerequisites for this application of antibodies are high specificity and high affinity to the target molecule. In a recent study, Tang and co-workers showed that high affinity of an antibody like anti-HER2/neu is associated with antibody-mediated cellular cytotoxicity (Tang et al. 2007). In addition, the neutralization ability of antibodies against infectious agents like HIV-1 (human immunodeficiency virus type 1) or SARS coronavirus have been demonstrated to simultaneously increase with their affinity (Nelson et al. 2007; Rogers et al. 2008), thus emphasizing the need for highly accurate binding data not only for basic research but also for diagnostics and therapy.

Optical biosensors provide an excellent tool for in-depth characterization of the interactions of protein-binding reagents in real time. For more than about 20 years now, chip-based Surface Plasmon Resonance (SPR) biosensors, such as those manufactured by Biacore (GE Healthcare), have been used for high-resolution, time-resolved interaction measurements of biomolecules. These instruments do not only allow the determination of affinities (e.g. equilibrium dissociation constant, K_D) but also provide distinct rate constants for association (k_a) and dissociation (k_d) with high accuracy. Intersystem comparisons of biosensors have demonstrated the reliability of these instruments (Katsamba et al. 2006). In a recent study, reproducibility was tested by Myszka and co-workers thus, demonstrating that differences

H.-M. Zenn, S. Hutschenreiter, and F.W. Herberg (✉)
Department of Biochemistry, University of Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany
e-mail: herberg@uni-kassel.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_42,

between users were negligible when testing the same set of immunosamples on different biosensors world-wide (Rich et al. 2008).

42.1.1 Application of SPR in Antibody Engineering

SPR-based Biacore technology was originally used to investigate antibody-antigen binding (Fagerstam et al. 1990; Karlsson et al. 1991). Since then, dozens of other applications combining antibodies and Biacore systems have been generated (for an overview, see Rich and Myszka 2008). One approach is to determine the antibody concentration in sera as shown by Pol et al. (2007) or the antibody isotyping as described (Swanson et al. 2004). Recently, these instruments have also been used to screen for high-affinity antibodies from crude cell lysates (Canziani et al. 2004; Steukers et al. 2006). Here, the authors used either covalently coupled anti-human-Fc IgG antibodies or immobilized protein A to capture antibodies from various hybridoma supernatants and subsequently determined the Fab concentration from extracts. With this strategy, Canziani and coworkers, using Biacore 2000 or 3000 instruments, were able to test three antibodies from three supernatants. In a similar study, it was demonstrated that Biacore A100 and Flexchip units (array format) were suitable for increased to high throughput applications (Safsten et al. 2006; Wassaf et al. 2006).

42.1.2 Principle of a Biacore Analysis

The principle work flow of a Biacore-based interaction analysis consists of (1) the immobilization of a ligand (molecule 1) on a sensor chip, (2) the injection of an analyte (molecule 2) in the flow phase over the sensor surface, and (3) the regeneration of the chip surface. Interaction of the analyte with the immobilized ligand causes a change in the refractive index close to the sensor chip surface which, in turn, is detected as a change in SPR-signal (in response units, RU) and plotted against the time in a so-called sensogram (for details, see Gesellchen et al. 2005).

For the immobilization of ligands, several strategies exist. The standard approach is the covalent coupling of a ligand via primary amines (free N-termini or lysine residues) utilizing NHS/EDC (*N*-hydroxysulfosuccinimide/*N*-ethyl-*N*′-dimethylaminopropyl-carbodiimide) chemistry or via thiol groups (Gesellchen et al. 2005). Non-covalent, reversible capturing can be achieved by employing genetically engineered fusion tags (for example, GST (Glutathion S-Transferase), FLAG (DYKDDDDK peptide) or Strep-tag (WSHPQFEK peptide)) once an appropriate binding reagent (antibody), specifically targeted against the fusion tag, has been attached to a sensor chip. Biotinylated molecules can also be captured site-directed on a streptavidin surface with high affinity; however, because of their high affinity, a complete regeneration applying rather mild conditions may

be problematic. For reversible capturing of GST-tagged fusion proteins, a commercially available anti-GST antibody (GST capture kit, BR-1002-23, Biacore) is commonly used (Zimmermann et al. 2008). Thus, the interaction pattern and/or the functionality of the fused protein can be influenced by the high molecular weight of the GST-fusion tag (~26 kDa). Proteolytic cleavage of the fusion tag using a specific protease may be a suitable strategy (Terpe 2003) to remove the fusion part still requiring extra steps and may result in loss of protein.

The commonly used polyhistidine-fusion tag is smaller in size than the GST-tag. Several strategies exist for site-specific capturing/immobilization. The first strategy is comprised of reversible, non-covalent binding to a specific NTA (Nitrilotriacetic acid) chip (BR-1004-07, Biacore; (Gershon and Khilko 1995) in the presence of Ni^{2+} ions; additionally, covalent coupling on those NTA chips can be performed (Willard and Siderovski 2006; Diskar et al. 2007; see also Method section). Slow dissociation from the polyhistidine capturing surface is fundamental for performing a sandwich type binding assay. Reversible binding has to be tested for every His-tagged protein in order to determine if the dissociation rate from the polyhistidine capturing surface is slow enough for subsequent interaction analysis. Insufficient capturing (fast off-rate) of polyhis-tagged proteins is especially problematic when using Ni^{2+}-NTA sensor chips (Herberg, personal communication). Immunotools, that are, antibodies against His_6-tagged fusion proteins, may provide an alternative that is superior to the Ni^{2+}-NTA-approach.

Besides immobilization, efficient regeneration is crucial in order to provide a defined ligand concentration for accurate binding analysis. There are two general modes of regeneration. One is to gently remove just the analyte from the ligand and the other is to dissociate both molecules from the sensor surface in a single step-procedure. In case of a covalently coupled antibody, acidic conditions are often useful to dissociate the ligand and analyte simultaneously (see Methods section for details).

In this study, we tested the binding of 12 RGS-$(His)_6$-tagged (Arg-Gly-Ser-$(His)_6$) proteins (Table 42.1) to two anti-$(His)_6$, one anti-$(His)_5$ and one anti-RGS-$(His)_4$

Table 42.1 Description, molecular weight, and Swiss-Prot accession number of the 12 RGS-$(His)_6$-tagged proteins

	MW (kDa)	SwissProt acc.	Description
His_9482_6E10	70	O43742	InaD-like protein
His_9146_6B2	50	O60239	SH3 domain-binding protein 5
His_9448_6E4	30	Q96H25	hypothetical protein (fragment)
His_9522_6F3	40	Q96F30	similar to src homology 3 domain-containing protein hip-55
His_9142_6B1	30	Q6PJ62	eEF-1B gamma (fragment)
His_9326_6C9	30	AAP35323	Eukaryotic translation elongation factor 1 gamma
His_9502_6F2	50	Q9UI47	Similar to catenin (cadherin-associated protein)
His_9605_6F10	110	P21333	Filamin A, alpha
His_9189_6B9	17	P43080	Guanylyl cyclase activating protein 1 (gcap 1)
His_9413_6D9	20	O08765	Ganglioside expression factor 2 (gef-2) (gate-16)
His_9427_6D11	26	O43639	Cytoplasmic protein nck2 (nck adaptor protein 2)
His_9617_6F11	48	P12277	Creatine kinase, b chain (ec 2.7.3.2)

antibody. The goal was to identify an antibody suitable for stable, but reversible capturing of His-tagged proteins in order to allow further interaction analysis with other proteins (sandwich assay).

42.2 Materials

42.2.1 Biacore Consumables

- CM5 sensor chips (research grade), store at 4°C
- NTA sensor chips, store at 4°C
- 100 mM NHS (*N*-hydroxysuccinimide), store at −20°C
- 400 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), store at −20°C
- 1 M Ethanolamine hydrochloride, pH 8.5, store at 4°C

42.2.2 Buffers

Coupling buffer
- 10 mM sodium acetate, pH 4–6, store at 4°C
- 100 mM N-hydroxysuccinimide (NHS), store at −20°C
- 400 mM *N*-ethyl-*N*′-(dimethylaminopropyl)-carbodiimide (EDC), store at −20°C
- 1 M ethanolamine hydrochloride, pH 8.5, store at 4°C

NaMOPS Biacore running buffer
- 20 mM MOPS (3-(N-morpholino) propanesulfonic acid), pH 7.0.
- 150 mM NaCl.
- 0.005% Tween 20 (Fluka) (store at 4°C and equilibrate to room temperature before use).

HBS-EP Biacore running buffer
- 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4
- 150 mM NaCl
- 0.005% Tween 20
- 0.05 mM EDTA (store at 4°C and equilibrate to room temperature before use)

Regeneration buffer for antibody surfaces
- 10 mM glycine-HCl, pH 1.5–2.5, store at 4°C (see also trouble shooting)

Regeneration buffer for NTA surfaces
- 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 8.3
- 150 mM NaCl
- 0.005% Tween 20
- 350 mM EDTA (storage at 4°C and equilibrate to room temperature before use)

42.2.3 Anti-His Tag Antibodies

- anti-$(His)_6$ antibody, directed against terminal $(His)_6$-epitope, monoclonal, mouse, IgG1, unconjugated, BSA-free, Dianova GmbH, Hamburg, DIA 910
- anti-$(His)_6$ antibody, Trend Pharma & Tech Inc., Canada, IgG2b
- Penta-His antibody, directed against terminal or internal $(His)_5$-epitope, monoclonal, mouse, IgG1, unconjugated, BSA-free, lyophilized from PBS buffer, Qiagen, Hilden
- RGS-His antibody, directed against RGS-$(His)_4$-epitope, monoclonal, mouse, IgG1, unconjugated, BSA (Bovine serum albumin)-free, lyophilized from PBS (Phosphate buffered saline) buffer, Qiagen, Hilden
- The antibodies from Qiagen were purchased as Anti-His Antibody Selector Kit (3×3 micrograms of Penta-His Antibody, Tetra-His Antibody, and RGS-His Antibody, respectively).

42.3 Methods

42.3.1 Purification of His-Proteins in a 96-Well Format

The codons for the RGS-$(His)_6$ tag/epitope (Arg-Gly-Ser-$(His)_6$) can be fused to a gene of interest using the pRSET-vectors (Invitrogen) or the pQE-vectors (Qiagen). N-terminal RGS-$(His)_6$-tagged proteins were overexpressed in *E. coli* SCS1 cells and purified with Ni^{2+}-NTA agarose in a 96-well plate format, as described by Büssow et al. (Bussow et al. 2004), except for substituting 50 mM Tris pH 8.0 by 50 mM Hepes pH 8.0. Eluates from four independent purifications were pooled for Biacore experiments to compensate for differences in expression level and purification yield. Proteins were concentrated with an Amicon Ultra 10 device to 2 mg/ml and the molecular weight of each protein was determined using SDS-PAGE, ranging from 17–110 kDa.

42.3.2 Amine Coupling of Anti-His Tag Antibodies

Analysis were performed with a Biacore 2000 instrument at 25°C and the data were evaluated with BIAevaluation 4.01 (Biacore) and Graphpad (Graphpad Prism 4.0,

San Diego). NHS, EDC, and CM 5 sensor chips were obtained from Biacore. All buffers were degassed and sterile filtered.

Anti-His tag antibodies were coupled to CM5 (carboxymethylated dextran) sensor chips on two flow cells. The other flow cells served as negative controls. One chip was used to test the two Qiagen antibodies. On the other chip, antibodies from Dianova and Trend Pharma were immobilized. To achieve comparable coupling densities, the antibodies from Qiagen had to be subjected to short-term dialysis (1 h, 4°C) against the coupling buffer (10 mM sodium acetate, pH 5.0) in order to adjust the concentration and pH of the solution. Antibodies (30 $\mu g\ ml^{-1}$) in the coupling buffer were injected for 7 min at a flow rate of 5 $\mu l\ min^{-1}$ over the NHS/EDC-activated surface to generate surface densities of 3060 RU (anti-RGS-$(His)_4$ antibody from Qiagen), 5970 RU (anti-Penta-His antibody from Qiagen) and 14310 RU (anti-$(His)_6$ antibody from Dianova), and 15370 RU (anti-$(His)_6$ antibody from Trend Pharma).

Procedure in detail:

- Insert CM5 sensor chip equilibrate to room temperature into a Biacore instrument and prime the system with running buffer.
- Start the sensogram and wait until the baseline is stable.
- Inject 15 µl 10 mM NaOH (three times) at a flow rate of 30 $\mu l\ min^{-1}$.
- Decrease the flow rate to 5 $\mu l\ min^{-1}$ and address only the flow cell where the antibody should be immobilized.
- In most cases, a pH scouting is necessary before trying to couple a protein. By this procedure, one determines the pH and protein concentration at which the electrostatic interaction to the dextran matrix works out best for subsequent immobilization. Therefore, dilute the ligand to concentrations ranging from 1 to 30 $\mu g\ ml^{-1}$ in the coupling buffer with different pH values. Generally, a pH from 4.5 to 5.5 is useful to couple antibodies.
- Perform short injections (1 min) of the diluted ligand and observe where the electrostatic attraction is suitable to obtain an increase in SPR-signal (RU).
- Mix the thawed NHS and EDC solution in a 1:1 ratio and inject the mixture for 7 min. After the injection, record an increase in the response signal of 180–250 RU (CM5 chip on a Biacore 2000 system) for the activation of the surface.
- Inject the ligand stepwise until the desired surface density is reached.
- Finally, inject ethanolamine hydrochloride for 7 min to quench unreacted esters and to remove non-covalently bound ligand.
- The control surface (in general flow cell 1) should at least be activated with NHS/EDC and treated afterwards with ethanolamine hydrochloride, as described above.

Application notes:

- The needle and microfluidics cartridge (IFC) have to be rinsed after each injection (extraclean) to avoid a cross-contamination.
- A sensor chip with an already coupled ligand can be stored at 4°C under buffer in a 50-ml screw cap tube.

42.3.3 Reversible Binding of His-Tagged Proteins to Ni^{2+}-NTA

For measurements on NTA sensor chips, HBS-EP (Biacore) running buffer can be used.

Detailed procedure:

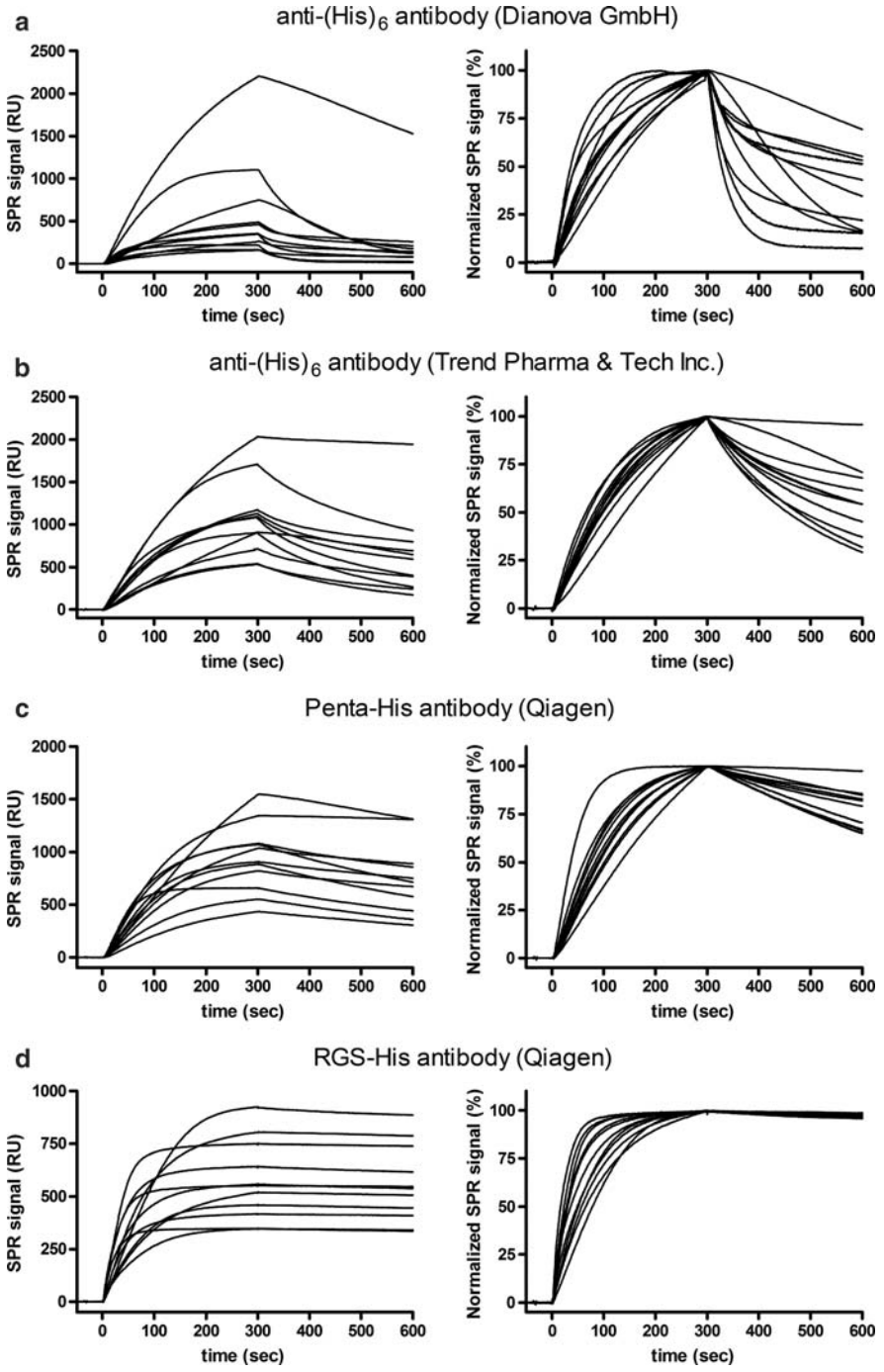
- The first step is to chelate Ni-ions by the NTA surface. Therefore, inject $NiCl_2$ (500 μM) in HBS-EP running buffer for 1 min at a flow rate of 20 μl min^{-1} into at least two flow cells.
- Switch the flow path to the flow cell where the polyhistidine-tagged protein will be captured is going to be bound and decrease the flow rate to 5–10 μl/min.
- Inject the purified histidine-tagged fusion protein diluted in HBS-EP buffer until the desired surface density is reached. Monitor the baseline to assure the stability of the bound ligand (as slow dissociation).
- Via short injections (several seconds) of the $NiCl_2$ solution, it is possible to strip minor amounts of the fusion protein from the surface, and by this, fine-tune the surface coverage.
- For subsequent interaction analysis, direct the non-His-tagged protein (analyte) over both flow cells (reference and the ligand-bound surface) at a flow rate of 30 μl min^{-1}.
- Inject the analyte, as described (Sect. 42.3.5), but omit subsequent cleaning steps.
- Remove the analyte and ligand by chelating Ni-ions with a pulse of 350 mM EDTA pH 8,3 (regeneration buffer for NTA surfaces). Several prolonged injections (2–5 min) might be necessary.

42.3.4 Covalent Coupling of His-Tagged Proteins on NTA

Covalent coupling of His-tagged fusion proteins via primary amine groups on a NTA sensor chip surface was originally described by Willard and Siderovsky (Willard and Siderovski 2006). This method, in contrast to the standard covalent coupling via primary amines, ensures a site directed immobilisation (N-terminal amine group) and can be performed in physiological buffer conditions (e.g. HBS). Initially, the NTA surface has to be saturated with Ni-ions such as described for the reversible binding of polyhistidine-tagged proteins to the NTA surface (see Sect. 42.3.3). In a second step, coupling via the primary amines is performed.

Detailed procedure:

- Inject $NiCl_2$ (500 μM) in HBS-EP running buffer for 1 min at a flow rate of 20 μl per min over only the flow cell where the ligand is to be covalently coupled.
- Decrease the flow rate to 5 μl/min and inject the 1:1 NHS/EDC mixture (see Sect. 42.3.2) for 7 min.


a
anti-(His)6 antibody (Dianova GmbH)
SPR signal (RU)
Normalized SPR signal (%)
time (sec)
b
anti-(His)6 antibody (Trend Pharma & Tech Inc.)
SPR signal (RU)
Normalized SPR signal (%)
time (sec)
c
Penta-His antibody (Qiagen)
SPR signal (RU)
Normalized SPR signal (%)
time (sec)
d
RGS-His antibody (Qiagen)
SPR signal (RU)
Normalized SPR signal (%)
time (sec)

- Subsequently, inject adequate dilutions of polyhistidine-tagged protein in HBS-EP buffer until the surface density of choice is reached.
- Inject ethanolamine hydrochloride for 7 min.
- Next, remove Ni-ions and non-covalently coupled proteins and inject the regeneration buffer for NTA surfaces for 3 min at a flow rate of 20 μl/min.

42.3.5 Surface Plasmon Resonance (SPR) Studies

Procedure for capturing RGS-$(His)_6$-tagged proteins: The 12 recombinant RGS-$(His)_6$-tagged proteins from the protein expression library were diluted 500-fold into running buffer and were injected sequentially over the antibody-decorated surfaces at a flow rate of 30 μl min^{-1}. Binding responses between 200 and 2,500 RU were reached after injection of the analyte (Fig. 42.1 *left panel*). Association and dissociation phases were monitored for 5 min each. Every cycle was completed with two injections of 10 mM glycine pH 1.9 (20 s) to remove the captured his-fusion proteins from the antibody surface.

Detailed procedure:

- Prior to injection, centrifuge all samples at 4°C for 10 min at 10,000×*g*. This procedure removes particles, possibly clogging the integrated fluidics of the instrument.
- Start a sensogram and switch the flow path to the reference flow cell and the antibody-decorated flow cell, respectively.
- Inject a series of different analyte concentrations over the ligand and reference surfaces.
- Regenerate the surface after each cycle by short injections (20–30 s) of glycine-HCl (pH 1.5–2.5). The appropriate pH must be determined previously for each and every antibody (see also trouble-shooting section).
- Additionally, a blank run with the running buffer can be carried out and subtracted from the binding curves (double referencing).

42.3.6 Sandwich Interaction Assay

A sandwich interaction analysis describes stable and reversible binding of one molecule (the ligand) to a chip surface via a specific antibody or Ni^{2+}-NTA in

Fig. 42.1 Binding of RGS-$(His)_6$-tagged proteins to immobilized anti-$(His)_6$ (a and b), anti-penta-His (c), and anti-RGS-$(His)_4$ (d) antibody. The uncorrected sensograms are shown in the left panel and the normalized data are in the right panel. Association and dissociation were carried out in HBS-EP for 300 s each. Normalization was performed with Prism 4.0 (Graphpad Prism 4.0, San Diego). The SPR signal (RU) before injection was set to 0% and the signal at the end of the association (300 s) was set to 100%. (**a**) anti-$(His)_6$ antibody (Dianova), (**b**) anti-$(His)_6$ antibody (Trend Pharma), (**c**) Penta-His antibody (Qiagen), and (**d**) RGS-His antibody (Qiagen)

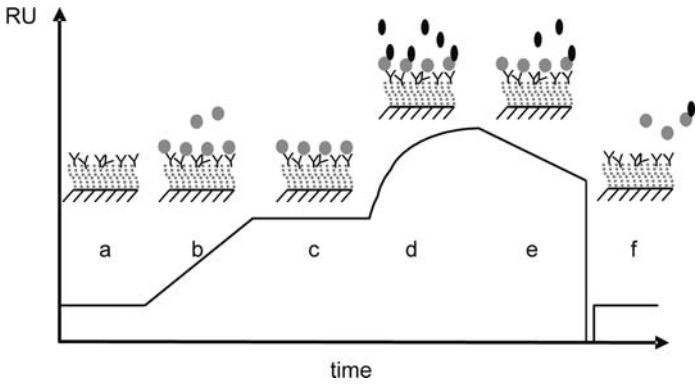


Fig. 42.2 Schematic workflow of a sandwich interaction analysis with RGS-His antibodies used for capturing. (**a**) Equilibration 1: Baseline prior to the injection of a ligand over the RGS-His antibody surface. (**b**) Step 1: Injection of the RGS-$(His)_6$-tagged ligand. (**c**) Equilibration 2: Baseline after ligand capturing. (**d**) Step 2: Association of the analyte. (**e**) Step 3: Dissociation of the analyte. (**f**) Step 4: Regeneration of the captured ligand and bound analyte from the antibody surface

order to be able to determine the binding kinetics for another molecule (the analyte) (see Fig. 42.2). To test for non-specific binding of the analyte to the capturing antibody, this antibody should also be covalently coupled to a reference flow cell. Subsequently, the ligand has to be captured only to one of the antibody surfaces, whereas the other surface serves as a reference.

Detailed procedure:

- To capture a determined amount of protein on an antibody surface, inject the ligand at low flow rates (5–10 µl min^{-1}) until the desired response (RU) is reached (Fig. 42.2b).
- When performing kinetic experiments, set the flow rate at a minimum of 30 µl/min not only to overcome mass-transport-limited interactions, but to reduce rebinding during the dissociation phase. In the same line, reducing the ligand density on the sensor surface may diminish mass transport limitation effects (for details, see Gesellchen et al. 2005).
- Inject the analyte over the reference surface and the analyzing surface (Fig. 42.2d).
- Remove the captured ligand and bound analyte with pulses of glycine-HCl (pH 1.5–2.5) (Fig. 42.2f). See also buffer-scouting procedure for regeneration (Sect. 42.5. trouble shooting).
- Always perform a blank run (buffer injection) for double referencing when carrying out a sandwich type interaction assay.
  Note: For a subsequent analysis cycle with a different analyte concentration, the same surface density has to be accomplished in order to allow a (pseudo first order) kinetic evaluation.

Table 42.2 Evaluation of the dissociation rate constants for the RGS-$(His)_6$-tagged proteins applied to the different antibody-decorated surfaces

RGS-His_6-tagged protein	Dissociation rate constant k_d ($\times 10^{-3}s^{-1}$)				Dissociation after 5 min (%)
	anti-$(His)_6$ antibody (Dianova)	anti-$(His)_6$ antibody (Trend Pharma)	anti-penta-His antibody (Qiagen)	anti-RGS-$(His)_4$ antibody (Qiagen)	
His_9482_6E10	16.4	4.82	1.4	0.13	4.3
His_9146_6B2	3.18	0.4	0.44	0.17	4.25
His_9448_6E4	0.84	0.18	0.064	0.03	1.2
His_9522_6F3	25.1	4.9	1.42	0.1	3.7
His_9142_6B1	18.9	4.11	1.37	0.11	3.5
His_9326_6C9	8.34	3.36	1.38	0.068	2.9
His_9502_6F2	8.06	2.46	0.64	0.052	2.5
His_9605_6F10	7.27	3.28	1.1	0.06	2.9
His_9189_6B9	8.02	3	0.81	0.044	2.2
His_9413_6D9	9.52	2.93	0.7	0.086	3.8
His_9427_6D11	6.96	3.52	0.89	0.016	1.3
His_9617_6F11	3.79	1.34	0.476	–	–

The dissociation rate constants were fitted within an interval of 30 s up to 100 s starting 4 s after the end of the injection assuming a 1:1 Langmuir dissociation model (BIAevaluation software version 4.01). Additionally, the percentage of protein dissociated from the anti-RGS-$(Hi)_{S4}$ antibody after 5 min is presented in the table.

42.4 Results

To select an optimum antibody for a sandwich type assay (Fig. 42.2), four different anti-His tag antibodies were covalently immobilized on CM5 sensor chips (Biacore GE Healthcare) and subsequently tested with 12 RGS-$(His)_6$-tagged proteins from a proteome-wide human expression library where proteins ranged from 17 to 110 kDa in molecular weight (Seitz et al. 2006) (Table 42.1). The overall requirement for this type of sandwich assay is a slow dissociation rate constant (k_d) of the captured his-tagged protein from the antibody to provide a stable ligand surface.

A specific interaction of the RGS-$(His)_6$-tagged proteins with each antibody was obtained suggesting that the fusion tag was accessible for binding to the capture antibody. Dissociation rate constants (k_d) for each antibody were calculated (see Table 42.2) assuming a 1:1 Langmuir model (BIAevaluation 4.01, Biacore). When comparing the dissociation rate constants of all proteins from the tested antibodies, a reduction in the k_d value in the order anti-$(His)_6$-antibody (Dianova) > anti-$(His)_6$-antibody (Trend Pharma) > anti-$(His)_5$-antibody (Qiagen) > anti-RGS-$(His)_4$-antibody (Qiagen) was observed demonstrating increased affinity for the epitope (Fig. 42.1). Only in case of the RGS-$(His)_4$-antibody from Qiagen, association with the RGS-$(His)_6$-tagged proteins was very rapid and dissociation very slow (Table 42.2). This suggested that the interaction with the RGS-$(His)_4$-antibody was extremely stable and suited best for a sandwich assay in order to determine binding kinetics with other, non-RGS-$(His)_6$-tagged proteins as analytes (Fig. 42.3). In contrast to the anti-RGS-$(His)_4$-antibody, the anti-penta-His antibody from

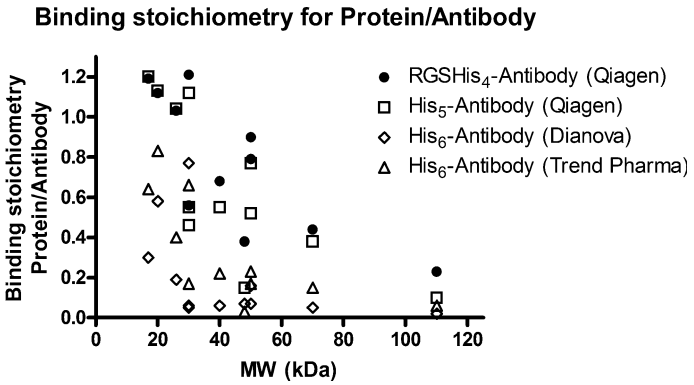


Fig. 42.3 Molecular weight correlation of the RGS-$(His)_6$-tagged protein with the binding stoichiometry for protein/antibody. Binding stoichiometries were calculated on the basis of the molecular weight of ligand and immunoglobulin G. The calculated binding stoichiometry was plotted for each anti-His antibody (depicted in the figure) against the molecular weight of the RGS-$(His)_6$-tagged protein

Qiagen showed a lower capability for capturing, mainly due to the faster dissociation rate (see Table 42.2 and Fig. 42.1). The Tetra-His antibody from Qiagen displayed an even faster off rate for the RGS-$(His)_6$-tagged proteins (Hahnefeld et al. 2004). The antibody distributed by Trend Pharma worked better than the anti-$(His)_6$ antibody from Dianova, that is, the dissociation rate for the RGS-$(His)_6$-tagged proteins was lower, but definitely higher when comparing it even with the anti-penta-His antibody. In general, more than 50% of the bound RGS-$(His)_6$-tagged proteins dissociated in a short period of time from the Dianova antibody (see Fig. 42.1a *right panel*), thus making the antibody less suitable to capture His-tagged proteins when compared with the other antibodies tested.

It could be generally deduced from the binding curves that, for the most part, the lower the molecular weight of a protein the higher the observed binding stoichiometry was (Fig. 42.3). Several RGS-$(His)_6$-tagged proteins were associated with a binding stoichiometry of ~1. However, this 1:1 binding stoichiometry with smaller proteins was only attained in case of the anti-His tag antibodies from Qiagen, probably also due to the steric conditions on the medium-dense antibody-surface. The anti-His antibodies from Dianova and Trend Pharma showed much lower binding stoichiometries for the RGS-$(His)_6$-tagged proteins, most likely due to the more dense antibody packing, thus imposing steric constraints (crowding effect).

Furthermore, we tested the stability of the covalently coupled antibodies after multiple regeneration steps based on residual binding capacity. Therefore, we injected a particular RGS-$(His)_6$-tagged protein on freshly decorated antibody surfaces and the same protein after more then 90 runs (data not shown). The binding capacity of the anti-RGS-His antibody versus the anti-penta-His antibody was significantly more altered. After 24 hours of interaction measurements and

regeneration steps, a ~60% reduction of the SPR signal (RU) was observed for the anti-RGS-His antibody. Still, the dissociation kinetics were unchanged (k_d (first run) = 3×10^{-5} s^{-1}, k_d (run after 24 h) = 3.2×10^{-5} s^{-1}). In contrast, the capacity of the anti-penta-His antibody surface was only reduced by ~25% with also unchanged off rates. The difference in binding capacity after more than 90 cycles of injections and regenerations most likely results from differences in aging of the antibodies under the regeneration conditions applied. Using milder regeneration conditions (i.e., higher pH), the stability of the antibody on the surface might be less impaired, thus allowing to perform even more injection cycles.

42.5 Troubleshooting

42.5.1 Covalent Coupling of Antibodies

The standard buffer for NHS/EDC-mediated covalent coupling via primary amines (free N termini or lysine residues) is 10 mM sodium acetate (pH 4–6). It is obligatory that primary amines (i.e. TRIS-containing buffers) are not present in the coupling buffer. Other options are phosphate or MES-buffers (2-(*N*-Morpholinoethanesulfonic acid)) in the same pH range.

42.5.2 Running Buffers

In some cases, the running buffer has to be optimized in order to reduce non-specific binding or electrostatic attraction to the dextran surface. These effects can be diminished by adding CM-dextran (i.e. 1 mg/ml or more) in the running buffer or by increasing the ionic strength (up to 500 mM NaCl). Furthermore, bovine serum albumin (e.g. 1 mg/ml or more) can be used to prevent adhesion of analyte, especially at low concentrations, to the sample tubes and the flow system of the instrument.

42.5.3 Regeneration of Antibody Surfaces

A general rule for regeneration of the antibody surface is to start with mild conditions and, if necessary, increase to harsher conditions. When using high-pH glycine-HCl (pH 2.5), continue with several short injections. Lowered pH can be tried to optimize the efficiency of the regeneration also by performing short injections (10–15 s) with 100 mM phosphoric acid (pH not adjusted). Short injections with up to 0.05% SDS may help to complete the regeneration. After using SDS

(Sodium dodecyl sulphate), apply several injections with running buffer to remove the residual detergent.

Acknowledgements We thank Dr. U. Bierfreund and Dr. W. Jäger from Biacore (GE Healthcare) for support. We thank Dr. H. Seitz (MPI for Molecular Genetics, Berlin, Germany) for providing RGS-$(His)_6$-tagged proteins. F.W.H.'s group is member of Proteome Binders. This work was supported by EU STREP Affinity Proteome to F.W.H.

References

Adams GP, Weiner LM (2005) Monoclonal antibody therapy of cancer. Nat Biotechnol 23:1147–1157

Bussow K, Quedenau C, Sievert V, Tischer J, Scheich C, Seitz H, Hieke B, Niesen FH, Gotz F, Harttig U, Lehrach H (2004) A catalog of human cDNA expression clones and its application to structural genomics. Genome Biol 5:R71

Canziani GA, Klakamp S, Myszka DG (2004) Kinetic screening of antibodies from crude hybridoma samples using Biacore. Anal Biochem 325:301–307

Diskar M, Zenn HM, Kaupisch A, Prinz A, Herberg FW (2007) Molecular basis for isoform-specific autoregulation of protein kinase A. Cell Signal 19:2024–2034

Fagerstam LG, Frostell A, Karlsson R, Kullman M, Larsson A, Malmqvist M, Butt H (1990) Detection of antigen-antibody interactions by surface plasmon resonance. Application to epitope mapping. J Mol Recognit 3:208–214

Gershon PD, Khilko S (1995) Stable chelating linkage for reversible immobilization of oligohistidine tagged proteins in the BIAcore surface plasmon resonance detector. J Immunol Methods 183:65–76

Gesellchen F, Zimmermann B, Herberg FW (2005) Direct optical detection of protein-ligand interactions. Methods Mol Biol 305:17–46

Hahnefeld C, Drewianka S, Herberg FW (2004) Determination of kinetic data using surface plasmon resonance biosensors. Methods Mol Med 94:299–320

Karlsson R, Michaelsson A, Mattsson L (1991) Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. J Immunol Methods 145:229–240

Katsamba PS, Navratilova I, Calderon-Cacia M, Fan L, Thornton K, Zhu M, Bos TV, Forte C, Friend D, Laird-Offringa I, Tavares G, Whatley J, Shi E, Widom A, Lindquist KC, Klakamp S, Drake A, Bohmann D, Roell M, Rose L, Dorocke J, Roth B, Luginbuhl B, Myszka DG (2006) Kinetic analysis of a high-affinity antibody/antigen interaction performed by multiple Biacore users. Anal Biochem 352:208–221

Nelson JD, Brunel FM, Jensen R, Crooks ET, Cardoso RM, Wang M, Hessell A, Wilson IA, Binley JM, Dawson PE, Burton DR, Zwick MB (2007) An affinity-enhanced neutralizing antibody against the membrane-proximal external region of human immunodeficiency virus type 1 gp41 recognizes an epitope between those of 2F5 and 4E10. J Virol 81:4033–4043

Pol E, Karlsson R, Roos H, Jansson A, Xu B, Larsson A, Jarhede T, Franklin G, Fuentes A, Persson S (2007) Biosensor-based characterization of serum antibodies during development of an anti-IgE immunotherapeutic against allergy and asthma. J Mol Recognit 20:22–31

Rich RL, Myszka DG (2008) Survey of the year 2007 commercial optical biosensor literature. J Mol Recognit 21:355–400

Rich RL, Papalia GA, Flynn PJ, Furneisen J, Quinn J et al (2008) A global benchmark study using affinity-based biosensors. Anal Biochem 386(2):194–216

Rogers J, Schoepp RJ, Schroder O, Clements TL, Holland TF, Li JQ, Li J, Lewis LM, Dirmeier RP, Frey GJ, Tan X, Wong K, Woodnutt G, Keller M, Reed DS, Kimmel BE, Tozer EC

(2008) Rapid discovery and optimization of therapeutic antibodies against emerging infectious diseases. Protein Eng Des Sel 21:495–505

Safsten P, Klakamp SL, Drake AW, Karlsson R, Myszka DG (2006) Screening antibody-antigen interactions in parallel using Biacore A100. Anal Biochem 353:181–190

Seitz H, Hutschenreiter S, Hultschig C, Zeilinger C, Zimmermann B, Kleinjung F, Schuchhardt J, Eickhoff H, Herberg FW (2006) Differential binding studies applying functional protein microarrays and surface plasmon resonance. Proteomics 6:5132–5139

Steukers M, Schaus JM, van Gool R, Hoyoux A, Richalet P, Sexton DJ, Nixon AE, Vanhove M (2006) Rapid kinetic-based screening of human Fab fragments. J Immunol Methods 310:126–135

Swanson SJ, Ferbas J, Mayeux P, Casadevall N (2004) Evaluation of methods to detect and characterize antibodies against recombinant human erythropoietin. Nephron Clin Pract 96: c88–c95

Tang Y, Lou J, Alpaugh RK, Robinson MK, Marks JD, Weiner LM (2007) Regulation of antibody-dependent cellular cytotoxicity by IgG intrinsic and apparent affinity for target antigen. J Immunol 179:2815–2823

Terpe K (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 60:523–533

Wassaf D, Kuang G, Kopacz K, Wu QL, Nguyen Q, Toews M, Cosic J, Jacques J, Wiltshire S, Lambert J, Pazmany CC, Hogan S, Ladner RC, Nixon AE, Sexton DJ (2006) High-throughput affinity ranking of antibodies using surface plasmon resonance microarrays. Anal Biochem 351:241–253

Willard FS, Siderovski DP (2006) Covalent immobilization of histidine-tagged proteins for surface plasmon resonance. Anal Biochem 353:147–149

Zimmermann B, Schweinsberg S, Drewianka S, Herberg FW (2008) Effect of metal ions on high-affinity binding of pseudosubstrate inhibitors to PKA. Biochem J 413:93–101

Chapter 43
Affinity Measurements Using Quartz Crystal Microbalance (QCM)

Thomas Johansson

43.1 Introduction

Affinity measurements are usually determined from titration experiments where the concentration of the reactants and products at equilibrium is established. A number of different techniques are available to determine or estimate the affinity values, the K_D-value, such as chromatography, isothermal titration calorimetry, radioimmunoassay (RIA) and the widely used enzyme-linked immunosorbent assay (ELISA). Many of the techniques rely on chemical modifications of one of the reactants or the use of indirect measurements, such as an enzyme, radio-isotope or a labeled antibody/probe, to study the affinity. In recent years, techniques have been developed to monitor molecules interacting in real time, without the need for labeling or chemical modifications. These techniques have been used to detect and study a vast variety of molecular events, such as characterization of antibody–antigen, nucleic acid, and protein interactions. The Quartz Crystal Microbalance (QCM) technology is, as many other biosensor techniques, label-free and permits a direct way to determine the affinity; the K_D. Biosensors can measure the rates by which two molecules associate, the k_{on}, as well as how rapidly they dissociate, the k_{off}. In addition, by determining the rate constants at different temperatures, it is possible to calculate thermodynamics and transition states, factors that can be used to reveal the molecular mechanism of an interaction.

Here, we present procedures that describe the basic experimental design of typical kinetic strategies as well as data processing and interpretation with the aim of calculating affinity and rate constants.

T. Johansson
Attana AB, Björnnäsvägen 21, Se-11347 Stockholm, Sweden
e-mail: thomas.johansson@attana.com

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_43,

43.1.1 Quartz Crystal Microbalance (QCM)

The piezoelectric effect seen in quartz was first discovered by Curie in the year 1880 (Curie and Curie 1880). The thin disk of quartz is cut using a specific angle (AT-cut) from a larger crystal, and by varying the thickness of the crystal, the oscillating frequency can be altered. If an oscillating voltage is applied over the AT-cut quartz crystal, it will resonate at a defined frequency. Sauerbrey discovered in the 1950s that there was a proportional relationship between frequency dampening and the mass added to an oscillating crystal (Sauerbrey 1959). The technique has the same principle as a tuning fork that resonates with a specific frequency. If mass is applied to the tuning fork, the resonance frequency changes. The resonating QCM crystal works the same way; if molecules are added to or removed from the surface, the resonance frequency is changed and can be measured.

Different surface chemistries, based on a self-assembled monolayer (SAM) of thiols, is applied on the gold-coated sensor surface. Commonly used chemistries include carboxy terminated and biotin terminated SAMs, mediating covalent coupling of amine containing ligands, and streptavidin-assisted coupling biotinylated ligands, respectively. Since the QCM technology can work with optically incompatible surfaces, Attana has developed the polystyrene chip with a sensor surface that permits physisorption of molecules, mimicking the coating performed in an ELISA plate. This sensor surface can therefore be used to optimize ELISA conditions or simply provide and serve as an alternative to the previously mentioned immobilization techniques.

There are only a few commercially available instruments that utilize the QCM properties, and not all of them support kinetic determination and affinity measurements. The Attana instruments are the QCM-instruments that are most widely used for kinetic determination, thanks to simple handling, robustness, sensitivity, and the ability to analyze crude samples. The low noise level allows for a rapid determination of very slow off-rates, as an example, a $1 \times 10^{-5}\ s^{-1}$ off-rate can be accurately determined within a couple of minutes.

Today, the Attana QCM instruments are used for a wide variety of measurements, including off-rate screening and ranking, affinity measurements, kinetics, concentration measurements, binding assays, thermodynamics, thermo selection, micro-purification, virus detection, molecular imprinting as well as optimization of ELISA protocols. ELISA-focused and in-vitro diagnostic companies use biosensors to get additional knowledge about the molecules they provide in their ELISA kits. To be able to get not only the affinity of an interaction but also additional information about the molecular mechanism, increases the possibility to select the antibody with the best properties for the kit, for example, with the shortest incubation times and the slowest off-rate or the highest affinity.

The crude sample tolerance of the Attana QCM systems permits reliable off-rate screening and ranking, balanced with a relatively high throughput, directly from hybridoma supernatants and serum samples. Moreover, the rapid and high accuracy temperature control provides a very good tool to conduct thermodynamic studies.

43.2 Ways of Calculating Affinities

The robust and refined fluidic system of the Attana instruments ensures that sample injections are delivered to the sensor surface in a reliable and reproducible manner allowing accurate on-rate measurement (k_{on}). At the injection end, the sample is exchanged for running buffer and the dissociation rate (k_{off}) can be monitored. The affinity (K_D) is calculated from these two constants by dividing the off-rate with the on-rate (k_{off}/k_{on}). By keeping the binding capacity of the immobilized molecule constant throughout an experiment, through optimized regeneration conditions, and collecting data using different concentrations of the analyte, increased precision in the analysis can be achieved. To achieve a good kinetic determination, at least three, but preferably five, different concentrations of the analyte have to be measured (Fig. 43.1).

A popular way of achieving kinetics and affinities is to use a capturing surface. This surface captures proteins by exploiting a tag (e.g. a His-tag) and part of a protein or an antibody (e.g. a Fc-moiety of an antibody). The benefit of using such a surface is that the molecules are captured in a specific way and with a homogeneous orientation, minimizing the risk of experimental artifacts. Furthermore, these surfaces do not require any regeneration optimization which often is tedious and consumes sensor chips and sample (see Fig. 43.2 for typical data). It is also possible to analyze many different proteins or antibodies on the same capturing surface, and either screen many samples, do detailed kinetics or a combination of these.

In this chapter, we will outline the general guidelines for generating the kinetic constants, as well as the affinity for an interaction using the Attana QCM biosensors. We will describe the procedures for immobilizing a ligand on a surface and running an analyte over this surface, as well as how to use different capturing surfaces using the commercially available Attana QCM technique.

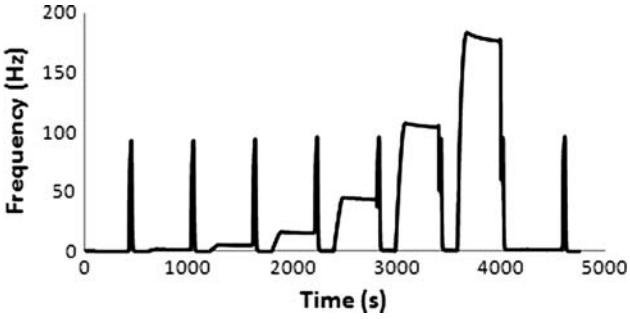


Fig. 43.1 Typical data from an assay using an immobilized molecule and six injections of increasing concentration of an analyte (T = 500–4,000 s) flanked by two buffer injections ($t = 0$ s and $t = 4{,}100$ s). After each injection a regeneration step is included to remove the analyte (appears as spikes at $t = 450$, 1,000, 1,600, 2,900, 3,400, 4,000 and 4,600 s)

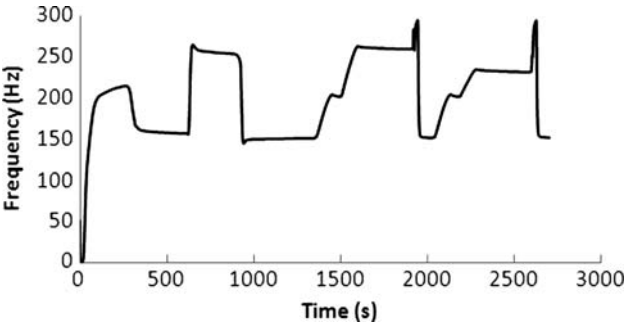


Fig. 43.2 Typical data collected from a capturing surface assay. The capturing agent is immobilized (at $t = 0$ s) followed by blocking of active carboxyl groups using etanolamine ($t = 600$ s). The first antibody is added ($t = 1{,}300$ s) followed by the antigen ($t = 1{,}500$ s). After removal of the antibody/antigen complex using regeneration solutions ($t = 1{,}900$ s), the next antibody and antigen is analyzed ($t = 2{,}000$ s)

43.3 Material

43.3.1 Equipment

Attana QCM biosensor

43.3.2 Surfaces

- Attana Carboxyl Sensor Chip *(product number 3616-3033)*
- Attana Biotin Sensor Chip *(product number 3613-3033)*
- Attana Polystyrene Sensor Chip *(product number 3611-3033)*

43.3.3 Reagent and Buffers

- HEPES-buffered saline running buffer (10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid], 150 mM NaCl, 0.005% Tween 20 *(HBS-T 10×, Attana product number 3506-3001)*
- Amine Coupling Kit, EDC (1-Ethyl-3-[3-dimethylaminopropyl]- carbodiimide hydrochloride), sNHS (N-hydroxysulfosuccinimide) and 1 M etanolamine pH 8.5 *(Attana product number 3501-3001)*
- Human IgG Capture kit *(Attana product number 3519-3001)*
- Mouse IgG Capture kit *(Attana product number 3518-3001)*

43.4 Protocols

43.4.1 Immobilizing the Ligand

A wide variety of different immobilization techniques can be used to create the active surface. An immobilization process in the Attana instrument is performed within 30 min and requires 5 μg or less of the analyte.

The most commonly used surface immobilization technique is the coupling of molecules with exposed primary amines to an activated carboxyl surface, covalently linking the ligand to the chip. Biotinylated molecules are also very common and provide a means for immobilizing molecules by utilizing the very strong interaction with Streptavidin (K_D is $1 \times 10\ E^{-15}$). In addition, site-specific biotinylation procedures allow for the immobilization of biotinylated molecules at a defined orientation.

43.4.1.1 Amine Coupling to a Carboxyl Chip

This method requires activation of the carboxyl groups on the surface by the addition of EDC/sNHS. The sulfo group on the sNHS introduces negative charges and lowers the pKa of the surface allowing immobilization of ligands with low pI. The basic immobilization protocol described below is also illustrated in Fig. 43.3.

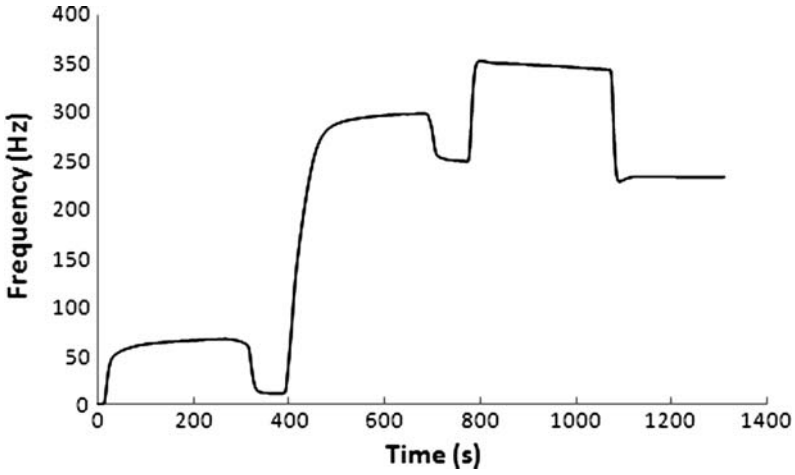


Fig. 43.3 Amine coupling of a ligand. First, the carboxyl groups are activated using EDC/sNHS (at $t = 0$ s). The ligand to be immobilized is then injected ($t = 400$ s), followed by blocking of remaining active carboxyl groups ($t = 800$ s). Here, a mass equivalent to 230 Hz of the molecule was immobilized

1. When the sensor surface is equilibrated in the running buffer, decrease the flow rate to 10 μl/min.
2. Mix equal volumes of 0.1 M sNHS and 0.4 M EDC.
3. Inject the mixture for 300 s to activate the carboxyl groups.
4. Prepare the ligand in an appropriate immobilization buffer (the recommended starting condition is 10 mM acetic acid, pH 4.5, and a ligand concentration of 50 μg/ml).
5. Clean the loop using the syringe with at least two loop volumes of high quality water.
6. Inject the ligand solution for 300 s.
7. Clean the loop using the syringe with at least two loop volumes of high quality water.
8. Inject 1 M etanolamine pH 8.5 for 300 s to deactivate the remaining active carboxyl groups.

43.4.1.2 Preparing a Surface Using a Biotinylated Molecule

By utilizing the very strong interaction between the *Streptomyces avidinii* protein streptavidin and biotin, a direct immobilization of the ligand can be achieved. One advantage with biotinylated ligands is that the Attana instruments provide the means to obtain avidity-free systems, also with bi- and multi-valent analytes. One drawback using biotinylated proteins can be that the biotinylation process could inactivate the binding site on the molecule. A typical sensorgram from an immobilization is shown in Fig. 43.4 and the procedure is as follows:

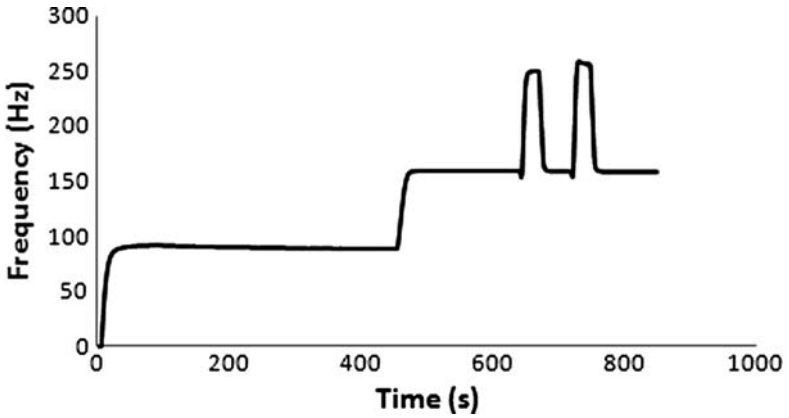


Fig. 43.4 Immobilization of a biotinylated ligand. Streptavidin is added to the sensor surface (at $t = 0$ s), followed by injection of the biotinylated ligand ($t = 450$ s). The surface is then primed twice ($t = 650$ s and 750 s) with the regeneration solution. Here, a mass equivalent to 60 Hz of the biotinylated molecule is added

1. When the sensor surface has stabilized in running buffer, set the flow rate to 25 μl/min.
2. Prepare streptavidin at 100 μg/ml diluted in the running buffer.
3. Inject the streptavidin for 80 s.
4. Dilute the biotinylated ligand in running buffer and inject for 80 s.
5. Prime the surface with a suitable regeneration condition.

The sensor surface will most likely be saturated if one injection of the ligand diluted to 50 μg/ml is performed (Step 4). Keep in mind that the high binding capacity of a saturated surface might lead to mass transport limitation effects. If such a phenomenon occurs, use a surface with a lower ligand density and/or an increased flow rate. If the analyte is bivalent or if there is a possibility of avidity effects, dilute the biotinylated ligand to 1 nM and do one 80 s injection. Then, do a high analyte injection to investigate if the signal level is high enough. If the frequency shift is too small, do another injection of the 10- ng/ml solution until you have reached a good avidity-free signal/noise level with the analyte. Avidity is easily recognized by a decreased k_{off} value.

43.4.1.3 Physisorption of a Ligand to a Polystyrene Chip

The immobilization is performed *ex situ*. Hence, the only way to obtain the desired immobilization level is by systematic testing. The benefit of using this type of surface is that the immobilization process very much resembles that of the ELISA technique. By elaborating with different immobilization buffers and ligand concentrations, optimal conditions can be quickly established. This chip type can also be tried for molecules that lose their binding capacity upon immobilization via another chemistry. The general procedure is described as follows:

1. Allow the chip to stabilize in room temperature *ex situ* for at least 10 min.
2. Dilute the ligand in the immobilization buffer (a recommended starting point is 250 μg/ml in a 10- mM NaAc buffer, pH 4.5–5, with an optional addition of 50 mM NaCl).
3. Fill the reaction chamber of the polystyrene chip with the diluted ligand using a pipette (~5 μl). To avoid evaporation, seal the sensor chamber inlet/outlet with tape (e.g. microtiter plate sealing tape) and incubate at room temperature for 3 h (or as specified by the application).
4. Prepare a suitable running buffer, such as Phosphate Buffered Saline (PBS) without surfactant, and filter through a 0.2 -μm filter. Add bovine serum albumin to a concentration of 0.1–1 mg/mL. Change the buffer in the Attana instrument using the change buffer command.
5. When finished, insert the polystyrene chip and let it run for 5 min at 100 μl/min.
6. Decrease the flow rate to 25 μl/min and condition the surface by injecting the regeneration solution.

43.4.2 Affinity Measurements on Surface-Bound Ligands

When the affinity between the immobilized molecule and an analyte is to be calculated, it is very important to establish the appropriate regeneration condition for the system. First, a high concentration of the analyte is injected to reach saturation of the surface. Thereafter, the regeneration scouting can commence. Unless you know the regeneration conditions, a rule of thumb is to go from mild to harsher conditions to avoid irreversible denaturation of the immobilized molecule. When the conditions have been established, continue by priming the surface by three cycles of analyte injection, followed by regeneration, using this high analyte concentration. If the frequency change is about the same for all three injections, then you have achieved a stable surface and found suitable regeneration conditions. To achieve reliable kinetic constants and calculate the affinity, a range of concentrations for the analyte has to be analyzed. A good starting point is to use a high concentration that saturates the surface (reaches the maximum capacity for the surface) and one concentration that only gives a small frequency response. Measure at least one, but preferably three or more analyte concentrations for the detailed kinetics. To achieve high quality data, it is good to start and end the series by buffer injections and use these as reference injections. The general procedure, after the establishment of regeneration conditions, is described below:

1. Set the flow rate to 25 μl/min.
2. Do a buffer injection for 80 s and monitor the off-rate for the same time or longer than the analyte injection.
3. Regenerate the surface.
4. Inject the analyte and monitor the on and off-rate.
5. Regenerate the surface.
6. Repeat Step 4 and 5 with various concentrations of the analyte.
7. Do a buffer injection, as in Step 2.

43.4.3 Capturing Surfaces

There are many benefits in using a capturing surface. One benefit is that the captured molecules are directed towards the solvent, resulting in a homogeneous surface. Also, no regeneration optimization is required, since a generic method has usually been established by the company that provides the capturing surface. It also increases the versatility and allows the screening of many different protein–protein or antibody–antigen interactions on the same surface. Many of these surfaces also have an increased robustness against non-specific interactions with the constituents of crude samples. In this section, two very common capturing surfaces are described, but the overall principle can be applied to close to all capturing molecules. A graphical overview of the experiment is found in Fig. 43.2.

43.4.3.1 Using the Attana mouse IgG Capturing Surface

This surface can be used to rapidly screen many different hybridoma supernatants or mouse antibodies for the same or different antigens. Following the preliminary screening, a detailed kinetic experiment can be performed by keeping the capturing level constant for one antibody and varying the antigen. The general procedure is described as follows:

1. Equilibrate and activate a carboxyl chip, as stated in Sect. 43.4.1.1.
2. Prepare the polyclonal rabbit anti-mouse IgG at a concentration of 50 μg/ml.
3. Inject the prepared polyclonal rabbit anti-mouse IgG, followed by the etanolamine, as in Sect. 43.4.1.1.
4. Change the flow rate to 25 μl/min.
5. Inject the hybridoma supernatant or the antibody to be captured at a concentration suitable for the experiment.
6. Inject the antigen at a suitable concentration and monitor the binding and dissociation.
7. Regenerate the surface using 100- mM HCl for 60 s, followed by 20- mM NaOH for 30 s.
8. Allow the surface to stabilize for 60 s.
9. Repeat Step 5–8.

For each antibody tested, a buffer injection has to be performed in order to be able to compensate for drift and to subtract the off-rate of the captured antibody. If detailed kinetics is performed, at least every fifth injection should be a buffer injection.

43.4.3.2 Using the Attana Human IgG Capturing Surface

This surface can be used to rapidly screen many different serum samples containing human antibodies or purified human/humanized antibodies for the same or different antigens. Following the preliminary screening, a detailed kinetic experiment can be performed by keeping the antibody to be captured constant and varying the antigen concentration. The general procedure is described as follows:

1. Equilibrate and activate a carboxyl chip, as stated in Sect. 43.4.1.1.
2. Prepare the polyclonal rabbit anti-human antibody at a concentration of 50 μg/ml.
3. Inject the polyclonal rabbit anti-human antibody, followed by the etanolamine, as in Sect. 43.4.1.1.
4. Change the flow rate to 25 μl/min.
5. Inject the antibody to capture at a concentration suitable for the experiment.
6. Inject the antigen at a suitable concentration and monitor the binding and dissociation.
7. Regenerate the surface using 100- mM HCl for 60 s, followed by 20- mM NaOH for 30 s.

8. Allow the surface to stabilize for 60 s.
9. Repeat Step 5–8.

For every antibody tested, a buffer injection has to be performed in order to be able to compensate for drift and to subtract the off-rate of the captured antibody itself. If detailed kinetics is performed, at least every fifth injection should be a buffer injection.

43.5 Data Analysis and Results

This part includes the processing of data, calculation of rate constants, and interpretation of results. Depending on the quality of the experimental setup, the processing of data, calculation of rate constants, and interpretation can be simple or more difficult.

43.5.1 Data Processing

The following are performed to process the raw data into a file that can be used to calculate the kinetic constants. Select the curves to be analyzed and subtract the reference injections to create a referenced subtracted dataset (Fig. 43.5). Then, calculate the kinetic constants from the dataset using the simplest model possible, usually a 1:1 model, or a mass transport model (Myszka 1997). Calculate the affinity by $K_D = K_{off}/K_{on}$

43.5.2 Data Interpretation

If the mass transport coefficient, k_m (Myszka 1997), is 10^8 ms^{-1} or lower, it is very likely that the experimental setup is mass transport limited. Depending on the

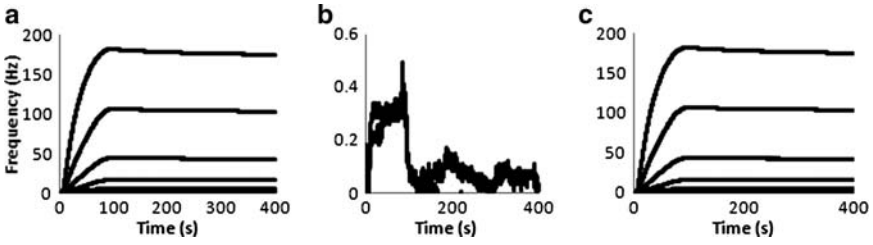


Fig. 43.5 Data processing. The analyte injections are selected (**a**) as well as the corresponding reference injections (**b**). The reference subtracted dataset is seen in (**c**)

severity of the mass transport limitation, it might be necessary to repeat the experiment with some changes. The most straightforward method is to either decrease the surface density or to increase the flow rate when the analysis is performed.

Another typical problem is a surface with too high a density, resulting in very fast analyte binding and response curves with limited kinetic information. The curvature contains more kinetic data than straight lines, and hence, if experimental data yields close to straight lines, it is recommended that the surface density be decreased and the analysis repeated. In order not to lose sensitivity, this can be combined with an increased contact time. The experiment will take a slightly longer time to execute, but the precision in the analysis will be higher.

43.6 Conclusion

Generation of data from kinetic interaction experiments and calculation of the rate constants and affinity values are matters that are considered to be relatively easy with today's advanced biosensors. However, the people using the advanced biosensors are not always skilled. It is often very easy to generate data but very hard to do a correct experimental setup and a correct interpretation of the result. These issues, how to solve them and how to create awareness about these problems, are discussed in a couple of reviews (Rich and Myszka 2007, Rich and Myszka 2008).

References

Curie J, Curie P (1880) Piezoelectric and allied phenomena in Rochelle salt. Comput Rend Acad Sci Paris 91:294–297

Myszka DG (1997) Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. Curr Opin Biotechnol 8:50–57

Rich RL, Myszka DG (2007) Survey of the year 2006 commercial optical biosensor literature. J Mol Recognit 5:300–366

Rich RL, Myszka DG (2008) Survey of the year 2007 commercial optical biosensor literature. J Mol Recognit 6:355–400

Sauerbrey G (1959) Verwendung von Schwinquarzen zur wägung dünner Schichten und zur mikrowägung. Zeitschrift für physic 155:206–222

Chapter 44
Affinity Measurements with Radiolabeled Antibodies

Verena Boschert and Peter Scheurich

44.1 Introduction

44.1.1 Radiolabeling

The method of radiolabeling typically involves the covalent attachment of radioisotopes to a particular protein. With this technique it is possible to quantify minute amounts of labeled protein, thereby elucidating, for example, its binding affinity for a specific binding partner. In general for antibodies, the affinity of antigen binding is an important characteristic. As might be anticipated, there is typically a direct correlation between the affinity of the antibody and its potency for drug targeting or tumor imaging purposes (Zuckier et al. 2000; Kennel et al. 1983).

Several established methods have been described to determine the affinity of an antibody. Most of them determine the affinity to its antigen in solution or when immobilized to a surface. Binding studies with radiolabeled antibodies are especially useful when the antigen is a cell-surface antigen, e.g., a cytokine receptor (Kontermann et al. 2008). It is then possible to use whole cells or membrane preparations in the binding assay ensuring proper folding and arrangement of the antigen. This is of particular importance because affinity determination with the antigen in solution may not correlate with the affinity to the antigen at the cell membrane. Similar constraints hold true when the antigen is immobilized on a surface.

The most commonly used radioisotope for covalent protein labeling is Iodine-125 (^{125}I). Due to the introduction of only a single atom into (typically, a tyrosine residue of) the protein, there is a good chance that biofunctionality is not affected. The nucleotide is easily detectable as it emits gamma-rays with maximum energies of about 35 keV, some of which are internally converted to X-rays. The half-life of ^{125}I is around 60 days, a suitable value for most applications. Labeling with ^{125}I is

V. Boschert and P. Scheurich (✉)
Institute of Cell Biology and Immunology, University of Stuttgart, Stuttgart, Germany
e-mail: peter.scheurich@izi.uni-stuttgart.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_44,

not very cost-intensive, however, special safety equipment is necessary. During the radioiodination process, volatile I_2 is formed and therefore the reaction must be conducted in an extractor hood containing a filter. Flat leads of 1 mm thickness are conferring sufficient protection against the released gamma and X-rays. Additionally, shields made of lead-endowed acrylic glass are useful.

The labeling reaction involves the oxidation of $^{125}I^-$ to $^{125}I^+$ by an oxidative reagent. The resulting $^{125}I^+$ is able to react with tyrosine groups of the protein at a pH value around 7.5. Alternatively, for example, if the antibody does not contain a suitable tyrosine group, histidine residues also become labeled at pH values higher than eight. When tyrosine residues must not be modified an alternative is labeling by ^{125}I-Boulton Hunter reagent which reacts with primary amino groups of the protein (Bolton and Hunter 1973).

After the labeling reaction, free $Na^{125}I$ has to be separated from the antibody. A simple method is the use of gel filtration columns like they are commercially available for desalting purposes. However, one has to be conscious about the fact that a mixture of differently labeled proteins is obtained. Dependent on the number of residues available for iodination in the protein, multiple forms of protein containing several iodine atoms are generated in addition to non-iodinated, and probably the most abundant, monoiodinated protein. If a homogenously iodinated protein is desired, high performance liquid chromatography (HPLC) may be applied for separating the differently labeled proteins (Rehm 2006).

44.1.2 Determination of Bioactivity

Radioiodination results in proteins containing modified amino acids. This could have an impact on the activity of the protein by influencing its structure, especially when amino acids in the active site or interaction sites of the protein are modified. In addition, the protein comes into contact with oxidatives and reductives during the labeling procedure. Therefore, if possible, one should compare the binding properties of the iodinated antibody with its unlabeled counterpart. Useful is, for example, a simple ELISA format. Alternatively, one can test in a cellular assay system to which degree the labeled antibody has lost its specific binding function. An antagonistic or agonistic radioiodinated antibody can be compared with its unlabeled counterpart using a competition assay system. The loss in activity determined by these methods should then be taken into account when calculating the binding affinity based on binding studies.

To quantify the percentage of the radioiodinated antibody which is still capable to bind its antigen with high affinity, it is also possible to perform binding studies as described below. However, in this case, a constant and low amount of the labeled antibody is incubated with increasing amounts of the antigen (cells in the case of a cell suface antigen or antigen coated plates with increasing antigen density). The resulting binding curve (bound cpm as a function of increasing antigen) and

the respective Scatchard plot should give a good estimate regarding the fraction of antibody still capable of high affinity binding.

If the antibody shows no binding at all after iodination, probably the reaction conditions were too harsh. The solution might be the use of milder oxidatives, like chloramine-T coupled to beads or lactoperoxidase together with H_2O_2. The advantage of the beads is that the reaction is stopped easily by separating the beads from the protein by centrifugation (Markwell 1982). No reductive has to be used, which is a more gentle procedure for the disulfide bridges of the antibody. When using lactoperoxidase one has to add H_2O_2 to the reaction. The enzyme reduces H_2O_2 to H_2O and can thereby oxidate $^{125}I^-$ (David and Reisfeld 1974). Another explanation for binding loss might be that the attached iodine atoms influence the structure of the antibody and can thereby prevent binding to the antigen. One can try to iodinate different amino acids by working at higher pH values (histidines) or by using ^{125}I-Boulton Hunter reagent (targeting then lysines; Bolton and Hunter 1973). It is also possible to change the radioisotope and to use tritium instead of ^{125}I. In a catalytic exchange, hydrogen atoms in the antibody can be exchanged with tritium. An advantage is the long half life of tritium (12.5 years), making the labeled antibody more stable than an iodinated one having a half life of only 60 days. Furthermore, the labeling reaction also takes place backwards which limits the shelf life of an iodinated protein to 3–4 weeks.

44.1.3 Different Forms of Binding Studies

Binding assays can be subdivided into equilibrium-binding studies (also called saturation binding studies) and determinations of binding kinetics. For both kinds of analyzes, it is crucial to determine unspecific binding. The radioactive protein always binds unspecifically to surfaces like the incubation tube or filters. Residual unbound protein, after separation of the bound and the unbound antibody, also accounts for unspecific binding. Unspecific binding is considered to occur in a linear, concentration-dependent manner. In contrast to specific binding, it is therefore not saturable. Accordingly, this background can be easily quantified by adding an unlabeled antibody in an about 100-fold excess related to the labeled one. This effectively blocks specific binding of the labeled antibody to its antigen. An unspecific binding of the radiolabel is linear with concentration, however, it remains unaffected, resulting in the determination of only unspecific binding. Subtracting these values from those of the total binding at the same label concentration reveals specific binding. If available, a different unlabeled protein can be used, which is known to compete with the antibody for antigen binding. A decline in binding on the level of unspecific binding would then assure that binding to the antigen of interest is measured and not the saturable binding to a different protein, for example to a membrane receptor binding the Fc (Fragment, crystallizable) region of an antibody (Keen 1999).

For saturation binding studies the binding reaction must be incubated until it is assumed to be very near to equilibrium, meaning that the rates of association and dissociation of the labeled antibody are equal. The time to reach equilibrium is not only temperature dependent but also concentration dependent; the lower the concentration of labeled antibody, the longer the equilibration times will be necessary. The time needed for equilibration can be determined by performing an association rate experiment as described below. When using whole cells, equilibrium binding studies are typically performed at 0°C, permitting largely secondary reactions induced by antibody binding like cluster formation, internalization or shedding.

When concentration of free labeled antibody [L] is plotted on the x-axis and specific binding of the antibody is plotted on the y-axis, a rectangular hyperbola described by the formula below can be obtained (Fig. 44.1). The data can be fit to the equation by nonlinear regression and one can obtain the dissociation constant K_d and B_{max}. K_d describes the antibody concentration where half of the antigen is bound. B_{max} stands for the whole amount of antigen molecules on the cell surface.

$$\text{Specific binding} = \frac{B\max \cdot [L]}{K + [L]} \tag{44.1}$$

It is also possible to linearize the data by plotting bound antibody on the x-axis and the ratio of bound antibody concentration and free antibody concentration on the y-axis, resulting in a diagram known as Scatchard plot. In the easiest case one should obtain a straight line, the x-intercept giving B_{max}, the slope $-1/K_d$

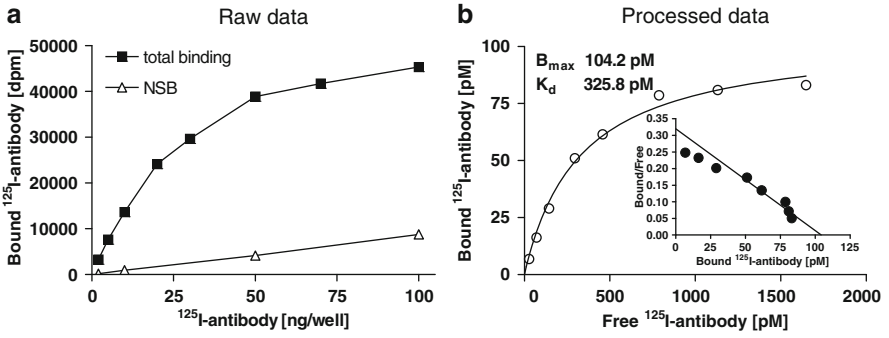


Fig. 44.1 Results from a typical equilibrium-binding study performed with a radioiodinated antibody on whole cells. Cells from the human rhabdomyosarcoma line Kym-1 (200,000 cells) were incubated at room temperature with the radioiodinated Tumor Necrosis Factor receptor type 2-specifc antibody 80 M2 (18,400 cpm/ng, stock concentration 1 µg/ml, bioactivity 42%) in 150 µl for 2 h. Labeled cells were separated from the unbound label. Raw data (**a**) show total and nonspecific binding (NSB) as a function of the amount of added ^{125}I-80 M2. To reveal specific binding, NSB values for each total binding value are calculated from the NSB regression line and then subtracted. Processed data (**b**) show specifically bound ^{125}I-80 M2 as a function of free (i.e., total input of bioactive ^{125}I-80 M2 minus bound material) antibody. Best fit of a one-site binding hyperbola is also shown. The inset depicts the respective Scatchard diagram. Here, the slope of a straight is related to the dissociation constant (K_d-value) of antibody binding, while the intercept on the abscissa reveals the number of available binding sites per cell (B_{max})

(Fig. 44.1). Cooperativity of binding or heterogenicity of binding sites will be visible by a divergence from the linearity of the data. Although it is a nice way of data display, one should not try to obtain K_d and B_{max} from a Scatchard plot by linear regression. By linearization of the data the scattering of the data, points does not follow a Gaussian distribution anymore. Furthermore, the experimental error is not defined to the y-axis, as the measured values also contribute to the x-coordinates. This violates the assumptions for linear regression and thus leads to inaccurate results (Hulme 1999).

Binding kinetic studies can be subdivided into association rate experiments and dissociation rate experiments. As association is concentration dependent, only association rates at a given concentration can be determined. The underlying association rate constant K_{on} must be calculated. To determine the association rate, a fixed concentration of radiolabeled antibody has to be added to the antigen, and specific binding is measured at various time points. Thereby, the observed rate constant K_{obs} is obtained, describing how fast the reaction reaches equilibrium at the concentration of the antibody used. For calculating the association rate constant, the dissociation rate K_{off} is needed, which can be derived from a corresponding dissociation rate experiment. Here, a fixed concentration of radiolabeled antibody is added to the antigen and incubated until equilibrium is reached. Afterwards, further binding of the antibody is blocked by adding a 100-fold higher concentration of unlabeled antibody, i.e., the dissociating label will be displaced by the unlabeled antibody. Bound radioactive antibody is then quantified as a function of time to reveal the dissociation curve. K_{off} can be obtained from this curve by nonlinear regression. As dissociation is not dependent on concentration, the observed K_{off} is identical with K_{diss}. As shown in the formula below, the dissociation constant K_d can be calculated by dividing K_{off} by K_{on} allowing determination of this value by two different methods.

$$K_d = \frac{K_{off}}{K_{on}} = \frac{K_{diss}}{\frac{K_{obs} - K_{diss}}{[L]}} \tag{44.2}$$

Several computer programs are available, which are suitable for the analysis of binding data, for example, GraphPad Prism®. On the webpage of the program, a nice introduction to the analysis of radioligand data can be found (www.graphpad.com/www/radiolig/radiolig.htm). A further insight into the analysis of data obtained by binding studies is given by the article of E. C. Hulme from "Methods in Molecular Biology, Vol. 106" (Hulme 1999).

44.1.4 Separating Bound from Unbound Antibody

In binding studies, different concentrations of the radioactively labeled antibody are incubated with the membrane preparations or the cells containing the antigen. To determine the amount of bound antibody, it has to be separated from the unbound antibody. Different methods are used for this purpose.

Because the free antibody and cells or membrane preparations differ in their physical properties, it is possible to separate them rather easily. Widely used is the technique of filtrating the reaction mixture containing membrane vesicles (Rehm 2006). The vesicles are detained by the filter and the free antibody molecules can pass. After a few washing steps, the radioactivity of the filter is quantified. With intact adherent cells, it is a useful method to seed the cells in multi-well plates and to incubate them with the radioactive antibody (Koenig 1999). The radioactive supernatant is afterwards removed and the cells are washed a few times. The cells can then be scraped off and the radioactivity of the cell suspension can be measured representing the amount of bound antibody.

Washing steps influence the equilibrium of the binding reaction by leading to dissociation of the antibody from its antigen. Especially if the antibody tends to dissociate rapidly from the antigen, other techniques might be more suitable. The technique described in the materials and methods part of this chapter uses a phthalate oil mixture through which the cells with the bound antibody molecules can be centrifugated. Unbound radioactive protein stays in the aqueous solution above the oil. The tip of the plastic tube is cut just above the cell pellet and its radioactivity is determined. Having no need for washing steps this method is probably more accurate and apart from that, less time consuming and far more comfortable.

44.2 Materials

44.2.1 Labeling of Ligand

- 10 μg of the antibody (70 μl), sterile
- Reaction buffer: 200 mM sodium phosphate buffer pH 7.4, sterile
- 37 MBq (1 mCi) Na ^{125}I
- Chloramine-T dissolved in ddH_2O, 0.5 mg/ml, prepared freshly, filter sterilize
- $Na_2S_2O_5$ dissolved in ddH_2O, 10 mg/ml, prepared freshly, filter sterilize
- NaI solved in ddH_2O, 0.6 M, sterile
- PBSB: sterile PBS with sterile 0.5% BSA and 0.02% NaN_3 (sodium azide can be omitted if not working with whole cells)
- Disposable PD10 desalting column (GE Healthcare), preequilibrated with 10 ml of PBSB

44.2.2 Equilibrium-Binding Studies and Kinetic Studies

- Phthalate-oil mixture: dibutylphthalate and dioctylphthalate mixed in a ratio leading to the desired density (*see Note 1*)

- PFA (PBS, FCS, Azide) buffer: PBS with 2% FCS and 0.02% NaN_3, for equilibrium binding studies
- Medium with 2% FCS and 0.02% NaN_3 for kinetic studies
- RIA (radio immuno assay)-tubes (Nr. 73.1055.050, Sarstedt)
- Aluminum rack for 96 RIA-tubes
- Micro tubes (Nr. 72.702, Sarstedt)
- Gamma-Counter
- For the kinetic studies: heat block or water bath
- Cells suspended in PFA buffer, for the kinetic studies in medium containing 2% FCS and 0.02% NaN_3 (2–20 Mio cells per ml, *see Note 2*)
- Labeled antibody
- Small bench top centrifuge for standard 1.5 ml reaction tubes

44.3 Methods

44.3.1 Radioiodination

A commonly used oxidative reagent is chloramine-T. $Na^{125}I$ and the antibody are incubated in the presence of $Na^{125}I$ with the oxidative reagent for a short period of time, then the reaction is stopped by the addition of a reductive agent. In addition, an excess of unlabeled NaI is added. The reaction mixture is then applied to a desalting column to remove free ^{125}Iodide, positive fractions are pooled and specific radioactivity is calculated.

1. Have 10 μg of the purified antibody in a standard 1.5 ml reaction tube under the hood in about 70 μl of reaction buffer
2. Add 37 MBq (1 mCi) of ^{125}I and mix a few times using the pipette
3. Add 10 μl of chloramine-T solution and mix a few times using the pipette
4. Allow 1 min to stand
5. Add 10 μl of sodium disulfite solution
6. Allow 5 min to stand
7. Add 70 μl of (cold!) NaI solution, mix with pipette
8. Load on PD-10 column and allow to enter the column
9. Add 1 ml of PBSB and collect Fraction 1
10. Add 1 ml of PBSB and collect Fraction 2
11. Repeat until Fraction 10
12. Count 10 μl aliquots of all Fractions

Two clearly separated peaks should appear, the first (around Fractions 3 and 4) representing the radiolabeled antibody and the second (around Fraction 7 and 8) representing residual ^{125}Iodide. The typical protein recovery is about 80%, i.e., 8 μg of radiolabeled antibody. Protein fractions are pooled, diluted to 8 ml, mixed well and three aliquots of 10 μl (10 ng) are counted. The resulting radioactivity is

divided by ten to give the specific radioactivity in dpm/ng. Values in the range of above 100,000 dpm/ng might result in quite a high instability of the protein, try other experiments with only 20 s of reaction time and/or reduced concentrations of $Na^{125}I$ and/or chloramine-T. Store at 4°C.

44.3.2 Equilibrium-Binding Studies

In this kind of study, the labeled antibody is added to a fixed amount of cells in a range of concentrations. After reaching equilibrium, the binding reaction is stopped by separating the cells from the unbound antibody. The radioactivity of the cells is a measure for the amount of bound antibody. Typically, around eight different antibody concentrations are measured in duplicates or triplicates. Additionally around four antibody concentrations have to be measured with an excess of unlabeled antibody for the determination of nonspecific binding (NSB).

1. Prepare the reaction mixture for each antibody concentration in RIA tubes on ice:
   (a) 50 µl cells suspended in PFA
   (b) Labeled antibody solution
   (c) for NSB-samples: unlabelled antibody at least 100-fold higher concentrated
   (d) Fill up to 150 µl with PFA
2. Incubate on ice for at least 2 h, mix at least one time during incubation using a pipette
3. Transfer the reaction mixture to a micro tube containing 150 µl of phthalate-oil mixture
4. Spin down in centrifuge for 20 s (~16,000 *g*)
5. Cut-off the tip of the micro tube containing the cell pellet (narrow region of the tube, oil phase) and measure its radioactivity in a gamma counter (*see Note 3*).

The best approach is probably to incubate all samples together, but perform steps 3–5 only with a subset of samples depending on the number of samples which can be measured with the counter at the same time. The rest of the samples can wait on ice for processing. Figure 44.1 shows an example of an equilibrium-binding study performed with the Tumor Necrosis Factor receptor type 2 specific antibody 80 M2 on the human rhabdomyosarcoma cell line Kym-1.

44.3.3 Kinetic Experiments

In these kinds of assays, the applied concentration of labeled protein is the same in each sample and a time kinetics of association or dissociation is prepared. For determination of NSB, some points of time have to be measured in the presence of

an excess of unlabeled antibody. Typically, these studies can be also performed at 37°C using whole cells, whereas equilibrium-binding studies performed at this temperature would allow secondary reaction like internalization and/or shedding.

44.3.3.1 Association Rate Kinetics

Samples differ in the time in which the antibody is able to interact with the cells.

1. Place 60 μl of the cell suspension in RIA tubes and equlibrate them at 37°C
2. Add 75 μl of labeled antibody solution (2× concentrated) to micro tubes containing 150 μl oil mixture (for NSB-values add unlabeled antibody, 100 times more concentrated than the labeled one in a volume of ~3 μl)
3. Equilibrate the micro tubes at 37°C
4. Start of the kinetics: Add 75 μl of the well-mixed cell suspension to the micro tubes containing the labeled antibody
5. Incubation of the micro tubes at 37°C for different time points (i.e., 0–10 min)
6. Spin down in centrifuge for 20 s (16,000 *g*)
7. Place the micro tubes on dry ice until all samples are ready
8. Cut-off the tip of the micro tube containing the cell pellet (narrow region of the tube, oil phase) and measure its radioactivity in a gamma counter (*see Note 3*).

44.3.3.2 Dissociation Rate Kinetics

The labeled antibody is incubated with the cells until binding reaction is in equilibrium, typically at 0°C when using whole cells, then it is allowed to dissociate by addition of an excess of unlabelled antibody. Dissociation can be done again at 37°C.

1. Place 30 μl of the cell suspension in RIA tubes
2. Add 30 μl of labeled antibody solution (6× concentrated) to the cells (for the NSB values add unlabeled antibody, 100 times more concentrated than the labeled one in a volume of ~3 μl)
3. Incubate on ice for at least 2 h, mix at least one time during incubation by pipette
4. Add 100 μl unlabeled antibody (100 times more concentrated than the labeled one) to micro tubes containing 150 μl oil mixture and switch if wanted to 37°C
5. Start the kinetics immediately: Add 50 μl of cell/antibody mixture to the micro tubes containing the unlabeled antibody
6. Incubation of the micro tubes at 37°C for different time points (i.e., 0–10 min)
7. Spin down in centrifuge for 20 s (~16,000 *g*)
8. Immediately place the micro tubes on dry ice until all samples are ready
9. Cut-off the tip of the micro tube containing the cell pellet (narrow region of the tube, oil phase) and measure its radioactivity in a gamma counter (*see Note 3*).

44.4 Notes

1. The oil mixture should have a density that allows the cells to pass it upon centrifugation while leaving the aqueous phase containing the unbound antibody at the top. The required density differs between cell lines: For HeLa cells and mouse fibroblasts a density of 1,014 g/cm^3 is required, for Kym-1 cells, a density of 1,000 g/cm^3. For other cell lines, the required density can be determined by staining a suspension of the cells with eosin. If the density of the oil mixture is suitable, the red dye should stay in the aqueous phase and should not stain the oil phase after centrifugation. The cells should be visible as a small pellet at the bottom of the tube (probably slightly red because of some stained cells).
2. Adherent cells should be detached by Trypsin-EDTA treatment, as scraping off could result in damaged cells. The cells should be incubated with the Trypsin-EDTA solution as shortly as possible to minimize proteolytic damage.
3. The centrifugation step separates free from cell-bound antibody. Therefore, it is important to cut the tube in the oil phase. To prevent the outflow of the residual oil and fluid of the upper part of the tube after cutting, seal the opening of the tube with one finger.

References

Bolton AE, Hunter WM (1973) The labelling of proteins to high specific radioactivities by conjugation to a 125I-containing acylating agent. Biochem J 133(3):529–539

David GS, Reisfeld RA (1974) Protein iodination with solid state lactoperoxidase. Biochemistry 13(5):1014–1021

Hulme EC (1999) Analysis of binding data. Methods Mol Biol 106:139–185

Keen M (1999) Definition of receptors in radioligand-binding experiments. Methods Mol Biol 106:187–196

Kennel SJ, Foote LJ, Lankford PK, Johnson M, Mitchell T, Braslawsky GR (1983) Direct binding of radioiodinated monoclonal antibody to tumor cells: significance of antibody purity and affinity for drug targeting or tumor imaging. Hybridoma 2(3):297–310

Koenig JA (1999) Radioligand binding in intact cells. Methods Mol Biol 106:89–98

Kontermann RE, Munkel S, Neumeyer J, Muller D, Branschadel M, Scheurich P, Pfizenmaier K (2008) A humanized tumor necrosis factor receptor 1 (TNFR1)-specific antagonistic antibody for selective inhibition of tumor necrosis factor (TNF) action. J Immunother 31(3):225–234

Markwell MA (1982) A new solid-state reagent to iodinate proteins. I. Conditions for the efficient labeling of antiserum. Anal Biochem 125(2):427–432

Rehm H (2006) Protein Biochemistry and Proteomics (The Experimenter Series). Academic Press, San Diego, CA

Zuckier LS, Berkowitz EZ, Sattenberg RJ, Zhao QH, Deng HF, Scharff MD (2000) Influence of affinity and antigen density on antibody localization in a modifiable tumor targeting model. Cancer Res 60(24):7008–7013

Chapter 45
Neutralization Tests

Philippe Thullier and Dorothea (Thea) Sesardic

45.1 Introduction

45.1.1 Neutralization Tests

Neutralization tests in vitro involve inhibition of critical stages of pathogenic activity, at a cellular or sub-cellular level. In the field of recombinant antibodies, the activity to be inhibited is generally deleterious to humans, and the putative inhibitors are antibodies or fragments thereof. The fact that recombinant antibodies are frequently obtained first in the form of antibody fragments, which cannot be reliably tested in vivo because of their short half lives, makes in vitro neutralization tests particularly important in the field of recombinant antibodies, at least for first-screening purposes.

Neutralization tests only scarcely apply to pathological conditions induced by whole bacteria or cancers, which are traditionally evaluated at the level of the whole organism by in vivo ("protection" tests), and it will not be covered in this chapter. In contrast, toxins (e.g., ricin, botulinum, diphtheria, clostridia, and pertussis toxins) and viruses neutralization tests have been widely applied, and sometimes they have replaced traditional in vivo protection models (Sesardic 1999; Sesardic et al. 2004) some of these tests have even been approved as regulatory alternatives (Sesardic 2007). Tests performed on cells, which are still in the form of an organ ("ex vivo" tests, see an example below), are included in the present definition of neutralization tests, as those realized at a subcellular level ("inhibition" tests) (Sesardic 2007).

P. (Thea) Thullier
Centre de Recherche du Service de Santé des Armées, Groupe de biotechnologie des anticorps, Département de biologie des agents transmissibles, La Tronche, France
e-mail: pthullier@yahoo.com

D. Sesardic (✉)
Division of Bacteriology, National Institute for Biological Standards and Control, Health Protection Agency, Potters Bar, Hertfordshire, UK
e-mail: thea.sesardic@nibsc.hpa.org.uk

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_45,

Three examples of toxin neutralization tests are presented in this chapter. They include (1) a viability assay after intoxication with ricin, performed at the cell level, (2) an inhibition assay of the protease activity of the botulinum neurotoxin (BoNT) light chain, performed at the sub-cellular level; and (3) an assay of BoNT induced paralysis inhibition performed at the ex vivo level. These examples cover a wide range of toxin neutralization tests that could be suitably modified to other toxins, provided the mode of action is known and suitable cells or intracellular targets have been identified. On the other hand, given the vast number of viruses that are pathogenic to Humans and the number of protocol variations, it is more difficult to cover the diverse virus neutralization tests. General considerations regarding these tests will thus be presented below, with only one example of neutralization test used in virology, i.e., plaque reduction neutralization test (PRNT) as applied to dengue virus (Thullier et al. 1999; Roehrig et al. 2008).

Those general considerations regarding virus neutralizing tests will also highlight the fact that the results of such tests may widely depend on their exact performance. For this reason, the scientists involved in antibody isolation should be well aware of the tests utilized to screen their molecules, and understand the potential limitations of neutralization models, even though they are may not always be specialists of the pathogen they target.

45.1.2 General Considerations Regarding Virus Neutralization Tests

Aggregation of viruses by dimeric IgGs may happen under particular virus-antibody ratios, but it is no longer regarded as a mechanism of virus neutralization. Structural changes of viral proteins (such as capsid stabilization) were also previously proposed but are now deemed improbable, because IgGs are flexible molecules that impose low constraints on the molecules they bind. Blocking of endocytosis, and that of viral fusion with cellular membranes, is a mechanism that follows interaction with the receptor, but it has not been frequently described. The main mechanism of virus neutralization is now widely regarded as the interference with cellular receptor binding. The blocking may be direct or indirect, by steric hindrance. Because antibodies are generally present in molar excesses, neutralization is regarded as being essentially a function of affinity, as antibodies must present an affinity that is sufficient to compete efficiently with the cell receptors. The exact location of the epitope is regarded as being of far less importance.

Testing the capacity of antibodies to neutralize viruses involves precautions regarding the origin and preparation of virions, host cells and antibodies. Primary isolates of viruses should be preferred to guarantee the integrity of surface proteins, or more practically, viruses should have been cultivated for a limited number of passages only. Master and working banks of viruses should be prepared and cell

passages limited to 5–10. This is of particular importance for viruses that mutate frequently, in particular, retroviruses (Parren et al. 1999). In another example, the human cytomegalovirus carries a gH surface protein that may be down-regulated after passaging, and even more rapidly in the presence of anti-gH antibodies. Such immune evasion of antibodies might thus be provoked, to prepare a second line of antibodies against escaping viruses (Martinez et al. 2009).

The quality of the virion preparation is also of importance, and for instance, utilizing virus stocks with significant quantities of inactive virions will cause underestimation of the neutralization potency. Viruses associated with cell membranes or viruses present inside aggregates, even after purification, have been shown to be responsible for incomplete neutralization (Prince 1994). Preparation of viruses may naturally contain several forms of viruses, such as in the case of poxviruses. The intracellular mature viruses (IMV) are responsible for cell-to-cell diffusion but not of infectivity at a greater distance. In preparations, they outnumber extracellular enveloped viruses (EEV), responsible for virus diffusion beyond the cell-to-cell level, which bear a fragile wrapping membrane. Because of that fragility, EEV produced by infected cells are not necessarily purified but let to infect distant cells through diffusion in a liquid medium, in a fashion opposite to the PRNT detailed below. Infection by EEV is thus conveniently revealed in the form of "comets," whose number decreases when anti-EEV antibodies are added to the medium for neutralization assessment (Chen et al. 2007).

To avoid the necessity of safety laboratories, pseudo-virions are utilized in neutralization tests. They represent virions whose surface proteins are retained, but other activities that are necessary for infectivity are deleted. In addition, these pseudo-virions may be conveniently quantified by an exogenous activity, such as antibiotic resistance or green fluorescent protein (GFP) fluorescence.

To replicate the in vivo infective steps, cells utilized in the test should indeed bear the receptors naturally utilized by the virus. Master and working banks of susceptible cells should be prepared to limit the number of passages, as with viruses. The use of cell lineage is very frequent for virus neutralization tests though it carries the risk that such "host cells" would be infected through a way of entry that would not be relevant to the disease so that discordance between in vitro and in vivo tests may happen.

The format and isotype of antibodies utilized in neutralization tests are also of importance. For instance, influenzae neutralization assays have to be used with full-size IgG or even with phages-antibodies, as corresponding fragments of antibodies would not keep susceptible cells at a sufficient distance from the spikes covering the virus, by which they interact with their host. Isotypes are also important in the particular case of intracellular neutralization by IgA. These immunoglobulins transit via epithelial cells where the secretory component is added, and they may bind viruses during this transit. To test such an effect, cells had been grown as pseudo-epithelial monolayers on permeable membranes and the transcytosis was blocked, (Bomsel et al. 1998) or neutralizing activity was detected at the apical pole after IgA were added at the basal pole.

Finally, one should not forget that non-neutralizing antibodies (in vitro) might still be protecting (in vivo), by immune effector recruitment. In order to predict such an effect, neutralization tests might be modified from the standard protocol to include these effectors, such as complement, for instance.

45.2 Materials

45.2.1 Neutralization of Ricin Cytotoxicity Assay

Equipments
- Flat-bottomed 96 well tissue culture plates (commercially available)
- Multichannel pipettes 50–200 µl
- Micropipettes 50–200 µl
- Polyester pressure sensitive film (commercially available)
- Tissue culture flasks, 75 cm^2 (or 150 cm^2)
- Haemocytometer (cell-counting chamber) with Neubauer rulings

Consumables
- Reference ricin toxin (vector labs) and antitoxin of known activity and stability
- Vero cells (ATCC or WHO collections) cultivated in minimal essential media (MEM) supplemented with fetal or newborn calf serum (5–10%), L-glutamine (2 mM), D-glucose (0.1% w/v), HEPES (0.015 M), penicillin (100 U/ml), and streptomycin (100 µg/ml) at a concentration of 4–10^5 cells/ml
- 0.25% trypsin/EDTA solution (commercially available)
- Trypan blue (0.4%) solution (commercially available)
- Other reagents:

  Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT (commercially available)
  Sodium lauryl sulfate, SDS (commercially available)
  N,N-dimethylformamide, DMF (commercially available)

45.2.2 Inhibition of the Botulinum Neurotoxin Light Chain Protease Activity

Equipments
- NUNC Maxisorp 96 well ELISA plate (Nunc, DIS-971-030 J)
- Gilson pipettes (P5000, P1000, P200, P100, P20) and tips
- Multi-channel pipette and tips

- Multiscan MS ELISA plate reader running Genesis software
- pH meter (calibrated from pH4 to 7, or pH7 to 10 as required)
- Electronic balance
- Pipetboy and graduated plastic disposable serological pipettes (50 ml, 25 ml, 10 ml)
- Pressure film for sealing ELISA plates (plate sealant)
- Large plastic sealable box
- Paper towels, weigh boats, lab timer and, marker pen
- Incubator set at 37°C
- Plate washer

Consumables

- Toxins: Reference Botulinum toxin A haemagglutinin complex (NIBSC A/19) and pure haemagglutinin-free toxin (NIBSC A/23) with 1831 and 650 LD_{50}/vial (Sesardic et al. 2003)

  Alternatively, use purified A and B toxins from Metabiologics Inc. (Madison, USA) @ 2.3 × 10^8 LD_{50}/mg, (1 mg/ml from Hall strain) or @ 2 × 10^8 LD_{50}/mg, (1 mg/ml from Okra strain) and pure haemagglutinin-free botulinum type E toxin from Metabiologics Inc., (Madison, USA), trypsinised and diluted in gelatin phosphate buffer (GPB, pH6.5).

  All toxins diluted to ~20 k LD_{50}/ml in gelatin (0.2% w/v) phosphate (50 mM di-sodium hydrogen orthophosphate) buffer pH6.5 (GPB) and stored frozen until use
- Subsrates:

  Synthetic peptide 70 aa substrate SNAP25137-206 (Immune System Ltd, Paignton, UK or a suitable alternative supplier) for A and E toxin neutralization (cleavage sited underlined):

  VTNDA-RENEM-DENLE-QVSGI-IGNLR-HMALD-MGNEI-DTQNR-QIDRI-MEKAD-SNKTR-IDEAN-QRATK-MLGSG >80% purity

  Synthetic peptide 50 aa substrate VAMP246-94 (Immune System Ltd, Paignton, UK or a suitable alternative supplier) for B toxin neutralization assay. (cleavage site underlined):

  MRVNVD KVLERDQKLS ELDDRADALQ AGASQFETSA AKLKRKY WWK NLK

- Primary detecting cleavage specific antibodies (Jones et al. 2008 or suitable alternative reagents available commercially)

  For A toxin antibodies to SNAP25190-197 – epitope CTRIDEANQ
  For E toxin antibodies to SNAP25193-206 – epitope CDEANQRAT
  For B toxin antibodies to VAMP2 69-76 – epitope CALQAGASQ

- Antitoxin reference standards for A, B and E calibrated in IU/vial (WHO or other suitable calibrated reagents as reviewed in Jones et al. 2006)
- Other reagents (including buffers):

DTT [DL-Dithiothreithol. Sigma, D5545]
Marvel [Dried skimmed milk powder, Premier Brands UK]
HEPES [Sigma, H3375]
Zinc chloride [Sigma, 211232]
Tween 20 [Sigma, P1379]
2,2′-Azino-bis (3-ethylbenzthiozoline 6-sulfonic acid) (ABTS) (Sigma A9941)
Hydrogen peroxide (30%w/v) [BDH, 101284 N]
Goat- anti-rabbit conjugated to HRP (Sigma A0545)
Carbonated Coating buffer (0.1 M $NaHCO_3/CO_3$, pH9.6)
HEPES/Zn/Tween Buffer (50 mM HEPES, 20 μM $ZnCl_2$, pH 7.0, 0.5% v/v Tween 20)
Blocking buffer (PBST/5% w/v Marvel): Prepared fresh for each assay by adding 5 g Marvel in 100 ml PBST and mixing thoroughly
Wash buffer PBST (PBS/0.05% v/v Tween-20)
Antibody buffer (PBST-M) (2.5% w/v Marvel in PBST)
Reaction buffer (HEPES/Zn/Tween buffer containing 5 mM DTT)
Citric Acid Substrate Buffer (0.05 M, pH 4.0)
ABTS substrate solution (0.05 M Citric Acid substrate buffer (pH 4.0), 10 mg/20 ml ABTS tablet, 10 μl/20 ml Hydrogen peroxide solution (30%w/v))

45.2.3 Inhibition of BoNT Induced Paralysis of Mouse Phrenic Nerve-Hemidiaphragm

Equipments
- Hemidiaphragm equipment/tissue baths/holders (FMI, Germany)
- Thermocirculator (Harvard Apparatus, UK)
- Computer: Dell Optiplex with 1.25 GB RAM
- PowerLab/4SP 4 channel recorder (ADInstruments, UK)
- Bridge Amp ML110 (ADInstruments, UK)
- 4 Channel Dual Impedence Stimulators (ADI, Digitimer, UK)
- Isometric force transducers, GM 2 type (FMI, Germany)
- 95% Oxygen/5% Carbon dioxide gas cylinder (BOC, UK)
- Gas regulator (BOC, UK)
- Extractor fan/filter Kit 2000 with hood and lamp (Nederman/VWR International)
- Low-energy light bulb 20w (equivalent to 100w) fitted to lamp (various)
- Small tissue clips/green braided polyester suture 2 metric 3-0 UPS (FMI, Germany)
- Calibration weight 5 g (Ohaus)
- Black thread (various)

Consumables:

– Toxins: Reference Botulinum toxin A haemagglutinin complex (NIBSC A/19) and pure haemagglutinin-free toxin (NIBSC A/23) with 1831 and 650 LD_{50}/vial (Sesardic et al. 2003)

  Alternatively, use purified A and B toxins from Metabiologics Inc. (Madison, USA) @ 2.3 × 10^8 LD_{50}/mg, (1 mg/ml from Hall strain) or @ 2 × 10^8 LD_{50}/mg, (1 mg/ml from Okra strain) and pure haemagglutinin-free botulinum type E toxin from Metabiologics Inc., (Madison, USA), trypsinised and diluted in gelatin phosphate buffer (GPB, pH6.5).

  All toxins diluted to ~20 k LD_{50}/ml in gelatin (0.2% w/v) phosphate (50 mM di-sodium hydrogen orthophosphate) buffer pH6.5 (GPB) and stored frozen until use. A fresh aliquot of each toxin must be used per assay, with the toxins diluted in pre-warmed gassed gelatin/Krebs buffer and pre-incubated for 30 min at 37°C before their application to the tissue.

  Antitoxin reference standards for A, B and E calibrated in IU/vial (WHO or other suitable calibrated reagents as reviewed in (Jones et al. 2006).

– Buffers:

  Krebs gelatin stock (4X) solution (Gelatin 0.8%, NaCl 472 mM, KCl 19.32 mM, KH_2PO_4 4.76 mM), prepared fresh on the day of assay

  Krebs Gelatine Ringer stock solutions

  Gelatine/NaCl/KCl/KH_2PO_4 stock (4X store sterile at 4–8°C, warm to room temperature (RT) before use)27.6 g/L or 55.2 g/2 L NaCl, 1.44 g/L or 2.88 g/2 L KCl, 0.64 g/L or 1.28 g/2 L KH2PO4, 8 g/L or 16 g/2 L gelatine. Heat until all the gelatine is completely dissolved, adjust volume with ion-exchanged, distilled water to exactly one and autoclave.

  Sodium hydrogen carbonate stock/$NaHCO_3$ (833 mM) – 21 g per 300 ml

  Magnesium sulfate heptahydrate stock/$MgSO_4.7H_2O$ (120 mM) – 2.96 g per100ml

  Calcium chloride 2-hydrate stock/$CaCl_2$ .2H2O (250 mM) – 3.68 g per 100 ml

  D-glucose/$C_6H_{12}O_6$ (2.22 M) – 39.6 g per 100 ml

  Store all stock solutions at 4–8°C.

  Make up 1 L of fresh Krebs solution daily from the stock solutions so that the final composition contains : Gelatine (0.2%), NaCl (118 mM), KCl (4.83 mM), KH_2PO_4 (1.19 mM), $NaHCO_3$(25 mM), $MgSO_4$.7H2O (1.2 mM), D-glucose/$C_6H_{12}O_6$ (11.1 mM), $CaCl_2$.$2H_2O$ (2.54 mM). This solution is continuously oxygenated with carbogen (95% Oxygen/5% Carbon dioxide) gas.

Animals

Male in-bred mice (strain Balb/c) weighing 19–27 g (Harlan, Olac, Bicester, UK) or Charles River (Margate, Kent, UK) for donation of tissue see (Rasetti-Escargueil et al. 2009).

45.2.4 Plaque Reduction Neutralization Test (PRNT), Applied to Dengue Virus

Equipment
- Cell incubator with CO_2 (Heraeus)
- Safety cabinet (Heraeus)

Consumables:
- Iscove's culture medium (Invitrogen, Cat 12440-046) supplemented with penicillin (20UI/mL) and streptomycin (20 μg/mL) (Invitrogen, Cat 15140-148), and calf fetal serum (3%) (Invitrogen, Cat 10091-148) (Iscove's medium, supplemented as indicated, is called culture medium in the protocol)
- Vero cells (ATCC or WHO collection), cultivated in culture medium and numbered at 2×10^5 cells/mL
- Dengue virions, representating the serotype of interest according to the expected neutralization range, and titrated at 250 Focus Forming Units (FFU)/mL in culture medium (400 mL of each). For instance, the following representative strains might be utilized: Hawaï 1944 for serotype 1, New Guinea C 1944 for serotype 2, H 87 for serotype 3, H 241 for serotype 4.
- Rabbit polyclonal antibody against dengue virus, serotypes 1, 2, 3 and 4 (BioSource, Cat MBS315003)
- Rabbit IgG-specific Goat polyclonal antibody, conjugated to peroxydase (Sigma, A6154)
- Diaminobenzidine (Sigma, Cat D4418)
- 20-wells culture plates (Costar, Cat 3513)
- 4% paraformaldehyde (PFA) (Sigma, Cat P6148) in PBS
- Carboxymethylcellulose (Sigma, Cat C9481)
- Triton X-100 0.5% (Sigma, Cat X100) in PBS
- 0.8 mL of antibody (or antibody fragment) to be tested, at a concentration of around 10 μg/ml (according to expected neutralization potency) in culture medium
- 0.8 mL of non relevant antibody (or antibody fragment) to be used as a negative control, at the same concentration than the antibody to be tested, in culture medium

45.3 Methods

45.3.1 Neutralization of Ricin Cytotoxicity Assay

Establishment and passage of Vero cells
- Prepare a cell suspension containing approximately 4×10^5 cells/ml in complete medium. Note: one 75 cm^2 tissue culture flask (~90% confluent) should

contain enough cells to prepare 3 × 96 well tissue culture plates for neutralization assay).

- To maintain the culture of Vero cells, seed approximately 1×10^6 cells into a new 75 cm^2 tissue culture flask. This should provide a confluent monolayer after a further 4–6 days of culture.
- On the day of use, remove the supernatant from a flask containing a confluent monolayer of Vero cells using a sterile pipette.
- Add 1 ml of sterile HBSS (or PBS) solution to the flask, rinse the cells, and then remove using a sterile pipette.
- Add 1 ml of sterile trypsin-EDTA solution to the flask and place in a 37°C incubator until the cells are detached from the flask (2–5 min).
- Add approximately 5 ml of complete medium to the flask to neutralize the trypsin, and resuspend the cell suspension using a sterile pipette to obtain a suspension of single cells for counting.
- Prepare a 1/5 dilution of the cell suspension in 0.4% trypan blue solution and complete medium (e.g., 100 µl cells + 100 µl 0.4% trypan blue solution + 300 µl complete medium). Note that the final dilution of the cell suspension will depend on the total number of cells present. The cell suspension should be diluted in such a way that the total number of cells counted exceeds 100 (minimum required for statistical significance).
- Prepare the haemocytometer by placing the coverslip over the mirrored counting surface.
- Using a pipette, introduce a small sample of the diluted cell suspension into the counting chamber in such a way that the mirrored surface is just covered. The chamber fills by capillary action. Fill both sides of the chamber to allow for counting in duplicate. Note: The total volume of each large square is 1×10^{-4} cm^3 (0.0001 ml).
- Count the number of cells in one large square and calculate the cell concentration per ml
- Dilute cells to approximately 4×10^5 cells/ml in a complete medium for assay.

Establishing a test dose of toxin

(The objective is to determine the toxin dose response curve and the minimum cytopathic dose of toxin (MCD), which is defined as the lowest concentration of toxin that is capable of causing a cytotoxic effect on Vero cells after defined time.)

Fill all the wells of Columns 2–10 (see Fig. 45.1 for a suitable layout) with 50 µl of complete medium using a multichannel micropipette.

Dilute the toxin in complete medium to give a starting concentration of approximately 1,000 pM concentrationl. Add 100 µl of the diluted toxin solution to each well in Column 1 using a micropipette.

Prepare serial ten or fourfold dilutions in 50 µl volumes starting at Column 1 through to Column 10 using a multichannel micropipette. Discard 50 µl from column 10.

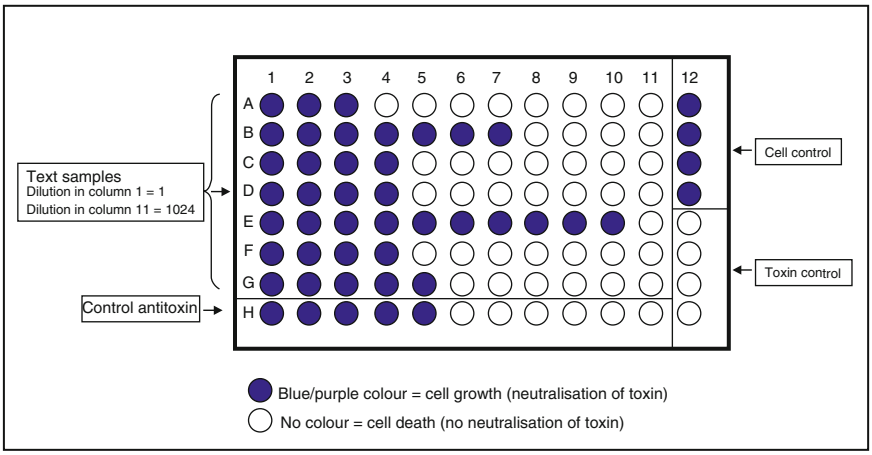


Fig. 45.1 An example of the Vero cell toxin neutralization assay following the MTT extraction. The presence of a dark blue color indicates viable cells because of the ability of mitochondrial dehydrogenase in viable cells to reduce the MTT to the colored formazan product. A light blue color indicates partial toxicity, while the absence of color indicates complete toxicity and cell death

Add 50 μl of complete medium to all wells in Columns 1–10 to bring the total volume to 100 μl.

Add 100 μl of complete medium to the "cell control" wells in Column 11.

Add 150 μl of complete medium to the "blank control" wells in Column 12.

Prepare a suspension of Vero cells in complete medium containing approximately 4×10^5 cells/ml.

Add 50 μl of the cell suspension to all wells of the microplate, except the "blank control" wells in Column 12. The total volume in all wells is 150 μl.

Shake the plates gently and cover with plate sealers to prevent the exchange of gas between medium and air. Note that the use of pressure film to seal plates is an important step for methods based on color changes in the culture medium to determine assay end points.

Incubate for 4–6 days at 37°C in 5% CO_2 incubator.

After 4–6 days of incubation at 37°C, remove the plate sealer and check the wells for microbial contamination. To improve reading of the cytotoxic end points it is recommended that spectrophotometric analysis be included by the addition of tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to stain selectively only viable cells.

Prepare a solution of MTT in PBS (5 mg/ml). Sterilize by passing through a 0.2 M syringe filter. Add 10 μl of the sterile MTT solution to each well of the microplate using a multichannel micropipette.

Return the microplate to the 37°C incubator for 2–4 h to allow metabolism of the MTT by viable cells and formation of the blue formazan product.

Remove the medium from all the wells Carefully using a multichannel micropipette.

Add 100 µl of extraction buffer to all wells and return the microplate to the 37°C incubator and leave overnight to allow extraction and solubilization of the formazan product. Once extraction and solubilization is complete, the color is extremely stable.

Examine the plates visually, and measure the absorbance at 550–570 nm on a microplate reader.

Determine MCD, which is defined as the dilution and lowest toxin concentration at which the absorbance (reflection of cell viability) is less than 50% of the mean optical density obtained in the wells containing Vero cells alone (control wells). The test dose of toxin will contain at least 5 to 10 MCD doses of the toxin to be suitable for the assay.

Neutralization test

Mark the 92 tissue culture plates to separate the test and control sections as suggested in Fig. 45.1. The suggested layout will allow testing of seven different antibody preparations.

Fill all the wells of Columns 2–11 with 50 µl of complete medium using a multichannel micropipette.

Fill the first four wells in Column 12 (12A–12D) with 100 µl of complete medium using a multichannel micropipette ("cell control").

Fill the last four wells in Column 12 (12E–12H) with 50 µl of complete medium using a multichannel micropipette ("toxin control").

Add 100 µl of each test serum sample into the appropriate well in Column 1 (test samples may be pre-diluted).

Prepare a suitable dilution of the control antitoxin in complete medium. Add 100 µl of the diluted control antitoxin to the appropriate well in column one in every plate.

Make a four or two -fold dilution series in 50 µl volumes starting at column 1 through to column 11 using a multichannel micropipette. Discard 50 µl from column 11 to equalize volumes.

Prepare a dilution of the toxin in complete medium with a predetermined concentration of approximately 10× the minimum cytopathic dose (10× MCD).

Add 50 µl of the diluted ricin toxin solution to all wells in columns 1–11 using a multichannel micropipette. Add 50 µl of the diluted ricin toxin solution to the last four wells in column 12 (12E–12H, toxin control).

Mix antitoxin with toxin by shaking gently and cover the plate with a lid.

Incubate at room temperature (20–25°C) for 1 h to allow toxin neutralization to occur.

Meanwhile, prepare a Vero cell suspension in complete medium containing approximately 4×10^5 cells/ml.

Add 50 µl of the cell suspension to all wells of the microplate. The total volume in all wells should now be 150 µl.

Shake the plates gently and cover with plate sealers to prevent the exchange of gas between medium and air.

Incubate for 4–6 days at 37°C in 5% CO_2 incubator.

After 4–6 days of incubation at 37°C, proceed by adding of MTT, as described above.

From the dose response curve of % cell survival against the log antitoxin concentration in the presence of a fixed dose of ricin toxin, it will be possible to calculate the antitoxin neutralizing potency of the antitoxin. For example, the amount of antitoxin required to inhibit a known amount of toxin in μg, can be calculated. For convenience, the reference antitoxin is stabilized and its activity is determined in arbitrary units, where a unit activity can be defined as the amount of antitoxin that neutralizes 1 μg of toxin. The neutralizing potency of unknown samples can thus be assayed in parallel with the reference antitoxin and their activity expressed in units, using the principle of parallel line assay.

45.3.2 Inhibition of the BoNT Light Chain Protease Activity

Immobilization of Synthetic peptide substrate to 96 well plates:

- Dilute appropriate substrate peptide (SNAP25 137–206 or VAMP 46–90) in carbonated coating buffer to give a final concentration of approximately 3–5 μg/ml and apply 100 μl/well to all the wells the of 96-well NUNC ELISA plate.
- Seal the plate with sealant and incubate at RT for 18 h (overnight).
- Decant and block plates with 300 μl/well of Blocking buffer.
- Seal the plate with sealant and incubate at RT, in a closed box, for 90 min.
- Wash the plate three times in distilled water, followed by tapping onto paper towels to remove all the excess liquids from the wells and air-dry.
- Once dried, the plate can either be used immediately or stored in a sealed bag with desiccators at −20°C for up to 3 months.

Establishing a test dose of toxin

- (To determine the test dose of botulinum toxin, and the range of toxin concentration, e.g., doubling, dilution between 0.1 and 200 mouse LD50 will be used in the assay. [2] The objective is to determine the toxin dose response range and the minimum dose of toxin producing near or 80% maximum optical density (OD) response in the assay.)
- Dilute Botulinum toxin in Reaction buffer to a suitable stock concentration (e.g., 200 mouse LD50/ml)
- Add 100 μl/well of Reaction buffer to all the wells of the peptide substrate-coated 96 wells
- Add 100 μl/well of the suitable stock concentration of Botulinum toxin to 100 μl/well of Reaction buffer in Column 1, giving the starting toxin concentration of ~200 mouse LD50/ml in the Column 1.
- Perform doubling dilution of the toxin across the plate up to Column 11, leaving Column 12 to act as an assay blank (contains buffer, detecting antibodies and no toxin)

- Seal the plate with sealant and incubate at 37°C for 18 h, in an open box.
- Wash the plate three times in PBST and remove all the excess liquids from the wells
- Add 100 μl/well of specific antibody suitably diluted in antibody buffer, to all the wells
- Seal the plate with sealant and incubate at room temperature for 90 min, in an open box
- Wash the plate three times in PBST, followed by, tapping onto paper towels to remove the excess liquids from the wells and add 100 μl to all the wells of the secondary detecting antibody, anti-rabbit IgG HRP (diluted as recommended in antibody buffer)
- Seal the plate with sealant and incubate at room temperature, in an open box, for 90 min
- Wash the plate three times in PBST, followed by tapping onto paper towels to remove the excess liquids from the wells
- Add 100 μl/well of ABTS substrate solution to all the wells and allow the color to develop for 30 min at RT (do not stack plates during color development)
- The absorbance is read at 405 nm using a suitable plate reader (Multiscan plate reader)

Neutralization test

- Use uncoated 96 NUNC ELISA plates to prepare the dilution of the antitoxin (test preparation or reference antitoxin) in Reaction buffer. A suitable dose range for reference antitoxin is between ~0.1 mIU/ml and 100 mIU/ml diluted from a suitable stock concentration, e.g., 200 mIU/ml^2.
- Perform 1 in 1.6 dilution of the antitoxin across the plate up to Column 10.

  (e.g., Antitoxin concentration across the plate is 20.0, 12.50, 7.81, 4.88.... mIU/ml)

- Dilute the toxin in Reaction buffer to a suitable stock concentration that produces near maximal OD for antitoxin neutralization (e.g., 150 mouse LD50/ml for A and E toxins and 20 mouse LD50/ml for B toxin), and add 75 μl to all wells except blanks.
- Seal the plate with sealant. Using a plate shaker, gently shake the plate for 1 min, to allow the antitoxin and toxin to mix, and incubate the plate at 37°C for 60 min in the open box.
- Transfer 100 μl/well of antitoxin/toxin mixture from the uncoated plate plates to SNAP25137-206 for A and E or VAMP2 46–90 coated plate for B assays, changing tips after each transfer to avoid contamination.
- Add 50 μl/well of Reaction buffer to the control wells (e.g., A–F 11–12) together with 50 μl/well of either antitoxin or toxin to act as controls.
- Add 100 μl/well of buffer to the blank wells (e.g., G–H 11–12).
- Proceed with the assay exactly as described above for toxin titration.
- Dose-dependent inhibition of enzyme cleavage reaction will be determined for WHO reference antitoxins in the range of 0.8–100 mIU/ml, and the protective

capacities of the anti-L chain scFVs or IgGs are confirmed if the appearance of the reaction product is prevented by the addition of the mixture of toxin/anti-L scFvs compared to the addition of the toxin alone, using at least four replicates for each antibody

The estimates of the relative protective activity of the antibody can be calculated using parallel line method.

45.3.3 Inhibition of BoNT Induced Paralysis of Mouse Phrenic Nerve-Hemidiaphragm

Dissection and set up

- Left phrenic nerve-hemidiaphragm preparations are excised from male, in-bred mice (Balb/c) and installed in a 6 ml organ bath maintained at 37°C containing Krebs-gelatin buffer gassed with 95% O2/5% CO2 bubbled.
- Kill the mice by cervical dislocation. Avoid overstretching the mice as this may damage the diaphragm's neuromuscular junction/nerve or severe the phrenic nerve.
- Place the mice under the lamp/extractor hood on the ice block. Lay the animal on its back, remove the fur and skin and muscle layers covering the chest, and expose the rib cage.
- Make lateral cuts on either side of the midline incision, parallel to the ribs, and fill the cavity with cold-gassed Krebs solution.
- Lift the caudal edge of the incised rib cage and remove all the ribs except the one attached to the diaphragm on the animals left side.
- Free the left phrenic nerve of the connective tissue/fat, and tie a short length of dark-colored thread around the uppermost part of the nerve and cut the nerve above the attached thread.
- Cut the wall of the abdomen to free the rib cage. Irrigated the diaphragm and phrenic nerve regularly with cold-gassed Krebs.
- Hold the xiphisternum with forceps and cut straight down through the diaphragm to the spine, take care not to cut or stretch the phrenic nerve.
- Cut the diaphragm laterally from its attachment to the body wall so freeing the preparation from the animal.
- Place the nerve and muscle in a Petri dish containing gassed Krebs and trim the ribs/diaphragm to a suitable size to fit in the organ bath.
- Attach a small tissue clip with an attached loop of thread to the tip of the tendinous tissue at the apex of the diaphragm preparation for the purpose of attaching to the transducer later.
- Locate the center of the rib on the platinum spike electrode of the tissue holder quickly but gently and impale to secure the preparation.

- Pull the thread attached to the nerve gently through the ring electrode. Hook the loop from the tissue clip/diaphragm onto the force transducer, and lower the mounted preparation into the organ bath containing gassed warmed Krebs.
- Maintain tissue baths at 37°C they must contain 6 ml of Krebs/gelatine oxygenated with 95% O2 + 5% CO2. Apply a short burst of direct (muscle) stimulation (~30 V, 1 Hz, lms) is applied shortly (5–10 min) before toxin addition and at the end of the experiment as an added control.
- Supply indirect stimulation (*via* the nerve) continuously using a supramaximal voltage (~3v, 1 Hz, 0.2 ms) and record the resultant muscle contractions using an isometric force transducer that is linked to a bridge amplifier ML110 and a Powerlab/4SP 4 channel recorder.
- Adjust the tension to give a baseline tension of 1–1.5 g (15 mN) and start recording and stimulatng of the nerve (~3 V, 1 Hz, 0.2 ms).
- Ensure that the preparation is adequately aerated, but over aeration will produce excessive frothing.
- Maintain nerve stimulation throughout the experiment, except during washing or direct stimulation.
- Adjust the tension until the baseline tension is consistent. Once the muscle-twitch responses to nerve stimulation gets stabilized and are of constant size for at least 30 min, the tissue is ready for use.

Establishing a test dose of toxin and neutralization step

- Prepared and stabilized tissue is used and Krebs buffer is replaced with a 6 ml of botulinum toxin (dependent on the size of the organ batch) diluted in Krebs solution.
- A fixed concentration of botulinum toxin, shown to induce quantifiable paralysis for each serotype will need to be determined from the dose response curves (e.g., 2–300 mouse LD50) (Rasetti-Escargueil et al. 2009). The decrease in contraction is calculated as a percentage based on the extent of the contractions just before toxin addition (Chart is shown as an example in Fig. 45.2).
- Once a test dose of each toxin serotype is determined, this fixed concentration is mixed with appropriate serial dilutions of WHO reference antitoxins.
- The time to reach 50% paralysis after the addition of the mixture of toxin and antitoxin is measured by fitting the linear part of the paralysis curve. Titration curves are obtained using data from at least four replicates for each antitoxin dilution and for each of the antitoxin serotype.
- The protective capacities of the antibodies are confirmed if the time to reach 50% paralysis after the addition of the mixture of toxin/antitoxin is longer than after addition of the toxin alone using at least four replicates for each preparation.

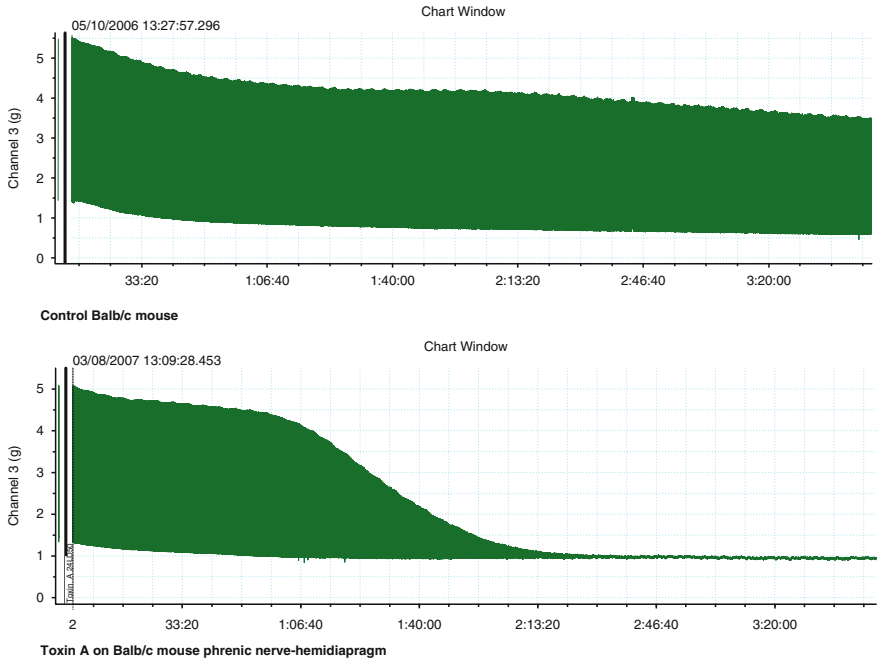


Fig. 45.2 The effect of botulinum type A toxin $12LD_{50}$/ml on the nerve induced twitch response is shown. Raw trace examples of one control preparation and one preparation exposed to type A toxin

45.3.4 Plaque Reduction Neutralization Test (PRNT), Applied to Dengue Virus

Day 1
- Dilute twofold the antibody and the negative control in culture medium, to obtain two series of five dilutions of 400 µL each
- Add 400 µL of virion suspension, and then add 300 µL of culture medium to each antibody dilution
- Incubate overnight at 4°C
- Plate 1 mL of the Vero cell suspension in each well of a culture plate and incubate overnight at 37°C with 5% CO_2

Day 2
- Discard the culture medium from the wells
- Incubate 500 µL of each product of incubation in a culture well, in duplicate
- Incubate the plate at 37°C, 5% CO_2 for 2 h
- Discard the product of incubation present in each well

- Cover each well with 500 μL of Iscove-carboxymethylcellulose 1.6%-CFS 3%
- Incubate the culture plates at 37°C, 5% CO_2 for 5 days (for serotypes 2 and 4) or 6 days (for serotypes 1 and 3)

Day 7 or 8
- Dicord iscove-carboxymethylcellulose 1.6%-CFS 3%.
- Fix cells with PFA 4% for 20 min., and then discarded
- Permeabilized cells by Triton X-100 0.5% for 4 min, which is then discarded
- Incubate 300 μL of primary antibody and diluted 1:100 in PBS-BSA 1% in each well for 2 h at RT.
- Wash each well once by 500 μL PBS.
- Incubate 300 μL of secondary antibody, diluted 1:1000 in PBS-BSA 1% for 1 h at RT
- Each well is washed once by 500 μL PBS
- 300 μL of diaminobenzidine is incubated at RT for 20 min., then discarded
- Each well is washed once by 500 μL PBS
- Perform an enumeration of foci manually, calculate the ratio of (the number of plaques observed for tested antibody)/(the number of plaques observed for nonrelevant antibody) for each antibody dilution. Name the concentration of the antibody giving 90, 50 or 30 plaque reduction $PRNT_{90}$, or $PRNT_{50}$, or $PRNT_{30}$ respectively.

Final notes:
- By definition, a "neutralizing epitopes" is the epitope of a neutralizing antibody that has the ability to bind to toxin or a virus in such a way as to prevent or block the entry, translocation, or intracellular activity.
- Dilutions of botulinum toxin should be performed within Class II safety cabinet
- The sensitivity of the cells to toxin may vary when different batches of cells are used. It is therefore important to use a set of standardized reagents to help in the routine performance of the assay and to select a robust concentration of toxin for the neutralization test.
- The dengue virus neutralization assay should be performed in a BSL3 safety laboratory
- Pipetting during the dengue virus neutralization test should be done carefully, so as to avoid damage of the cell monolayer
- Iscove-carboxymethylcellulose 1.6%-CFS 3% is a semi-liquid culture medium, utilized to prevent the diffusion of viruses from infected cells, as these following rounds of infections would render the test more difficult to interpret
- A standardized neutralizing antibody provided by other laboratories or by WHO, for instance, might be utilized in some cases. The neutralization potency of this positive control and of the tested antibody might then be compared.
- Neutralization tests may be applied to polyclonal sera induced by vaccines, to indirectly evaluate the vaccine potency through the neutralization property of the induced antibodies.

Acknowledgements The authors would like to thank Drs RGA Jones, P Stickings, C Rasetti-Escargueil and Ms Y Liu for help with protocols on ricin and botulinum neutralization assays.

References

Bomsel M, Heyman M, Hocini H, Lagaye S, Belec L, Dupont C, Desgranges C (1998) Intracellular neutralization of HIV transcytosis across tight epithelial barriers by anti-HIV envelope protein dIgA or IgM. Immunity 9:277–287

Chen Z, Earl P, Americo J, Damon I, Smith SK, Yu F, Sebrell A, Emerson S, Cohen G, Eisenberg RJ, Gorshkova I, Schuck P, Satterfield W, Moss B, Purcell R (2007) Characterization of chimpanzee/human monoclonal antibodies to vaccinia virus A33 glycoprotein and its variola virus homolog in vitro and in a vaccinia virus mouse protection model. J Virol 81:8989–8995

Jones RG, Corbel MJ, Sesardic D (2006) A review of WHO International Standards for botulinum antitoxins. Biologicals 34:223–226

Jones RGA, Ochiai M, Ekong T, Sesardic D (2008) Development of improved SNAP25 endopeptidase immuno-assays for botulinum type A and E toxins. J Imm Methods 329:92–101

Martinez O, Tsibane T, Basler CF (2009) Neutralizing anti-influenza virus monoclonal antibodies: therapeutics and tools for discovery. Int Rev Immunol 28:69–92

Parren PW, Moore JP, Burton DR, Sattentau QJ (1999) The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. Aids 13(Suppl. A):S137–S162

Prince AM (1994) Challenges for development of hepatitis C virus vaccines. FEMS Microbiol Rev 14:273–277

Rasetti-Escargueil C, Jones RG, Liu Y, Sesardic D (2009) Measurement of botulinum types A, B and E neurotoxicity using the phrenicnerve-hemidiaphragm: Improved precision with in-bred mice. Toxicon 53:503–511

Roehrig JT, Hombach J, Barrett AD (2008) Guidelines for plaque-reduction neutralization testing of human antibodies to dengue viruses. Viral Immunol 21:123–132

Sesardic D (1999) Alternatives to the use of animals for bacterial toxins and antitoxins. Dev Biol Stand 100:75–82

Sesardic D (2007) Potency and safety assessment of vaccines and antitoxins: Use of cell-based assays. In: Davies G, Davies J (eds) Medicines from animal cell culture. Wiley, Chichester, pp 497–503

Sesardic D, Leung T, Gaines Das R (2003) Role for standards in assays of botulinum toxins: International collaborative study of three preparations of botulinum type A toxin. Biologicals 31:265–276

Sesardic D, Jones RG, Leung T, Alsop T, Tierney R (2004) Detection of antibodies against botulinum toxins. Mov Disord 19(Suppl 8):S85–S91

Thullier P, Lafaye P, Megret F, Deubel V, Jouan A, Mazie JC (1999) A recombinant Fab neutralizes dengue virus in vitro. J Biotechnol 69:183–190

Chapter 46
Functional Characterization of Antibodies Neutralizing Soluble Factors *In Vitro* and *In Vivo*

Geertruida M. Veldman, Zehra Kaymakcalan, Renee Miller, Leena Kalghatgi, and Jochen G. Salfeld

46.1 Introduction

Therapeutic antibodies against soluble factors are generated almost entirely for the purpose of neutralizing the biologic effects of these factors. Neutralization assays are incorporated into the screening paradigm early on for the selection of lead candidate antibodies. If a program of affinity maturation or humanization of lead candidates is in place, neutralization assays must run in parallel to antigen-binding assays. Affinity measurement assays for these soluble factors are often based on an enzyme-linked immunosorbent assay (ELISA) (Underwood 1993) or a specialized instrument, such as BIAcore (BIAcore, Inc, Piscataway, NJ) (Myszka et al. 1998), KinExA (Sapidyne Instruments, Inc., Boise, ID) (Darling and Brault 2004), AlphaScreen® (PerkinElmer, Waltham, MA) (Guenat et al. 2006), or fluorescence resonance energy transfer (Glickman et al. 2002). The progress of affinity maturation or humanization is determined by an increase in or preservation of initial affinity values, respectively. The terms affinity and avidity of antibodies are often used indiscriminately. For most factors for which there is only one epitope per molecule, the distinction between affinity and avidity does not matter practically and monitoring the affinity by any sensitive method is adequate. Affinity of an antibody for a ligand (antigen) is an intrinsic value and does not change in different experimental settings, such as different antigen or antibody concentrations. On the other hand, neutralization potency, often expressed as a 50% inhibitory concentration (IC_{50}) value, is a relative term. Its value for an antibody changes when determined at different antigen concentrations. It is, therefore, necessary to cite the IC_{50} value of an antibody within the context of a distinct antigen concentration.

G.M. Veldman (✉), Z. Kaymakcalan, R. Miller, L. Kalghatgi, and J.G. Salfeld
Abbott Bioresearch Center, Department of Biologics, 100 Research Drive, Worcester, MA 01605, USA
e-mail: trudi.veldman@abbott.com

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_46,

Neutralization of a soluble factor by an antibody can be achieved in two ways. The first is straightforward and involves binding of the antibody to the soluble factor in such a way that the soluble factor can no longer bind to its receptor. This could be achieved either by direct competition with the receptor-binding sites on the soluble factor or by inducing allosteric changes in the factor that prevent receptor binding. This mode of neutralization affords a variety of neutralization assays. In addition to a gold-standard bioassay, several receptor-binding assays can be used. If cell lines expressing the receptors for the soluble ligand are available, a receptor-binding assay can be developed with the labeled soluble ligand to determine the ability of the antibody to inhibit its binding. In cases where no such cell line exists, the corresponding receptor could be transfected into a suitable cell line. The receptor-binding assay could even be extended to a purely biochemical assay in which the receptor can be expressed as a soluble molecule either by itself (minus the transmembrane and intracellular domains) or as a chimeric molecule fused to an immunoglobulin constant domain (Fc region) (Li et al. 2004). With such reagents, different platforms such as ELISA (Hautanen et al. 1989), BIAcore (Miyashita et al. 2005), KinExA (Darling and Brault 2004), AlphaScreen (Wilson et al. 2003), and fluorescence resonance energy transfer (Llopis et al. 2000) can be used to determine the inhibition of ligand receptor binding by the candidate antibodies.

Neutralization of a soluble ligand by an antibody could also be achieved by interfering with the signaling mechanism. In this scenario, the ligand binds to its receptor on the cell surface, but simultaneous binding of the antibody interferes with the productive engagement of receptor-ligand pairing and the transmission of the signal through the complex. The proper way to assess such a neutralization mechanism is to use a traditional bioassay. The bioassay should be chosen with great care. The vast majority of ligands are pleiotropic in terms of their biologic actions. The biologic read-out and the cells or cell lines of the selected bioassay should reflect the intended use of the therapeutic antibody. If several bioassays are possible, then concentration-response curves of soluble factor in these assays should be compared, and the one closest to the physiologic concentration should be considered.

Functional characterization of antibodies that inhibit soluble cytokines or chemokines requires the availability of robust *in vitro* bioassays with sufficient sensitivity to accurately determine the neutralization potency of the antibodies. Protocols for many cytokine or chemokine biologic response assays can be found in *Current Protocols in Immunology* (Foster et al. 2007; Pappu and Dong 2007; Zeng et al. 2007), and Meager (2006) has provided a list of references for bioassays. In addition, *in vitro* bioassays suitable for assessment of neutralizing antibodies to key cytokine targets of therapeutic interest, such as tumor necrosis factor (TNF) (Jeffes et al. 1991; Pauli et al. 1994), interleukin-13 (IL-13) (Miller et al. 2008), IL-17 (Yao et al. 1995), IL-18 (Konishi et al. 1997), and IL-23 (Aggarwal et al. 2003), have been described.

In the selection and development of an *in vitro* bioassay for antibody characterization, several factors that ultimately determine the accuracy and relevance of the data that will be obtained need to be considered. Some of the factors are:

1. Antigen source and integrity
2. Antigen concentration
3. Magnitude of biologic response (stimulation over background)
4. Assay interference by components in antibody test sample
5. Correlation of *in vitro* activity with *in vitro* activity

46.1.1 Antigen Source and Integrity

An important first step in choosing a biologic response assay is to select a suitable form of the antigen. In most cases, recombinant antigen can be used and is often purchased in purified form. The expression, folding, and stability of the recombinant antigen may be improved by modifying the natural amino acid sequence of the antigen. Examples include introducing selective cysteine to alanine mutations in the IL-18 sequence to prevent disulfide bond scrambling, producing the heterodimeric cytokine IL-23 as a single-chain protein or expressing the mature forms rather than the pre-pro forms of the IL-1 family members. The expression system that is chosen for antigen expression (e.g., COS cells, Chinese hamster ovary cells, *Escherichia coli*, and baculovirus) may also affect antigen potency and antibody affinity, and host cell-derived impurities can have significant effects on assay outcomes.

Because a recombinant antigen typically is used for *in vitro* assay validation and evaluation of antibody neutralization potency, it is necessary to confirm that the antibody has a similar neutralizing potency for the native antigen. Ideally, native antigen is isolated and purified from natural sources and tested in the bioassay. In cases in which native antigen concentrations are too low, crude cell supernatants containing the native antigen are used in the bioassay and the antigen-dependent readouts are used to determine antibody potency. This is especially important when comparing the neutralization potency of the antibody for the antigen in species that are used in preclinical pharmacokinetic and toxicologic studies. A bioassay that allows cytokines from several species to be tested in parallel using the same assay format is of great benefit. An example protocol for such an assay is provided in Sects. 46.2.2 and 46.3.2. Alternatively, an *in vitro* assay can be developed by stimulating native cytokine production in whole blood and measuring the downstream cytokine response in the whole blood sample. An example of a whole blood assay for IL-18 is provided in Sects. 46.2.3 and 46.3.3.

46.1.2 Antigen Concentration

For selection of the appropriate antigen concentration in the bioassay, a concentration-response curve of the cytokine in the assay should be established. Usually, 50–70% of the maximum effect concentration (EC_{50}–EC_{70}) is an adequate starting point to develop a bioassay. Inhibition at antigen concentrations around EC_{50} are very sensitive to small changes in concentrations of antagonists, and thus result in

deriving consistent IC_{50} values. Ideally, the EC_{50}–EC_{70} concentration of antigen should be similar to the concentration of target antigen found in tissues, especially in disease states for which the therapeutic antibody is intended for administration. For example, if the concentration of the antigen used in the bioassay is much greater (several logs greater) than the concentrations encountered in tissues, then the potency of the antibodies selected in the *in vitro* bioassay may not be appropriate to neutralize the biologic effects of the target factor *in vitro*. Although the strength of the affinity determines the potency of neutralization, the kinetic parameters of affinity (especially the association rate constant) are very critical factors in the neutralization of low concentrations of target antigens. Antibodies with faster association rate constants bind a greater amount of the antigen at low antigen concentrations and thus neutralize more antigen when compared with antibodies with slower association rates. At high-antigen concentrations, the sensitivity of the assay to rank the neutralization potencies of a panel of antibodies is limited, because the concentration of the antibodies required to result in inhibition is primarily determined by the antigen concentration and less by the affinity of the antibodies for the antigen.

46.1.3 Magnitude of Biologic Response (Stimulation Over Background)

The biologic stimulation over background obtained at the EC_{50}–EC_{70} antigen concentration should be ideally five-fold to allow for a large enough response window to assess inhibition by the antibodies and to calculate IC_{50} values accurately. However, more important than the magnitude of biologic response are other parameters that indicate robustness of the assay, such as acceptable standard deviations and reproducibility. For obtaining good neutralization data, samples need to be tested at least in duplicate, if not in triplicate or quadruplicate, to reduce variability. Some antibodies may not be able to inhibit fully the activities of their target cytokines even at very high concentrations. This partial inhibition is often because of the epitope of the antibody. In general, a good neutralizing antibody should be able to inhibit more than 90% of the biologic activity of the target antigen.

46.1.4 Assay Interference by Components in Antibody Test Sample

In cases in which the candidate antibodies are generated by the hybridoma technology, components in hybridoma supernatants may interfere with the bioassay by either causing a non-antigen-specific stimulation of cellular response or by decreasing the antigen-specific response. One should always test a reference antibody spiked into spent hybridoma supernatant to ensure that neutralization can be observed under the assay conditions chosen. If the interference is significant, the antibody may need to be

purified prior to testing in the bioassay. Other interfering substances include serum, host cell proteins, and *E. coli*–derived endotoxin in the case of recombinantly generated antibodies or antibody fragments.

46.1.5 Correlation of In Vitro *Activity with* In Vivo *Activity*

Evaluation of the neutralizing potency of an antibody *in vivo* requires either cross-reactivity of the antibody with the target antigen in the test species or introduction of the human antigen into the test species either by injection, transgenic mouse technology, or transient grafting of human cells into immunocompromised mice. The first two approaches require that the administered or expressed human protein is functional in the test species. In the third approach, the grafted human cells can serve as antigen producers and responder cells. *In vivo* testing offers some advantages, including testing the antibody against the native antigen in its natural biologic environment in which the antigen is present at physiologic concentrations. However, *in vivo* testing also presents a number of challenges, the impact of which is sometimes difficult to quantify. Testing an antibody *in vivo* requires consideration of the extent of cross-reactivity with the target antigen in test species, selection of the dosage range and the route of administration, analyses of the pharmacokinetics and tissue distribution of the antibody, and evaluation of the effect of a host antibody response to the antibody to be administered. An example of a simple, short-term *in vivo* bioassay is provided in the protocol in Sects. 46.2.4 and 46.3.4 (Wu et al. 2007). In this assay, human peripheral blood mononuclear cells (PBMCs) were injected into severe combined immunodeficient (SCID) mice and then activated *in vivo* to produce human IL-18, which in turn led to the production of interferon-γ (IFN-γ). Antibodies to IL-18 were administered the day before the activation of human cells in the SCID mouse-inhibited IFN-γ production in a dosage-dependent manner, confirming that the antibodies were able to inhibit native human IL-18 in an *in vivo* setting.

46.2 Materials

46.2.1 A Typical Bioassay to Determine Antibody Neutralization Potency (KG-1 Assay for IL-18)

– Complete medium: RPMI 1640 medium (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, and 0.075% sodium bicarbonate

- Human acute myelogenous leukemia cells, KG-1 (CCL 246; American Type Culture Collection, Rockville, MD): culture KG-1 cells in complete medium and split at a density of 1–3 $\times$ 10^6 cells/mL every 3–4 days.
- Recombinant human IL-18 (R&D Systems, Minneapolis, MN): Store at −20°C at a concentration of 50 μg/mL in phosphate-buffered saline (PBS), 0.1% bovine serum albumin (BSA). Prepare fresh stock solutions of 2,000 ng/mL and 8 ng/mL of human IL-18 in complete medium.
- Recombinant human TNF (R&D Systems): Store at −20°C at a concentration of 50 μg/mL in PBS, 0.1% BSA.
- Anti+−human IL-18 antibody (R&D Systems): Prepare a fresh stock solution of 45 μg/mL (300 nM) in complete medium.
- Abgene 96-well dilution plates (Thermo Fisher Scientific, Waltham, MA)
- 96-well tissue culture plates (flat and V-bottom; Costar, Cambridge, MA)
- Human IFN-γ ELISA kit (R&D Systems)

46.2.2 L929 Cytotoxicity Assay to Determine Species Cross-Reactivity of Anti-TNF Antibodies

- L929 culture medium: RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 0.075% sodium bicarbonate, 1 mM MEM sodium pyruvate, 0.1 mM MEM nonessential amino acids, and 20 mM HEPES buffer (Life Technologies).
- Murine fibrosarcoma cell line, L929 (CCL 1 NCTC clone 929; American Type Culture Collection): Maintain L929 cells in L929 culture medium and split 1:10 and 1:20 twice a week using standard Trypsin-(EDTA) treatment. Split L929 cells at 1:2 dilution 1 day prior to the assay.
- RPMI 1640 medium without phenol red (Life Technologies)
- Trypsin (0.05%)-EDTA (0.53 mM) (Life Technologies)
- Dulbecco's PBS (D-PBS; Gibco, Grand Island, NY)
- Hank's balanced salt solution (Gibco)
- Lipopolysaccharide (LPS), from *E. coli*; serotype 0111:B4 (Difco Laboratories, Detroit, MI)
- Actinomycin-D, NaCl solution, sodium dodecyl sulfate, 20% (Sigma, St. Louis, MO)
- 3-[4, 5-dimethythiazol-2-yl] 2, 5-diphenyltetrazolium bromide (MTT; Sigma)
- Human immunoglobulin G_1 (IgG_1) (Sigma)
- Anti-TNF antibody (R&D Systems)
- Sodium-heparinized vacutainer tubes (BD; VWR, West Chester, PA)
- Primate blood collected in sodium-heparinized tubes (New England Regional Primate Center, Southborough, MA)
- Canine blood collected in sodium-heparinized tubes (TSI Mason Laboratories, Worcester, MA)

- Human blood collected in sodium-heparinized tubes (in-house donors)
- 1-Step animal PBMC separation media (Accurate Chemicals, Westbury, NY)
- 24-well and 96-well flat bottom tissue culture plates (Costar)
- SpectraMax® 340PC microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) equipped with the SoftMax® program.

46.2.3 *Whole Blood Assay: Inhibition of Natural Human IL-18–Induced Human IFN-γ*

- Human blood (100 mL) drawn from multiple donors (minimum three) and collected in sodium-heparinized vacutainer tubes (BD; VWR): Store at room temperature and use within 2 h.
- Anti-human IL-18 antibody (Abbott Laboratories, North Chicago, IL) or other IL-18 inhibitor, such as human IL-18 binding protein (R&D Systems): Prepare a fresh stock solution of 75 μg/mL (500 nM) in PBS.
- LPS, *E. coli* strain 0127:B8 (Sigma): Dissolve in PBS at 1 mg/mL and store at −20°C.
- Recombinant human IL-12 (R&D Systems): Dilute to 5 μg/mL in PBS containing 0.1% BSA (source) and store at −20°C.
- Abgene 96-well dilution plates (Thermo Fisher Scientific)
- 96-well tube racks with 1.2 mL tubes (Costar).
- 96-well flat-bottom plates (Costar)
- Rocker: Speci-Mix Thermolyne shaker, M26125 (Thermo Fisher Scientific): Place in a 37°C, 5% CO_2 incubator.
- Human IFN-γ ELISA kit (R&D Systems)

46.2.4 *Human PBMC SCID* In Vivo *Assay Model*

- PBMCs (Biological Specialty Corp [BSC, Colmar, PA]): Wash cells twice in PBS and then resuspend at a cell density of 1×10^8 cells/mL in PBS.
- Female CB-17 SCID mice, 6–8 weeks old (Taconic Labs, Germantown, NY): House animals in rooms with constant temperature and humidity under a 12 h light/dark cycle and feed with normal rodent chow (Lab Diet 5010; PharmaServ, Framingham, MA) and water ad libitum.
- *Staphylococcus aureus* dried cells (SAC; Sigma): Suspend in 0.9% isotonic saline at a concentration of 0.67 mg/mL and then further dilute tenfold to 67 μg/mL.
- Human anti-human IL-18 monoclonal antibody: Prepare stock solutions of 2.5 mg/mL (for intravenous [IV] injection) and 0.83 mg/mL (for intraperitoneal [IP] injection) by dilution with sterile saline. Prepare ten-fold serial dilutions in sterile saline to obtain 250 μg/mL, 25 μg/mL, and 2.5 μg/mL for IV injection and 83 μg/mL, 8.3 μg/mL, and 0.83 μg/mL for IP injection.

- Control human IgG (Polygam® S/D; Baxter Healthcare Corp, Westlake Village, CA): Prepare stock solutions of 2.5 mg/mL (for IV injection) and 0.83 mg/mL (for IP injection) by dilution with sterile saline.
- Microtainer tubes (BD Biosciences, Franklin Lakes, NJ)
- Human IFN-γ ELISA kit (R&D Systems)

46.3 Methods

46.3.1 Typical Bioassay to Determine Antibody Neutralization Potency (KG-1 Assay for IL-18) (Konishi et al. 1997; Fernandez-Botran and Větvička 2001)

The first step in setting up a bioassay is to determine the biologic response of the KG-1 cell line to different concentrations of human IL-18 by measuring the production of IFN-γ. The concentration of IL-18 that results in an EC_{50}–EC_{70} is then selected as the concentration to be used in the second part of the assay, in which anti-human IL-18 antibody is prepared at different concentrations. The potency of the neutralizing antibody is expressed as the IC_{50}, which is the antibody concentration that results in 50% inhibition of the IL-18-induced IFN-γ response.

46.3.1.1 Determine the Biologic Response Curve for IL-18

1. Prepare a standard curve for IL-18. In a 96-well dilution plate, prepare a serial 1:2 (16 points) or 1:4 (8 points) dilution of human IL-18 (starting at 2,000 ng/mL) in complete medium.
2. In a 96-well flat-bottom tissue culture plate, add 100 μL/well human IL-18 dilutions in quadruplicate. Include eight control wells containing 100 μL/well complete medium without IL-18.
3. Harvest KG-1 cells, centrifuge at 1,500 rpm for 5 min, aspirate off media, and resuspend at a cell density of 3×10^6 cells/mL in complete medium containing 20 ng/mL human TNF.
4. Add 100 μL KG-1 cells to each well in the 96-well plate and incubate for 24 h at 37°C, 5% CO_2.
5. Continue with Sect. 46.3.1.3.

46.3.1.2 Antibody Neutralization

1. Prepare a 1:2 (16 points) or 1:4 (8 points) serial dilution of IL-18 antibody in complete medium (starting at 300 nM) in a 96-well dilution plate.
2. To a 96-well flat-bottom tissue culture plate, add 50 μL/well diluted IL-18 antibody in quadruplicate. Include eight control wells containing 50 μL/well

complete medium without IL-18 antibody (IL-18-only control wells) and eight control wells containing 100 μL/well complete medium without IL-18 antibody (medium-control wells).
3. Add 50 μL/well human IL-18 (8 ng/mL), except to medium-control wells. Preincubate the plate containing IL-18 and IL-18 antibody for 1 h at 37°C, 5% CO_2.
4. Harvest KG-1 cells, centrifuge at 1,500 rpm for 5 min, aspirate off media, and resuspend at a cell density of 3×10^6 cells/mL in complete medium containing 20 ng/mL human TNF.
5. Add 100 μL KG-1 cells to each well in the 96-well plate and incubate for 24 h at 37°C, 5% CO_2.
6. Continue with Sect. 46.3.1.3.

46.3.1.3 Determination of IL-18 Bioactivity and Antibody Neutralization

1. Transfer plate contents to 96-well V-bottom tissue culture plates and centrifuge plates at 1,500 rpm for 10 min. Transfer 150 μL of the culture supernatants to 96-well flat-bottom tissue culture plates. Do not disturb the cell pellets. At this point, the supernatants can be stored at −20°C.
2. Determine human IFN-γ concentrations in the supernatants by using a human IFN-γ ELISA kit. Further dilution of supernatants may be necessary to ensure that data points fall within the standard curve of the ELISA.
3. Calculate the EC_{50} or EC_{70} for IL-18 by plotting the IL-18 concentration on the x-axis versus human IFN-γ on the y-axis.
4. Calculate the IC_{50} of the IL-18 antibody by plotting the antibody concentration on the x-axis versus human IFN-γ on the y-axis. The maximum IFN-γ response can be determined from the IL-18-only control wells and the background IFN-γ response can be determined from the medium-control wells. The IC_{50} of the antibody is the concentration that causes 50% inhibition of the maximum IFN-γ response over the background response.

46.3.2 Determination of Species Cross-Reactivity of Anti-TNF Antibodies in the L929 Cytotoxicity Assay

The L929 assay is amenable to determining the species cross-reactivity of anti-TNF antibodies because TNF from many different species is biologically active in this assay. Furthermore, culture supernatants containing natural TNF induced by LPS activation of blood leukocytes can be used in the L929 assay without prior purification to test the efficacy and potency of anti-TNF antibodies. LPS and other factors present in the culture supernatants do not interfere with antibody neutralization. The first step is to produce natural TNF by activating PBMCs isolated from primate blood (Sect. 46.3.2.1) or whole human or canine blood with LPS (Sect. 46.3.2.2).

The second step is to determine the amount of TNF activity in the culture supernatants from LPS-activated PBMCs or whole blood in the L929 assay (Sect. 46.3.2.3). The third step involves titration of anti-TNF antibody with a fixed amount of TNF-containing supernatants to determine the 50% inhibitory concentrations for each species' TNF in the L929 assay (Sect. 46.3.2.4).

46.3.2.1 Production of Natural TNF from PBMCs Isolated from Primate Blood

1. Dilute heparinized blood from chimpanzee, marmoset, cynomolgus, and rhesus monkey 1:1 with D-PBS. Layer the diluted blood over the animal PBMC separation media and centrifuge according to the manufacturer's instructions.
2. Collect the cells and wash in Hank's balanced salt solution. Lyse the remaining red blood cells by resuspending the cell pellet in 0.2% NaCl, and 30 s later, adjust the salt concentration to 1.6% NaCl.
3. Wash the cells and resuspend at 2×10^7 cells/mL in L929 culture medium.
4. Pipette the cell suspension into 24-well plates, 2 mL/well.
5. Add LPS to a final concentration of 100 ng/mL, and incubate the plate in a 37°C, 5% CO_2 incubator for 24 h.
6. Collect the supernatant, spin to remove remaining cells, aliquot, and store at −80°C.

46.3.2.2 Production of Natural TNF from Whole Blood

1. Dilute heparinized canine or human blood 1:10 in RPMI 1640 medium without phenol red.
2. Pipette the diluted blood into 24-well plates, 2 mL/well.
3. Add LPS to a final concentration of 10 μg/mL, and incubate the plate in a 37°C, 5% CO_2 incubator for 24 h. Include some wells without LPS to serve as controls.
4. Collect the natural TNF-containing and control supernatants, spin to remove remaining cells, aliquot, and store at −20°C.

46.3.2.3 Determining the Amount of Natural TNF in Culture Supernatants in the L929 Assay

1. Serially dilute the natural TNF-containing and control supernatants 1:2 (6 points) with L929 culture medium; place into 96-well tissue culture plates. Prepare each experimental point in duplicates.
2. Serially dilute recombinant human TNF 1:5 (12 points) in L929 culture medium, starting at 10 ng/mL.
3. In a 96-well flat-bottom tissue culture plate, add 100 μL/well L929 culture media. Do not use the outer wells for the assay samples to avoid plate-edge effects due to evaporation.
4. Add 50 μL/well diluted natural TNF or recombinant human TNF in duplicate.

5. Wash and trypsinize L929 cells and resuspend cells at 1×10^6 cells/mL in L929 culture medium containing 1 μg/mL actinomycin-D and add 50 μL/well L929 cells (50,000 cells/well).
6. Incubate overnight at 37°C, 5% CO_2.
7. Remove 100 μL supernatant from each well and discard. Use separate tips for all rows.
8. To the remaining cells/supernatant in the wells, add 50 μL/well MTT (5 mg/mL). Incubate for 4 h at 37°C, 5% CO_2.
9. Add 50 μL/well 20% sodium dodecyl sulfate to lyse cells.
10. Incubate overnight at 37°C, 5% CO_2.
11. Read the plate on the spectrophotometer at 570–630 nm using the SoftMax® program.
12. Construct the recombinant human TNF standard curve by plotting the optical density (OD) values on the y-axis and TNF concentrations on the x-axis. Calculate the human TNF bioequivalent units for each dilution of TNF-containing supernatant from the standard curve. Determine the dilution factor corresponding to 70–90% cell death caused by recombinant human TNF.

46.3.2.4 Neutralization of Natural TNF by Anti-TNF Antibody in the L929 Assay

1. Serially dilute anti-TNF antibody or human IgG_1 (negative/isotype control) 1:2 (12 points) with L929 culture medium, starting at 300 ng/mL.
2. In a 96-well flat-bottom tissue culture plate, add 100 μL/well diluted antibody. Do not use the outer wells for the assay samples to avoid plate edge effects due to evaporation.
3. Add 50 μL/well recombinant TNF (2,000 ng/mL) or 50 μL/well natural TNF (at 4× the dilution previously determined to cause 70–90% cell death). Incubate the plate at 37°C, 5% CO_2 for 30 min.
4. Continue the assay set-up as described in Sect. 46.3.2.3, Steps 5–11.
5. Generate a plot of OD (y-axis) versus anti-TNF antibody concentration (x-axis).
6. Determine the IC_{50} values from the plot using the nonlinear 4-parametric curve-fitting analysis method. A low OD (range of 0.070–0.150 nm) is indicative of cell death.

46.3.3 Whole Blood Assay: Inhibition of Natural Human IL-18–Induced Human IFN-γ (Kim et al. 2000; Nakanishi et al. 2001)

1. Prepare a fresh solution of 10 μg/mL LPS plus 20 ng/mL recombinant human IL-12 in PBS.
2. To a 96-well rack with 1.2-mL tubes, add 50 μL/well LPS/IL-12 solution. Include PBS-control wells containing 50 μL/well PBS.

3. Prepare a 1:3 (8 points) serial dilution of IL-18 antibody in PBS (starting at 500 nM) in a dilution plate. Add 50 μL/well in triplicate to the 1.2 mL tubes. Include no-antibody–control wells containing 50 μL/well PBS.
4. Mix and dispense 400 μL/well of human blood to the 1.2-mL tubes for a total volume of 500 μL/well. The final concentration of LPS and IL-12 per well is 1 μg/mL and 2 ng/mL, respectively. The final IL-18 antibody concentration range is 50 nM–20 pM.
5. Cover the 1.2-mL tube rack, place it on a rocker, and incubate at 37°C, 5% CO_2 for 24 h.
6. Centrifuge the tubes at 1,600 rpm for 10 min, harvest serum in a 96-well round-bottom plate, and store at −20°C until ready to run the human IFN-γ ELISA.
7. Calculate the IC_{50} by plotting antibody concentration on the x-axis versus human IFN-γ on the y-axis. Determine neutralization by the percent decrease in IFN-γ levels in the presence of IL-18 antibody. Determine the maximum IFN-γ response from the no-antibody–control wells and the background IFN-γ response from the PBS–control wells. The IC_{50} of the antibody is the concentration that causes 50% inhibition of the maximum IFN-γ response over the background response.

46.3.4 Human PBMC SCID In Vivo *Assay Model*

Severe combined immunodeficient mice lack functional T or B lymphocytes and, therefore, accept xenografts of human lymphoid cells, which can then be stimulated *in vitro* with SAC to produce human cytokines such as IL-12 and IL-18. The concentrations of human IL-12 and IL-18 are still low and often remain below the detection concentration. However, the induced IL-18 does give rise to production of IFN-γ, which can be measured by ELISA. Thus, the potency of a neutralizing antibody for the endogenous human IL-18 can be evaluated *in vivo* by administering different doses of antibody either IV or IP prior to the stimulation of the human PBMCs by IP administration of SAC and measuring the concentration of IL-18–dependent IFN-γ as a secondary readout.

1. Inject female CB-17 SCID mice ($n = 7$) with anti-human IL-18 antibody (250 μg, 25 μg, 2.5 μg, or 2.5 μg per mouse) or control human IgG (250 μg per mouse) in a volume of 100 μL for IV injection or 300 μL for IP injection.
2. One hour later, inject 3×10^7 human PBMCs intraperitoneally in a volume of 300 μL.
3. Fifteen minutes after human PBMC engraftment, inject 1 mg/kg SAC (300 μL of 67 μg/mL suspended in 0.9% isotonic saline) IP to stimulate engrafted human PBMCs to produce human IFN-γ.
4. Twenty-four hours after SAC stimulation, bleed the mice via cardiac puncture and collect the blood in Microtainer serum separator tubes.
5. Spin tubes for 5 min at 15,000 rpm in an Eppendorf tabletop centrifuge and collect serum.

6. Determine human IFN-γ concentrations in the serum using a human IFN-γ ELISA kit.
7. Determine the *in vivo* neutralization potency of the antihuman IL-18 antibody, determining the dose that results in 50% inhibition of the IFN-γ response compared with the IFN-γ concentrations in mice treated with control human IgG.

46.4 Case Studies and Additional Points to Consider

In a recent study, two neutralizing antibodies for human IL-13 that acted by two different mechanisms (Kasaian et al. 2008) have been described. Biologic effects of IL-13 are mediated by signaling through a heterodimeric receptor comprising IL-13 receptor alpha-1 (IL-13Ra1) and IL-4 receptor alpha (IL-4Ra). One antibody blocked the IL-13 interaction with the IL-13Ra1 chain and the other antibody, with the IL-4Ra chain, thereby each preventing the high-affinity signaling complex from forming. The study showed that both antibodies potently inhibited IL-13 signaling in an *in vitro* bioassay and had comparable anti-inflammatory efficacy *in vivo*.

It is important to keep in mind, however, that *in vitro* neutralization activity does not always correlate with *in vivo* neutralization. Although the total concentration of the target cytokine in the circulation usually increases upon antibody administration owing to the longer half-life of the antibody/antigen complexes, these complexes are for the most part not biologically active. However, several cases have been reported in which antibodies with neutralizing activity *in vitro* actually enhanced the biologic activity of the target cytokine *in vivo*. For instance, neutralizing antibodies to IL-2 increased the *in vivo* biologic activity of pre-existing IL-2 through the formation of immune complexes (Boyman et al. 2006), and administration of antibodies to IL-6 resulted in higher levels of biologically active IL-6 in the periphery owing to a chaperoning effect (May et al. 1993). In a clinical study with a neutralizing antibody to CCL2/MCP-1, lack of efficacy may have been attributed to a dosage-related increase of up to 2,000-fold in antibody-complexed total CCL2/MCP-1 concentrations, and antibody treatment in this case may have been associated with worsening of rheumatoid arthritis symptoms in patients treated with the greatest dose (Haringman et al. 2006). A number of other studies describe the enhanced *in vivo* effects of exogenous cytokines such as IL-3, IL-4, IL-5, and IL-7 by injection of neutralizing antibody/cytokine complexes but not by injection of the antibody alone (Finkelman et al. 1993; Zabeau et al. 2001; Boyman et al. 2008). It is of interest that this phenomenon is mostly observed with neutralizing antibodies and not with non-neutralizing antibodies, indicating the importance of the epitope of the antibody.

In addition to the impact of the antibody/cytokine complex on the clearance and bioactivity of the cytokine, one needs to keep in mind that many cytokines are regulated *in vivo* by binding proteins and soluble receptors. Antibodies that bind to cytokine/binding protein complexes or those that prevent natural binding proteins from interacting with the cytokine may lead to quite different therapeutic results *in vivo*.

Acknowledgements Editorial support was provided by Robin L. Stromberg, PhD, of Arbor Communications, Inc. (Ann Arbor, MI, USA) and funded by Abbott Laboratories.

References

Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL (2003) Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 278:1910–1914

Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J (2006) Selective stimulation of T cell subsets with antibody-cytokine immune complexes. Science 311:1924–1927

Boyman O, Ramsey C, Kim DM, Sprent J, Surh CD (2008) IL-7/anti-IL-7 mAb complexes restore T cell development and induce homeostatic T Cell expansion without lymphopenia. J Immunol 180:7265–7275

Darling RJ, Brault PA (2004) Kinetic exclusion assay technology: characterization of molecular interactions. Assay Drug Dev Technol 2:647–657

Fernandez-Botran R, Větvička V (2001) Assays of cytokines. In: Methods in cellular immunology, 2nd edn. CRC Press, Boca Raton, FL. pp 87–158

Finkelman FD, Madden KB, Morris SC, Holmes JM, Boiani N, Katona IM, Maliszewski CR (1993) Anti-cytokine antibodies as carrier proteins. Prolongation of in vivo effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes. J Immunol 151:1235–1244

Foster B, Prussin C, Liu F, Whitmire JK, Whitton JL (2007) Detection of intracellular cytokines by flow cytometry. Curr Protoc Immunol Chap. 6:Unit 6.24

Glickman JF, Wu X, Mercuri R, Illy C, Bowen BR, He Y, Sills M (2002) A comparison of AlphaScreen, TR-FRET, and TRF as assay methods for FXR nuclear receptors. J Biomol Screen 7:3–10

Guenat S, Rouleau N, Bielmann C, Bedard J, Maurer F, Allaman-Pillet N, Nicod P, Bielefeld-Sévigny M, Beckmann JS, Bonny C, Bossé R, Roduit R (2006) Homogeneous and nonradioactive high-throughput screening platform for the characterization of kinase inhibitors in cell lysates. J Biomol Screen 11:1015–1026

Haringman JJ, Gerlag DM, Smeets TJ, Baeten D, van den Bosch F, Bresnihan B, Breedveld FC, Dinant HJ, Legay F, Gram H, Loetscher P, Schmouder R, Woodworth T, Tak PP (2006) A randomized controlled trial with an anti-CCL2 (anti-monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis. Arthritis Rheum 54:2387–2392

Hautanen A, Gailit J, Mann DM, Ruoslahti E (1989) Effects of modifications of the RGD sequence and its context on recognition by the fibronectin receptor. J Biol Chem 264:1437–1442

Jeffes EW 3rd, Schmitz K, Yamamoto R, Tomich JM, Beckman M, Nep R, Knauer M (1991) A simple nonisotopic in vitro bioassay for LT and TNF employing sodium fluoride-treated L-929 target cells that detects picogram quantities of LT and TNF and is as sensitive as TNF assays done with ELISA methodology. Lymphokine Cytokine Res 10:147–151

Kasaian MT, Tan XY, Jin M, Fitz L, Marquette K, Wood N, Cook TA, Lee J, Widom A, Agostinelli R, Bree A, Schlerman FJ, Olland S, Wadanoli M, Sypek J, Gill D, Goldman SJ, Tchistiakova L (2008) Interleukin-13 neutralization by two distinct receptor blocking mechanisms reduces immunoglobulin E responses and lung inflammation in cynomolgus monkeys. J Pharmacol Exp Ther 325:882–892

Kim SH, Eisenstein M, Reznikov L, Fantuzzi G, Novick D, Rubinstein M, Dinarello CA (2000) Structural requirements of six naturally occurring isoforms of the IL-18 binding protein to inhibit IL-18. Proc Natl Acad Sci USA 97:1190–1195

Konishi K, Tanabe F, Taniguchi M, Yamauchi H, Tanimoto T, Ikeda M, Orita K, Kurimoto M (1997) A simple and sensitive bioassay for the detection of human interleukin-18/interferon-gamma-inducing factor using human myelomonocytic KG-1 cells. J Immunol Methods 209:187–191

Li J, Tomkinson KN, Tan XY, Wu P, Yan G, Spaulding V, Deng B, Annis-Freeman B, Heveron K, Zollner R, De Zutter G, Wright JF, Crawford TK, Liu W, Jacobs KA, Wolfman NM, Ling V, Pittman DD, Veldman GM, Fouser LA (2004) Temporal associations between interleukin 22 and the extracellular domains of IL-22R and IL-10R2. Int Immunopharmacol 4:693–708

Llopis J, Westin S, Ricote M, Wang Z, Cho CY, Kurokawa R, Mullen TM, Rose DW, Rosenfeld MG, Tsien RY, Glass CK (2000) Ligand-dependent interactions of coactivators steroid receptor coactivator-1 and peroxisome proliferator-activated receptor binding protein with nuclear hormone receptors can be imaged in live cells and are required for transcription. Proc Natl Acad Sci USA 97:4363–4368

May LT, Neta R, Moldawer LL, Kenney JS, Patel K, Sehgal PB (1993) Antibodies chaperone circulating IL-6. Paradoxical effects of anti-IL-6 "neutralizing" antibodies in vivo. J Immunol 151:3225–3236

Meager A (2006) Measurement of cytokines by bioassays: theory and application. Methods 38:237–252

Miller R, Sadhukhan R, Wu C (2008) Development of an in vitro potency bioassay for therapeutic IL-13 antagonists: the A-549 cell bioassay. J Immunol Methods 334:134–141

Miyashita M, Shimada T, Miyagawa H, Akamatsu M (2005) Surface plasmon resonance-based immunoassay for 17beta-estradiol and its application to the measurement of estrogen receptor-binding activity. Anal Bioanal Chem 381:667–673

Myszka DG, Jonsen MD, Graves BJ (1998) Equilibrium analysis of high affinity interactions using BIACORE. Anal Biochem 265:326–330

Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H (2001) Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. Cytokine Growth Factor Rev 12:53–72

Pappu BP, Dong C (2007) Measurement of interleukin-17. Curr Protoc Immunol Chap. 6:Unit 6.25

Pauli U, Bertoni G, Duerr M, Peterhans E (1994) A bioassay for the detection of tumor necrosis factor from eight different species: evaluation of neutralization rates of a monoclonal antibody against human TNF-alpha. J Immunol Methods 171:263–265

Underwood PA (1993) Problems and pitfalls with measurement of antibody affinity using solid phase binding in the ELISA. J Immunol Methods 164:119–130

Wilson J, Rossi CP, Carboni S, Fremaux C, Perrin D, Soto C, Kosco-Vilbois M, Scheer A (2003) A homogeneous 384-well high-throughput binding assay for a TNF receptor using AlphaScreen technology. J Biomol Screen 8:522–532

Wu C, Ying H, Grinnell C, Bryant S, Miller R, Clabbers A, Bose S, McCarthy D, Zhu RR, Santora L, Davis-Taber R, Kunes Y, Fung E, Schwartz A, Sakorafas P, Gu J, Tarcsa E, Murtaza A, Ghayur T (2007) Simultaneous targeting of multiple disease mediators by a dual-variable-domain immunoglobulin. Nat Biotechnol 25:1290–1297

Yao Z, Painter SL, Fanslow WC, Ulrich D, Macduff BM, Spriggs MK, Armitage RJ (1995) Human IL-17: a novel cytokine derived from T cells. J Immunol 155:5483–5486

Zabeau L, Van der Heyden J, Broekaert D, Verhee A, Vandekerckhove J, Wu SJ, Chaiken I, Heinrich P, Behrmann I, Tavernier J (2001) Neutralizing monoclonal antibodies can potentiate IL-5 signaling. Eur J Immunol 31:1087–1097

Zeng R, Spolski R, Leonard WJ (2007) Measurement of interleukin-21. Curr Protoc Immunol Chap. 6:Unit 6.30

Chapter 47
Competitive ELISA

Torsten Meyer

47.1 Introduction

The enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann 1971) is used as a diagnostic tool and is well established in diagnostic laboratories. Due to cross-reactivity between closely related pathogens or nonpathogens, which lead to false positive ELISA results, it is necessary to increase the specificity and sensitivity of the diagnostic procedure. Because of the simplicity and sensitivity, the competitive ELISA (cELISA) is a suitable system for diagnosis and is established for a multiplicity of diseases like bluetongue virus (Mecham and Wilson 2004), vesicular stomatitis virus (Afshar et al. 1993), cattle-plague virus (Renukaradhya et al. 2003), and epizootic hemorrhagic disease of deer virus (Mecham and Jochim 2000). Due to the fact that the detection system is directed to a generic competitive monoclonal antibody, the cELISA is species independent. This generic competitive antibody is combined with the defined fractions of virus particles, cell lysates or recombinant-produced proteins immobilized to suitable 96-well plates. Crude cell lysate can be used as an antigen provided that the competitive antibody is specific to the immunogenic target antigen and shows no cross-reactivity with other proteins. Nevertheless, to increase the specificity and sensitivity of the cELISA, recombinantly produced and purified protein or synthesized immunogenic peptides are preferred as antigen. Serum samples and a competing antibody are added, allowing the monoclonal antibody and the antigen-specific IgGs from the analyzed serum to compete for the binding sites of the antigen. With an increasing amount of serum IgGs, less monoclonal antibodies can bind the antigen. So, a decreased ELISA signal compared to the maximum control indicates the presence of specific antibodies in the sample. The monoclonal antibody or antibody fragment used for competition may be

T. Meyer
Technische Universität Braunschweig, Institute of Biochemistry and Biotechnology, Spielmannstr. 7, Braunschweig 38106, Germany
e-mail: torsten.meyer@tu-bs.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_47,

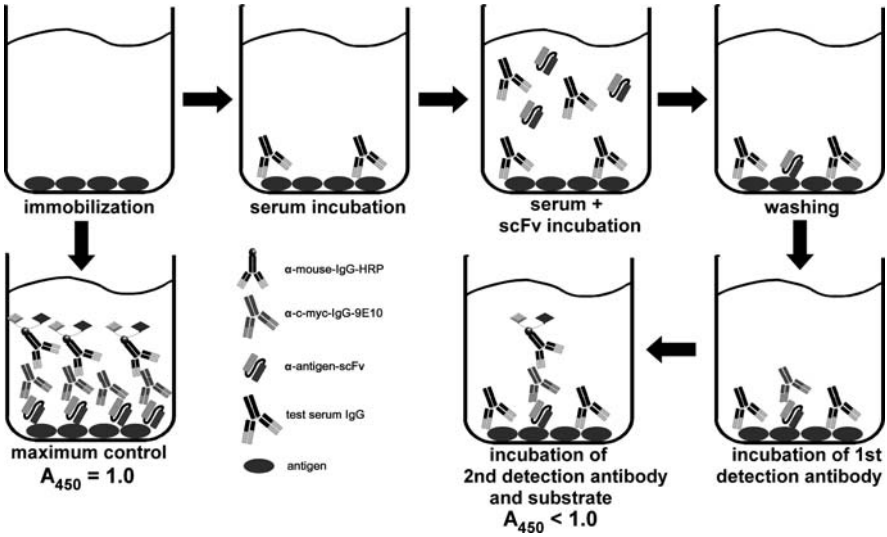


Fig. 47.1 Principle of the competitive ELISA

isolated from the hybridoma cultures of immunized animals or by in vitro selection systems like antibody phage display. To date, mainly monoclonal IgGs used in cELISA, but other antibody formats like Fab or scFv, can be used as well.

The result of the cELISA is given as the percentage of inhibition compared to the maximum control calculated according to the following formula: $\%$ inhibition $=$ $([OD_{450}$ test serum$]/[OD_{450}$ positive control$]) \times 100$ (Afshar et al. 1987).

The following method describes a competitive ELISA using recombinant scFv antibody fragments produced in *Escherichia coli* for competition. This requires an additional step using an anti-tag antibody, which is then detected by a respective serum. The principle of the cELISA is given in Figure 47.1

47.2 Materials

- Phosphate-buffered saline with Tween 20 (PBS-T): Solve 8.5 g/L NaCl, 1.34 g/L $Na_2HPO_4 \times 2\ H_2O$, 0.35 g/L NaH_2PO_4 in 1 L distilled water, pH 7.4, autoclave and store at room temperature. Add 0.1% Tween 20 before use
- Coating buffer: Solve 35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.7, autoclave and store at room temperature
- 2% MPBS-T: Solve 2% skim milk powder in PBS-T. Prepare fresh to avoid degradation
- First-detection antibody, e.g., mouse-α-c-myc IgG 9E10 (anti-tag)
- Second-detection antibody, e.g., goat-α-mouse-IgG Fab-specific HRP-conjugate
- Subtrate solution A: 30 mM potassium citrate. Adjust pH to 4.1 with 1% (w/v) citric acid

- Substrate solution B: 10 mM tetramethylbenzidine, 80 mM H_2O_2 (30% v/v), 90% (v/v) EtOH, 10% (v/v) acetone
- Stop solution: 0.5 M H_2SO_4
- Polysorp 96-well microtiter plates (Nunc™ Denmark)
- ELISA washer Columbus Pro (Tecan, Crailsheim)
- Sunrise ELISA reader (Tecan, Crailsheim)

47.3 Methods

Perform all incubation steps in a wet chamber to avoid evaporation of the samples. Determine the optimal antigen concentration by titration ELISA with high concentrations of the isolated specific competitive antibody. Use an antigen concentration with a maximum of $A_{450} \approx 1.5$ in the titration ELISA; 100 ng of antigen was the optimal concentration in this ELISA.

Determine the optimal concentration of the competitive monoclonal antibody by titration ELISA with 100 ng/well antigen to achieve a maximum of $A_{450} \approx 1.5$. The concentration depends on the affinity and quality of antibody preparation and must be analyzed for every new batch.

1. Dilute the antigen in coating buffer to the optimal concentration (e.g. 100 ng/well) and incubate it in the 96-well microtiterplate at 4°C overnight.
2. Wash the wells 3× with 0.05% PBS-T.
3. Block unspecific binding with 2% MPBS-T for 1 h at room temperature.
4. Wash the wells 3× with 0.05% PBS-T.
5. Dilute sample in 2% MPBS-T and incubate 100 µL/well for 1 h at room temperature in the coated wells without the competitive antibody. Antigen-specific IgGs in the sample bind to the immobilized antigen. For negative control, use serum from healthy individuals.
   Note: The dilution of the serum was set to 1:4. Dilution up to 1:200 or higher is possible when using hyperimmune sera. This step is additional to the standard cELISA to block most of the binding sites of the antigen with serum IgGs before the competitive antibody is added.
6. Wash the wells 3× with 0.05% PBS-T.
7. Dilute the serum again in 2% MPBS-T and add the competitive antibody in an optimal concentration to the sample. Incubate the serum and the competitive antibody (final volume 100 µL) on the immobilized antigen for 1 h at room temperature. For maximum control, incubate the competitive antibody without any serum.
8. Wash the wells 3× with 0.05% PBS-T.
9. Add 100 µL of the first detection antibody diluted in 2% MPBS-T for 1 h at room temperature.
10. Wash the wells 3× with 0.05% PBS-T.
11. Add 100 µL of the second detection antibody (HRP conjugated) diluted in 2% MPBS-T for 1 h at room temperature.

12. Wash the wells 3× with 0.05% PBS-T.
13. Prepare the substrate solution by mixing 20 parts of Solution A and one part of Solution B (20+1). Add 100 μL of the substrate solution to each well and incubate for 20 min.
    Note: The ELISA can be shortened using a competitive antibody that is directly labeled with horseradish peroxidase or alkaline phosphatase.
14. Stop the reaction by adding 100 μL of stop solution.
    Note: Color turns from blue to yellow.
15. Read out the absorbtion of each well at 450 nm (reference wavelength: 620 nm) in the ELISA reader.

47.4 Typical Results

For determination of the maximum signal, only antigen-specific scFv and the detection system were added and led to an A_{450} from 0.9 to 1.1. The same A_{450} values should be achieved when using negative samples. A competitive effect is only achieved when samples contain antibodies against the antigen The A_{450} value is reduced compared to the maximum control because the binding sites are occupied by the sample's antibodies; preventing the competing scFv fragments to bind to the antigen. The positive-negative cut-off has to be determined by using a panel of well-analyzed samples.

References

Afshar A et al (1987) Comparison of competitive and indirect enzyme-linked immunosorbent assays for detection of bluetongue virus antibodies in serum and whole blood. J Clin Microbiol 25(9):1705–1710

Afshar A, Shakarchi NH, Dulac GC (1993) Development of a competitive enzyme-linked immunosorbent assay for detection of bovine, ovine, porcine, and equine antibodies to vesicular stomatitis virus. J Clin Microbiol 31(7):1860–1865

Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8(9):871–874

Mecham JO, Jochim MM (2000) Development of an enzyme-linked immunosorbent assay for the detection of antibody to epizootic hemorrhagic disease of deer virus. J Vet Diagn Invest 12(2):142–145 Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc

Mecham JO, Wilson WC (2004) Antigen capture competitive enzyme-linked immunosorbent assays using baculovirus-expressed antigens for diagnosis of bluetongue virus and epizootic hemorrhagic disease virus. J Clin Microbiol 42(2):518–523

Renukaradhya GJ et al (2003) Competitive enzyme-linked immunosorbent assay based on monoclonal antibody and recombinant hemagglutinin for serosurveillance of rinderpest virus. J Clin Microbiol 41(3):943–947

Chapter 48
Quantification of Human IgG and Related Fc Fusion Proteins by a Human IgG/Fc Capture ELISA

Torsten Rülker, Doris Meier, and Thomas Schirrmann

48.1 Introduction

The quantification of human immunoglobulins in serum or other body fluids is an important factor for certain diseases (Peebles et al. 1995; Speight et al. 1990). Moreover, diagnostic or therapeutic antibodies have to be quantified to specify the needed amounts. Recombinant human antibodies represent the biggest and fastest growing group of therapeutic proteins on the market. In contrast to animal-derived antibodies, the research use of recombinant human immunoglobulin G (IgG) and IgG-like derivates such as the single chain Fv-Fc (scFv-Fc) antibody format offers the option for future therapeutic development. The Fc moiety of IgGs and scFv-Fc fusion proteins is responsible for homodimerisation, including enhanced antigen binding by avidity effect, increased serum stability, and the mediation of effector functions such as the recruitment of immunological factors that include complement or immune cells. Today, the replacement of the cytoplasmic and transmembrane domains of different receptors with the human IgG-Fc domain has been successfully used to improve their soluble expression as well as pharmacokinetic properties. For example, Etanercept (Enbrel®, Wyeth, now Pfizer) is a fusion of the tumor necrosis factor (TNF) receptor 2 with the human IgG1 Fc fragment that neutralizes TNFα and is indicated for the treatment of rheumatoid or psoriatic arthritis (Fuchs and Hadi 2006). Other promising therapeutic reagents include Fc domains in more complex fusion proteins such as ImmunoRNAses (Menzel et al. 2008, Schirrmann et al. 2009).

T. Rülker, D. Meier, and T. Schirrmann (✉)
Technische Universität Braunschweig, Institut für Biochemie und Biotechnologie, Department of Biotechnology, Spielmannstr. 7, Braunschweig, 38106, Germany
e-mail: th.schirrmann@tu-bs.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_48,

Typically, human immunoglobulins and their subtypes are quantified by enzyme-linked immunosorbent assay (ELISA), which replaced older techniques such as rocket immunoelectrophoresis, radial immunodiffusion, and nephelometry because of higher sensitivity, robustness, cost efficiency, and reliability. Particularly, sandwich or capture ELISAs allow high sensitivities of less than 1 ng/mL in solution and working ranges of more than two orders of magnitude (Hamilton et al. 1988). Recently, microsphere-based flow cytometric assays promise easier multiplexing and a greater dynamic range than ELISAs (Dasso et al. 2002), but the costs for both instrumentation and microspheres are much higher.

In the following protocols, we describe an inexpensive, accurate, and robust method to quantify human IgG and derived Fc fusion proteins by a capture ELISA with a sensitivity of less than 1 ng/mL.

48.2 Material

- Phosphate buffered saline (PBS): 8.0 g/L sodium chloride (NaCl), 0.2 g/L potassium chloride (KCl), 1.44 g/L di-sodiumhydrogenphosphate-di-hydrate ($Na_2HPO_4 \times 2\ H_2O$) and 0.24 g/L potassium-di-hydrogenphosphate (KH_2PO_4), pH 7.4 (Note that a 10× PBS stock has a higher pH!)
- PBST: PBS with 0.05% Tween® 20 (Sigma)
- 96-well Maxisorp® microtiter plates (Nunc)
- Dilution buffer: 1% (v/v) fetal calf serum (PAA) in PBS
- Capture antibody: Goat-anti-human immunoglobulin (polyvalent) (Cat.-No. I1761, Sigma, Munich, Germany). Predilute antibody to obtain 1 mg/mL in dilution buffer and store small aliquots at −20°C. Do not freeze thawed aliquots again. Store thawed aliquots at 4°C and use them within 6 weeks.
- Blocking buffer: 50% fetal calf serum (PAA) in PBS. Skim milk powder or caseinate is not recommended for blocking because it results in a high background.
- Wet chamber
- ELISA washer (e.g., TECAN)
- Human IgG standard: N-Protein SL (Dade Behring, now Siemens Healthcare Diagnostics, Eschborn, Germany) contains standardized concentrations of human serum proteins, including 9.91 mg/mL human IgG.
- Detection antibody: Horseradish peroxidase conjugated goat-anti-human-IgG (Fc-specific) antibody (Cat.-No. A0170, Sigma). Predilute the antibody to 1 mg/mL in dilution buffer. Store small aliquots at −20°C. Do not freeze thawed aliquots again. Store thawed aliquots at 4°C, and use them within 6 weeks.
- Tetramethyl benzidine (TMB) substrate solution: Prepare freshly by mixing 20 volumes of TMB solution A (30 mM potassium citrate, 0.5 mM citric acid, pH4.1) and 0.5 volumes of TMB solution B (Dissolve 10 mM 3,3′,5,5′-TMB in 10% (v/v) Aceton. 89% (v/v) ethanol, 1% (v/v) of 30% H_2O_2). Store TMB solutions dark at 4°C.

48.3 Protocol

1. 96-well Maxisorp® microtiter plates are coated with 100 µL per well and 100 ng/mL of capture antibody for 1 h at 37°C. The antibody is diluted 1:1,000 from the prediluted stock in the dilution buffer. Perform all incubations in a wet chamber to prevent desiccation of the plates.
2. Block all wells by adding 200 µL per well blocking buffer and incubate for 1 h at 37°C.
3. Wash all wells 3× with PBST using an ELISA washer. Use a standard wash protocol.
4. The coated and blocked microtiter plates can be stored under wet conditions at 4°C for several weeks. We typically prepare more plates and seal the plates in plastic bags with some drops of water.
5. Prepare seven serial half dilutions of the human IgG protein standard (N-protein SL) in dilution buffer starting with an initial dilution of 1:200,000 and a final dilution of 1:12,800,000, which gives a concentration range of 50–0.78 ng/mL human IgG.
6. Dilute samples containing human IgG or human IgG-Fc fusion protein in dilution buffer according to the expected concentration (see Table 48.1 for details). Test several dilutions of each sample to make sure to be in the linear range of the standard curve.
7. Incubate 100 µL per well of the prediluted samples and the standard series on the same plate for 1 h at 37°C.
8. Wash all wells 3× with PBST using an ELISA washer. Use a standard wash protocol.
9. Dilute detection antibody 1:1,000 in dilution buffer and add 100 µL to each well. Incubate for 1 h at 37°C.
10. Wash all wells 3× with PBST using an ELISA washer. Use a standard wash protocol.
11. Add 100 µL per well TMB substrate solution and incubate at room temperature until the blue color reaction of the samples and the standard series are well detectable (usually within 10–30 min).
12. Stop the reaction by adding 100 µL/well of 0.5 N H_2SO_4.

Table 48.1 Preparation of the human IgG/Fc protein sample and the standard series

Expected human IgG/Fc concentration in the sample [µg/mL]	Recommended sample dilution
0.01	1:1–1:5
0.1	1:5–1:50
1	1:50–1:500
10	1:500–1:5,000
100	1:5,000–1:50,000
1,000	1:50,000–1:500,000
10,000	1:500,000–1:5,000,000

13. Measure the absorbance at 450 nm against a reference wavelength of 620 nm ($A_{450–620}$) using an ELISA-Reader (e.g., Sunrise, TECAN). Optimal results will be obtained if the samples are within the linear range of the standard series and below $A_{450–620} = 1$.

48.4 Results

Human embryonic kidney cells (HEK293T) were transiently transfected with the mammalian expression vector pCMV-scFv-hIgG1Fc-4E3 (Menzel et al. 2008) using polyethylenimine (PEI) in three independent samples in 24-well plates. HEK293T cell control samples were incubated with PEI alone. The medium was completely exchanged 24 h later. A day after transfection, samples were taken and diluted 1:500. Triplicates of each sample were analyzed using the human IgG/Fc capture ELISA according to the described protocol. In Fig. 48.1, the complete analysis of the data is shown. First, the human IgG standard series was analyzed. The linear range of the standard curve was accurate (correlation coefficient $R^2 > 0.99$) for dilutions of more than 1:800,000 and within the absorbance range of $0.1 < A_{450–620} < 0.5$ (Fig. 48.1a). Since the absorbances of the samples were in the linear range of the standard curve, the resulting linear regression equation was used to evaluate the scFv-Fc concentrations. Supernatant samples collected from HEK293T cells transfected with pCMV-scFv-hIgG1Fc-4E3 contained about 5.4 μg/mL scFv-Fc protein, whereas the control samples did not contain any scFv-Fc protein. However, it should be noticed that yields of less than 0.5 μg/mL are not accurately detectable with the used sample dilution of 1:500 and may require lower sample dilutions (see troubleshooting).

48.5 Troubleshooting

Optimal results are obtained if the samples are within the linear range of the standard series and with an $A_{450–620}$ between 0.1 and 1.0, but as shown in the results section, the linear range is often smaller. Samples and standard series must be on the same plate to have exactly the same conditions. It is recommended that several dilutions of each sample or two standard series that can be stopped at different time points with the respective samples be used. Other capture and detection antibodies (even other lots) may require a new titration of the working concentrations. It is useful to test the incubation time of the color reaction, which should be at least 10 min. The human capture IgG/Fc ELISA does not replace additional assays for the functionality of the IgG or Fc fusion protein in the sample. For example, proteolytically cleaved human IgGs and Fc fragments are still detected by this capture ELISA.

N Protein SL Standard

Standard		$A_{450-620}$				
Dilution	ng/mL	1	2	3	Mean	SD
1:200,000	50	1,067	1,025	1,048	1,047	0,021
1:400,000	25	0,690	0,676	0,698	0,688	0,011
1:800,000	12,5	0,402	0,400	0,413	0,405	0,007
1:1,600,000	6,25	0,219	0,216	0,217	0,217	0,002
1:3,200,000	3,125	0,109	0,109	0,107	0,108	0,001
1:6,400,000	1,5625	0,061	0,058	0,059	0,059	0,002
PBS	0	0,016	0,013	0,015	0,015	0,002

Dilution factor of the samples = 500

Samples

Sample	$A_{450-620}$									Mean	SD	scFv-hIgG1Fc [µg/mL]	
	Sample 1			Sample 2			Sample 3						
	1	2	3	4	5	6	7	8	9			Mean	SD
Control	0,011	0,011	0,012	0,011	0,013	0,013	0,011	0,013	0,018	0,013	0,002	–0,01	–0,17
scFv-hIgG1Fc	0,345	0,345	0,349	0,335	0,365	0,358	0,367	0,344	0,344	0,350	0,011	5,35	–0,04

Regression formula:

hIgG/Fc [ng/mL] = (A_{mean} – 0,0131) [ng/mL] / 0,0315 x dilution factor

hIgG/Fc [µg/mL] = (A_{mean} – 0,0131) [ng/mL] / 0,0315 x dilution factor x 10^{-3}

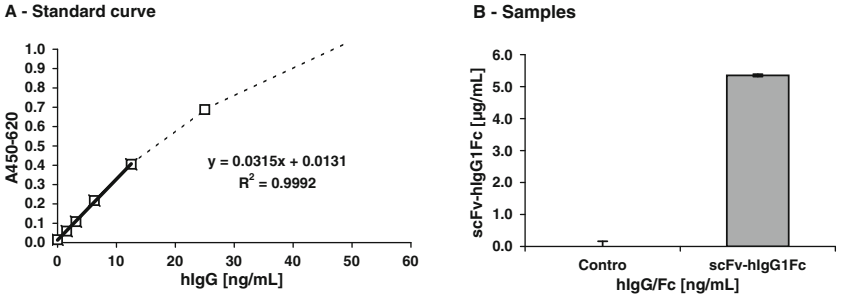


Fig. 48.1 A detailed analysis of a human IgG/Fc capture ELISA. Culture supernatant of three independent transfections of HEK293T cells with the mammalian expression vector pCMV-scFv-hIgG1Fc-4E3 in a 24-well scale using PEI were analyzed as triplicates by the human IgG/Fc capture ELISA. Control cells were only incubated with PEI alone. All samples were diluted 1:500. The half dilution standard series of the human serum standard N Protein SL was started with 1:200,000 (=50 ng/mL). However, only standard concentrations of less than 12.5 ng/mL (1:800,000) were in the linear range (*thick line*) with a correlation coefficient of $R^2 > 0.99$ (**a**). After linear regression, the mean absorbances of the samples were used to evaluate the scFv-Fc protein concentration (**b**)

References

Dasso J, Lee J, Bach H, Mage RG (2002) A comparison of ELISA and flow microsphere-based assays for quantification of immunoglobulins. J Immunol Methods 263:23–33

Fuchs BS, Hadi S (2006) Use of etanercept in the treatment of psoriasis and psoriatic arthritis. Rev Recent Clin Trials 1:259–263

Hamilton RG, Wilson RW, Spillman T, Roebber M (1988) Monoclonal antibody-based immunoenzymetric assays for quantification of human IgG and its four subclasses. J Immunoassay 9:275–296

Menzel C, Schirrmann T, Konthur Z, Jostock T, Dübel S (2008) Human antibody RNase fusion protein targeting CD30+ lymphomas. Blood 111:3830–3837

Peebles RS Jr, Liu MC, Lichtenstein LM, Hamilton RG (1995) IgA, IgG and IgM quantification in bronchoalveolar lavage fluids from allergic rhinitics, allergic asthmatics, and normal subjects by monoclonal antibody-based immunoenzymetric assays. J Immunol Methods 179:77–86

Schirrmann T, Krauss J, Arndt MA, Rybak SM, Dübel S (2009) Targeted therapeutic RNases (ImmunoRNases). Expert Opin Biol Ther 9:79–95

Speight PM, Cruchley A, Williams DM (1990) Quantification of plasma cells in labial salivary glands: increased expression of IgM in Sjogren's syndrome. J Oral Pathol Med 19:126–130

Chapter 49
Determination of Fc-Mediated Antibody-Effector Functions by Chromium Release Assay

Tina Otz

49.1 Introduction

During the last few years, monoclonal antibodies (mAbs) gained substantial importance in the treatment of diseases such as viral infections or cancer (Glennie and van de Winkel 2003; Sanz et al. 2004; Waldmann 2003). MAbs can exert their biological functions by a large variety of mechanisms. For example, they can act by the extraordinary epitope specificity of the variable domain. Hereby, they can directly block interactions between receptors and their ligands or trigger biological responses such as apoptosis or cell proliferation. Such target-specific mechanisms are usually independent of the Fc constant domain and the isotype of the antibody, and are likely to account for much of the efficacy of mAbs in cancer therapy (Strome et al. 2007). Moreover, mAbs can act "indirectly" via their Fc-region. These host-killing mechanisms activated by specific sites located in the Fc constant domain include antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). For the extent of activation of such host-killing mechanisms, the type of human constant region (antibody isotype) of the mAb plays a crucial role. Therefore, mAbs directed against the same antigenic target may, theoretically, differ in their clinical profile depending on whether or not they effectively activate immune-mediated effector functions (Strome et al. 2007).

This chapter focuses on ADCC and CDC mediated by mAbs and provides a simple method for detecting whether a mAb is able to induce ADCC or CDC.

T. Otz
Institute for Cell Biology and Immunology, University of Stuttgart, Allmandring 31, Stuttgart, Germany
e-mail: tina.otz@izi.uni-stuttgart.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_49,

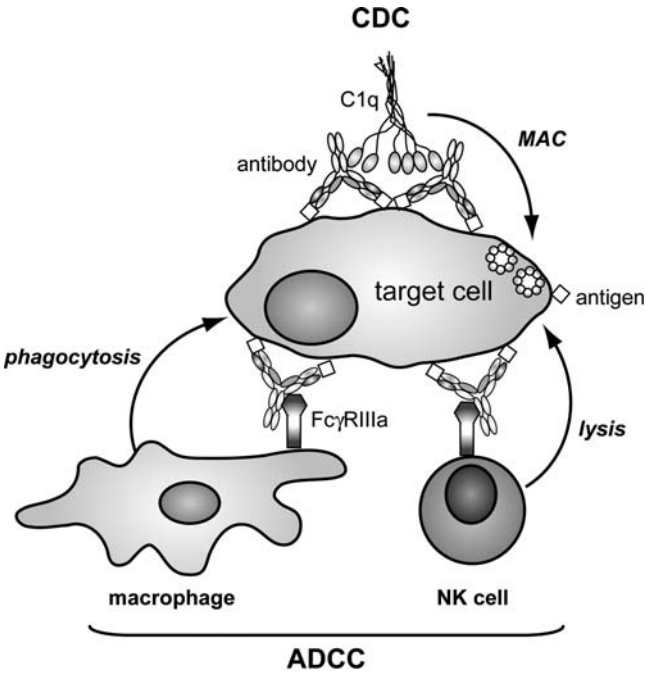


Fig. 49.1 Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Specific sites located in the Fc constant domain of an antibody bound to the surface of a pathogenic or tumorigenic cell, labels the target cell for destruction. This is either through binding to an activating antibody receptor (FcγR) on the surface of an immune-effector cell, resulting in lysis or phagocytosis (ADCC), or to the complement component C1q (CDC) leading to the induction of a membrane attack complex (MAC)

ADCC is an effector mechanism of the immune response in which IgG antibodies bind to antigens on the surface of pathogenic or tumorigenic target cells (Fig. 49.1). This label renders the target cell susceptible to destruction through binding of the antibody Fc region to an activating antibody receptor (Fc gamma receptor, FcγR) on the surface of an immune-effector cell such as a natural killer (NK) cell, a macrophage, or a granulocyte (Iannello and Ahmad 2005). The mAb-FcγR interaction leads to phagocytosis or triggers a lytic attack of the target cell (Fig. 49.1). Especially NK cells play a crucial role in this mechanism of antibody effector functions because they express the low-affinity activating Fc receptor FcγRIIIa (CD16) and are able to mediate an effective lysis.

Subsequent to the interaction of FcγRIIIa with the Fc region of an antibody bound to its target on a cell, NK cells release cytolytic substances such as perforin and granzymes, which leads to lysis of the target cell.

Another important effector mechanism of therapeutic antibodies is CDC. MAbs of the IgM or IgG isotype possess complement-binding sites in the Fc portion and

are therefore able to activate the complement system through binding of the complement protein C1q (Fig. 49.1).

Unattached C1q is inactive and innocuous. After binding to an antibody C1q changes its molecular shape, which triggers a cascade of complement, and by this, their sequential activation. This activation of the classical complement pathway ultimately leads to the formation of a "membrane attack complex" (MAC) that mediates lysis of the target cell (Bhakdi and Tranum-Jensen 1991).

Activation of the complement cascade requires at least a simultaneous binding of C1q to two Fc regions. Thus, IgM antibodies are the most potent inducers of CDC. However, other antibody classes (IgG1 and IgG3) are also able to activate the complement cascade through C1q if the antigen density is sufficient (Cooper 1985).

Most of the existing therapeutic antibodies that have been licensed and developed as medical agents exhibit an Fc-region of the IgG1 subclass so that they can induce effector functions such as ADCC or CDC.

In the last few years, it could be shown that several amino acid residues in the C_{H2} region (Clark 1997; Greenwood et al. 1993; Lazar et al. 2006; Morgan et al. 1995; Presta et al. 2002; Shields et al. 2001, 2002) and a sugar chain linked to the Asn 297 of the C_{H2} region of the antibody (Clark 1997; Clynes 2006; Kaneko et al. 2006; Presta 2006; Raju 2008; Shinkawa et al. 2003) play an essential role for binding of mAbs to FcγRs or the complement component C1q. As studies in mice and results from human clinical trials have demonstrated that ADCC plays an essential role for the efficiency of a therapeutic antibody (Cartron et al. 2002; Clynes and Ravetch 1995; Clynes et al. 2000; Dall'Ozzo et al. 2004; Nimmerjahn and Ravetch 2005; Nimmerjahn and Ravetch 2006; Shields et al. 2001; Weng et al. 2004; Weng and Levy 2003), there is strong interest in generating antibodies with improved binding to activating FcγRs.

Therefore, a lots of efforts have been made over the last few years to optimize monoclonal antibodies for an improved interaction with FcγRs on immune effector cells or C1q. For example, there exist Fc-engineered (Lazar et al. 2006) and glycoengineered antibodies (Shinkawa et al. 2003) showing a dramatically enhanced ADCC-efficiency.

The standard assay for determining whether an antibody induces ADCC is the so called chromium release assay (Brunner et al. 1968). This assay can also be used for the analysis of the extent of CDC which an antibody is able to induce.

The chromium release assay is based on the measurement of radioactive ^{51}Cr released from metabolically labeled target cells by incubation with an appropriate mAb in the presence of FcγR bearing effector cells (ADCC) or serum (as a source of complement, CDC) in a given period of time.

For labeling, target cells are incubated with $Na_2{}^{51}CrO_4$, which is cell permeable and is easily taken up by the cells. Intracellularly it binds to cytoplasmic proteins and is thus spontaneously released only in small amounts. Disruption of the cell membrane – as caused by ADCC and CDC – allows a release of cytoplasmic proteins and hereby of bound ^{51}Cr. The amount of released radioactive ^{51}Cr

can subsequently be detected easily in the cell culture supernatant by gamma counting.

^{51}Cr fulfills the requirements of a good label as the chromium release assay is very sensitive and $Na_2{}^{51}CrO_4$ does not change the characteristics or the morphology of the target cells. Although there are also other assays that can be used for detecting ADCC and CDC – also non-radioactive assays – they will not be further discussed here as most of them lack sensitivity.

49.2 Materials

49.2.1 Buffers and Solutions

- Cell culture medium RPMI-1640, supplemented with 10% heat-inactivated (56°C, 45 min) fetal bovine serum (FBS), 2 mM glutamine, and, if desired, antibiotics such as 100 U/ml Penicillin and 100 μg/ml Streptomycin; this medium is stored at 4°C.
- Phosphate buffered saline (PBS) 0.137 M NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4. Adjust pH to 7.4 and filter through a 0.2 μm filter.

49.2.2 Preparations

49.2.2.1 Preparations for ADCC Assay

- Effector cells: Fresh heparinised blood from healthy donors or a buffy coat can be used for the isolation of peripheral blood mononuclear cells (PBMCs) which serve as effector cells. Buffy coats can be obtained from a blood bank.
- Density gradient solution for PBMC preparation (density 1.077 g/ml; Biocoll™ Separation Solution; Biochrom, Berlin, Germany). It is stored in the dark at 4°C.
- Cell culture medium RPMI-1640.
- PBS.

49.2.2.2 Preparations for CDC Assay

- Human serum: In CDC assays, human serum is used as a source of complement components. For preparation of human serum, blood samples from healthy donors can be processed using serum separator tubes (S-Monovetten; Sarstedt, Nümbrecht, Germany). Such tubes contain particles that cause fast blood clotting as well as a special gel that separates blood cells from serum after centrifugation. Alternatively, commercially available serum can be used.

49.2.3 Target Cell Labeling

- Target cells: Theoretically, any suspension culture or semi-adherent cell line expressing the antigen of interest can be used as a target. An optimal target cell line for ADCC assays should be insensitive to spontaneous cytotoxicity and for CDC assays, it should not be susceptible to complement-mediated lysis. Furthermore, target cells expressing high amounts of FcγRs may compete with the effector cells for binding of the antibody Fc-region, leading potentially to false results.

  In case semi-adherent cells are used as targets it is advisable not to use trypsine for harvesting the cells because the antigen of interest can be lost or hampered by trypsine. Here, it is advisable to detach the cells mechanically from the culture flask, for example, by using a cell scraper.

  For the assay only cells from cultures of more than 80% viability should be used. A lower viability indicates that cells are stressed by the culture conditions. This leads to a higher spontaneous release of ^{51}Cr because of spontaneously dying cells. If this spontaneous ^{51}Cr-release is too high, the assay cannot be evaluated!
- Chromium-51 di-sodium chromate ($Na_2{}^{51}CrO_4$), 5 mCi/ml in saline (GE Healthcare, München, Germany): The radionucleide must be stored lead-protected at 4°C and should be used within 8 weeks due to its short half-life of 28 days. Due to the short half-life, the amount of $Na_2{}^{51}CrO_4$ has to be adjusted based on actual radioactivity levels at any given time in order to guarantee a sufficient activity of the labeled cells. However, using too large volumes for labeling may hamper labeling efficiency because of adverse effects of the high salt concentrations on the cells.
- Cell culture medium RPMI-1640.
- PBS.
- 37°C water bath or incubator.

49.2.4 Cytotoxicity Assay

- Cell culture 96-well flat-bottom microtiter plates (Greiner Bio-one, Frickenhausen, Germany).
- Antibodies: The ability of a mAb to stimulate ADCC depends on its isotype and its ability to bind to activating FcγRs. The IgG subclasses IgG1 and IgG3 possess the highest affinity for binding to the activating FcγRIIIa on NK cells and therefore are the most potent inducers of ADCC. In contrast, IgG4 and IgG2 antibodies bind only weakly to FcγRIIIa (Goldsby 2003) and thus have a reduced lytic activity.

  For induction of CDC, the used mAb must be able to fix complement. Thus, only mAbs of the IgM or IgG isotype are able to induce CDC. CDC effector function is high for human IgG1 and IgG3 and low for IgG2 (Goldsby 2003). IgM antibodies are potent inducers of CDC, whereas in the case of IgG antibodies, the antigen density must be sufficient to induce CDC-activity.

Many different purified antibodies are nowadays commercially available; all antibodies reported in the literature should, in general, be available from individual investigators. The antibody concentration that should be used in the ^{51}Cr-release assay depends on the affinity of the antibody Fc-part to bind to activating FcγRs.

- Target cells labeled with Chromium-51 di-sodium chromate (see Sect. 49.2.3.).
- In case of ADCC-assay PBMCs as effector cells (see Sect. 49.2.2.1.).
- In case of CDC-assay human serum as a source of complement components (see Sect. 49.2.2.2.).
- Triton X-100 solution: Prepare a 3% solution in H_2O. Triton X-100 is used for determining the maximum release (100% release) of ^{51}Cr from the target cells which is an essential control for the evaluation of the assay. Alternatively other reagents leading to cell lysis can be used (H_2O, 1% sodium dodecyl sulfate (SDS)). These reagents are stored at room temperature.
- LumaPlate™-96 (Packard BioScience, Dreieich, Germany).
- MicroBeta Counter 1450 Plus (PerkinElmer Wallac, Turku, Finnland).
- Cell culture medium RPMI-1640.
- 37°C incubator.

49.3 Methods

This protocol describes a method for measuring both ADCC effector activity in PBMCs and CDC activity using human serum as source of complement, and employs ^{51}Cr-labeled target cells (Fig. 49.2).

Therefore, preparations for ADCC and CDC assays are described in separate sections. As the target cell labeling and the procedure for the cytotoxicity assay are mostly identical, they are described in a joint section with special announcements for the ADCC and CDC assays.

49.3.1 Preparations

49.3.1.1 ADCC

Preparation of Effector Cells

PBMC serving as effector cells are isolated by density gradient centrifugation. After centrifugation, lymphocytes and monocytes can be found at the gradient-plasma interface whereas erythrocytes along with granulocytes pellet to the bottom of the tube. The PBMC layer can subsequently be removed with a pipette.

This protocol describes a procedure for isolating PBMCs either from fresh heparinised blood or a buffy coat. As human blood is biohazardous, reasonable precautions for proper handling should be taken.

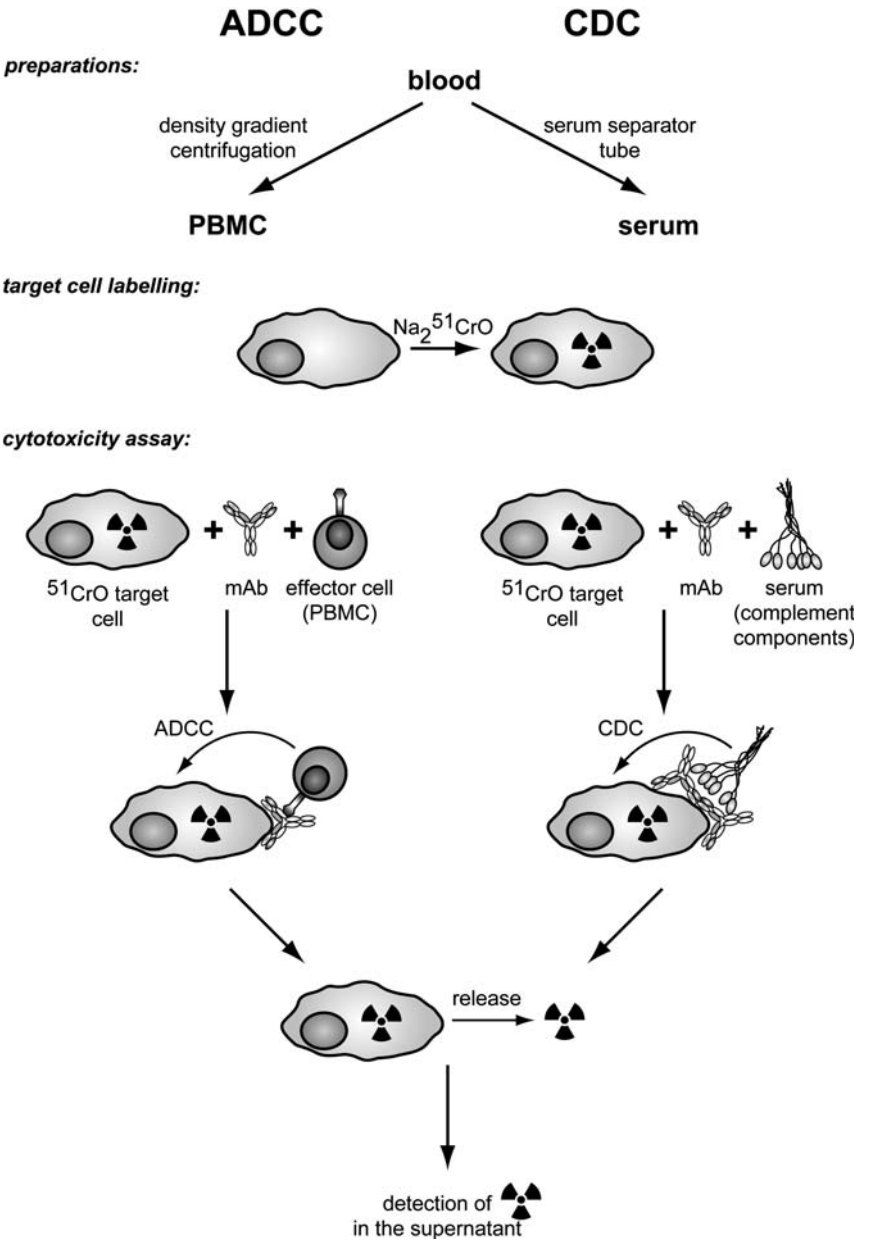


Fig. 49.2 Survey of the different working steps of a ^{51}Cr-release assay. In the ADCC assay peripheral blood mononuclear cells (PBMC) are used as effector cells whereas in the CDC assay human serum serves as a source of complement

Procedure

1. Let Biocoll™ solution warm to room temperature (in the dark) and pipette 12 ml Biocoll™ solution in 50 ml tubes. For each 30 ml of sample, one tube with a 12 ml Biocoll™ solution is needed.
2. Transfer buffy coat from its storage bag into a 250 ml cell culture flask and dilute 1:5 with sterile PBS. When using freshly isolated whole blood, a 1:2 dilution with PBS is sufficient.
3. Take a 25 ml pipette, and gently overlay 30 ml of the diluted buffy coat/blood onto the Biocoll™. Keep the centrifuge tube at a 45° angle, and allow the sample to run down along the side of the tube.
4. Centrifuge at 800 *g* for 20 min at room temperature. It is essential to switchoff the brakes of the centrifuge off in order to avoid disturbing the PBMC layer.
5. Remove tubes from the centrifuge carefully so that the gradient is not disturbed. The mononuclear leukocytes should appear as a cloudy ring at the interface. Harvest them from the interface with a 5 ml pipette, and transfer the cells to a fresh 50 ml tube avoiding carryover of the lower clear layer.
6. Fill the tube to 50 ml with room temperature PBS, and mix gently by inversion.
7. Centrifuge at 400 *g* for 5 min at room temperature to pellet the leukocytes.
8. Gently resuspend the lymphocyte pellet in 50 ml PBS.
9. Re-centrifuge at 400 *g* for 5 min to pellet the PBMCs.
10. Aspirate the supernatant and resuspend the cell pellet in culture medium (for 1 ml of fresh blood, 1×10^6 PBMC could be expected).
11. Determine the actual cell number of the isolated PBMCs, and adjust the concentration. Keep in mind that for the assay the desired effector cell number has to be provided in 50 µl /well (see Sect. 49.3.1.1.E:T-ratio).

E:T-Ratio

To determine the best effector – target cell ratio (E:T ratio) and to interpret the results correctly it is advised to test different effector cell numbers (e.g. 1:2 dilutions) – an E:T-ratio of 50:1 can be a good starting point

For example, using 5×10^3 target cells/well in 50 µl, the amount of effector cells must be adjusted to 5×10^6/ml – resulting in 2.5×10^5 effector cells per well, which gives an E:T-ratio of 50:1.

49.3.1.2 CDC

Preparation of Human Serum

Human serum required as a source of complement components for the CDC assay can be purchased or prepared using fresh blood. One can expect nearly half of the drawn blood volume as serum.

Procedure
1. Draw blood in serum-separator tubes.
2. Mix gently by inverting and let the blood coagulate for 1 h at room temperature.
3. Centrifuge the tube for 15 min, 1,000× *g* at 4°C to separate the serum from the coagulated blood components.
4. Carefully collect the serum and store at 4°C until usage.

Serum Concentration

It is advisable to titrate the serum to find out the best working dilution in the ^{51}Cr-release assay, a dilution of 1:3 with PBS can be a reasonable starting point. If the serum concentration is too high, this may interfere with the detection of released ^{51}Cr. In case the serum is diluted too much the induction of CDC will be impaired.

49.3.2 Target Cell Labeling

Target cells are labeled with Chromium-51 di-sodium chromate ($Na_2{}^{51}CrO_4$). The labeling step of the target cells is performed at 37°C for 1–2 h, depending on the cell line. All precautions for working with radionuclides have to be taken.

Procedure
1. Harvest the cells and determine the cell number. Centrifuge the required number of target cells (0.5×10^6 cells/plate if using 5×10^3 target cells/well) at about 400 g for 5 min. Discard the supernatant and resuspend the cells in 1 ml culture medium.
2. Add 50–100 μCi $Na_2{}^{51}CrO_4$ /10^6 cells and incubate at 37°C (either in a water bath or an incubator) for 1–2 h.
3. Fill up the tube with at least 10 ml of medium and centrifuge (400 g, 5 min).
4. Thoroughly resuspend the cells in at least 10 ml medium and centrifuge at about 400 *g* for 5 min (= washing step).
5. This washing step is repeated three times.
6. Count the cells and resuspend them at 1×10^5/ml in culture medium. In the cytotoxicity assay, you will plate 50 μl of this target cell suspension in each well, leading to 5×10^3 target cells per well.

49.3.3 Cytotoxicity Assay

The cytotoxicity assay is performed in a 96-well flat-bottom microtiter plate. It is recommended that flat-bottom plates be used because of a higher contact surface between the target and effector cells or the target cells and complement. The components are mixed and lysis of target cells is detected by measuring the release of radioactivity into the cell supernatant.

Procedure

1. Calculate the amount of wells needed for the assay and prepare the antibody dilutions. The assay is performed for each antibody concentration and E:T ratio in triplicate.
2. Pipette 50 µl of each antibody dilution to be tested in triplicate per well of a 96-well flat-bottom microtiter plate. Leave three triplets (nine wells) empty for controls.
3. For controlling the spontaneous release of the labeled target cells plate 100 µl of culture medium in one triplet of the nine empty wells. For detection of the maximum release, plate 100 µl of 3% Triton X-100 in the next three empty wells. In most cases, Triton X-100 induces release of about 90% of the incorporated radioactivity. Be careful not to contaminate the spontaneous control wells with Triton X-100. In the last three control wells pipette 50 µl of culture medium to account for unspecific effector cell/serum effects in the ADCC or CDC assay

   ADCC-assay: To determine whether the effector cells have an effect on the rate of spontaneous ^{51}Cr-release, incubate the effector cells in the absence of antibodies with target cells.

   CDC assay: In order to control the effect of serum on the rate of spontaneous ^{51}Cr-release, a control containing only target cells and serum in the absence of antibodies is required.
4. Add 50 µl of the radiolabeled target cell suspension to all wells required for the assay, including the control wells.
5. Now pipette 50 µl of the effector-cell suspension for the ADCC-assays or 50 µl of diluted serum for the CDC-assays in all wells, except the wells containing the maximum release control and the spontaneous release control.
6. Spin down the cells for 1 min at about 100 g.
7. Incubate the plate(s) for 4 h in an incubator (5% CO_2, 95% humidified atmosphere, 37°C). If there is a need for longer incubation, it is possible to incubate even overnight (about 21 h) depending on the cell line used and the efficiency of the labeling.
8. Take out the plates from the incubator and centrifuge for 1 min at about 400 *g* to spin down the cells.
9. Collect 50 µl supernatant and be careful not to take off the cells. Otherwise, the results will be falsified (see 49.5.1.1).
10. Count in a gamma counter at a rate of 1 min/sample).

49.4 Data Evaluation

Each value for ^{51}Cr-release is calculated as the mean of its respective triplicate. The percentage of specific ^{51}Cr-release is calculated as follows:

$$\%\ specific\ lysis = \frac{cpm_{sample} - cpm_{spontaneous\ release}}{cpm_{max\ release} - cpm_{spontaneous\ release}} \times 100\%$$

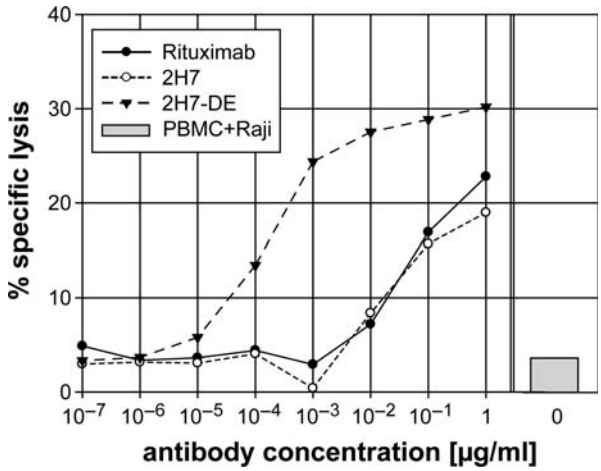


Fig. 49.3 Example for the evaluation of an ADCC assay showing the ADCC effector activity of three different anti-CD20 antibodies in peripheral blood mononuclear cells (PBMCs)

Figure 49.3 shows an example for ADCC effector activity of three different anti-CD20 antibodies using PBMC as effector cells and the CD20 expressing B-lymphoma cell line Raji as target cells (E:T = 10:1) (Otz 2008). The antibodies were titrated and the ^{51}Cr-release was detected after 4 h of incubation. The antibody 2H7-DE shows the highest ADCC-efficacy. This is due to a modification in the Fc-part of the 2H7 antibody, which results in a much higher affinity for binding to activating FcγRs.

The bar on the right shows the control for sensitivity of the target cells to spontaneous cytotoxicity. Here, Raji cells were incubated with PBMC in the absence of antibodies. This control should, in general, not be higher than 10%.

The evaluation for CDC-assays is performed respectively.

49.5 Troubleshooting

49.5.1 General Notes for ADCC and CDC-Assays

49.5.1.1 High Variance in Triplicates

To avoid triplicates with a high variance it is essential to

- Have a homogeneous single target cell suspension for labeling. Otherwise, the cells will not be labeled homogeneously and plating clusters of cells will falsify the results extremely.
- Be very careful and precise when plating the target and effector cells. Prevent the formation of air bubbles.
- Avoid taking out any target cells when collecting the supernatant. If labeled target cells that have not been lysed are collected with the supernatant, this will

pretend a too high level of lysis. Therefore, it is advisable to centrifuge the plates accurately before harvesting the supernatant. Do not harvest too big volumes and keep the tip against the wall of the well at a 45° angle to take off the supernatant.

49.5.1.2 Low ^{51}Cr Incorporation by the Target Cells

A low ^{51}Cr incorporation of the target cells can have several reasons:

- Some cells (especially primary cells) are difficult to label or release the uptaken ^{51}Cr only in small amounts. To check the ability of such "unusual" target cells to incorporate and release ^{51}Cr, it is advised to label a small portion of the cells and to count the spontaneous release of ^{51}Cr. Furthermore, the maximum release as an indicator for the amount of releasable ^{51}Cr should be tested. This pre-testing should be performed in the same manner as the actual assay will be performed including the volume of collected supernatant.
- For determining the best labeling protocol, test different cell concentrations and incubation times.

49.5.1.3 High Spontaneous Release

A too high spontaneous release can be due to:

- Incorrect collection of supernatant like discussed in Sect. 49.5.1.1.
- Bacterial contamination which results in toxicity and maximal lysis of the target cells. Such a contamination might be due to the non-sterile conditions during the ^{51}Cr release assay performance.
- Target cells stressed by the culture or labeling conditions. For labeling of target cells, only cells from cultures that are of more than 80% viability should be used. For optimal labeling, target cells should be in their proliferative phase. If cells are stressed by the culture conditions or by the labeling procedure, this leads to a higher spontaneous release because of spontaneously dying cells. If the spontaneous release is too high (more than 25% of the maximum release), the assay cannot be correctly evaluated.

49.5.2 Special Notes for ADCC-Assays

49.5.2.1 High ^{51}Cr Release in all Wells Containing Target and Effector Cells

- Target cells may be sensitive to spontaneous cytotoxicity. Therefore, a control consisting of target and effector cells without any antibody being added is essential. In case of spontaneous cytotoxicity the number of effector cells

could be decreased. In case this does not help, testing another blood donor for effector cell preparation might help. In the worst case, the target cells are too sensitive to spontaneous cytotoxicity so that they cannot be used in this kind of assay.

49.5.2.2 Suboptimal Killing

- The antibody is not able to induce ADCC due to a lack of the Fc-part to bind to activating FcγRs.
- The E:T-ratio is not optimal. So increase the effector-cell concentration.
- The antibody concentrations are either too low or too high. Therefore, a wider range of antibody concentrations should be tested.

49.5.2.3 E:T-Ratio

- Using purified or cultured NK cells as effector cells, the initial E:T-ratio must be much lower than using PBMCs (e.g. 1:1). The optimal E:T-ratio has to be determined experimentally.

49.5.3 Special Notes for CDC-Assays

49.5.3.1 Suboptimal Killing

- The antibody is not able to induce CDC due to an inability to fix C1q.
- The antigen density on the target cell surface is not high enough. Therefore, test another cell line expressing the same antigen in higher amounts.
- Some cell lines show an intrinsic resistance to complement-induced cytotoxicity. In this case the only possibility is to change the target cell line.
- The amount of serum used is not optimal. So titrate the serum in order to determine the optimal serum dilution.

References

Bhakdi S, Tranum-Jensen J (1991) Complement lysis: a hole is a hole. Immunol Today 9:318–320

Brunner KT, Mauel J, Cerottini JC, Chapuis B (1968) Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunology 2:181–196

Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, Watier H (2002) Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood 3:754–758

Clark MR (1997) IgG effector mechanisms. Chem Immunol 65:88–110
Clynes R (2006) Antitumor antibodies in the treatment of cancer: Fc receptors link opsonic antibody with cellular immunity. Hematol Oncol Clin North Am 3:585–612
Clynes R, Ravetch JV (1995) Cytotoxic antibodies trigger inflammation through Fc receptors. Immunity 1:21–26
Clynes RA, Towers TL, Presta LG, Ravetch JV (2000) Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nat Med 4:443–446
Cooper NR (1985) The classical complement pathway: activation and regulation of the first complement component. Adv Immunol 37:151–216
Dall'Ozzo S, Tartas S, Paintaud G, Cartron G, Colombat P, Bardos P, Watier H, Thibault G (2004) Rituximab-dependent cytotoxicity by natural killer cells: influence of FCGR3A polymorphism on the concentration-effect relationship. Cancer Res 13:4664–4669
Glennie MJ, van de Winkel JG (2003) Renaissance of cancer therapeutic antibodies. Drug Discov 11:503–510 Today
Goldsby RA, Kindt TJ, Osborne BA, Kuby J (2003) Immunology, Fifth Edition, WH Freeman and Company, New York, 1–551
Greenwood J, Clark M, Waldmann H (1993) Structural motifs involved in human IgG antibody effector functions. Eur J Immunol 5:1098–1104
Iannello A, Ahmad A (2005) Role of antibody-dependent cell-mediated cytotoxicity in the efficacy of therapeutic anti-cancer monoclonal antibodies. Cancer Metastasis Rev 4:487–499
Kaneko Y, Nimmerjahn F, Ravetch JV (2006) Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science 5787:670–673
Lazar GA, Dang W, Karki S, Vafa O, Peng JS, Hyun L, Chan C, Chung HS, Eivazi A, Yoder SC, Vielmetter J, Carmichael DF, Hayes RJ, Dahiyat BI (2006) Engineered antibody Fc variants with enhanced effector function. Proc Natl Acad Sci USA 11:4005–4010
Morgan A, Jones ND, Nesbitt AM, Chaplin L, Bodmer MW, Emtage JS (1995) The N-terminal end of the CH2 domain of chimeric human IgG1 anti-HLA-DR is necessary for C1q, Fc gamma RI and Fc gamma RIII binding. Immunology 2:319–324
Nimmerjahn F, Ravetch JV (2005) Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science 5753:1510–1512
Nimmerjahn F, Ravetch JV (2006) Fcgamma receptors: old friends and new family members. Immunity 1:19–28
Otz (2008) New approaches for an antibody-based treatment of $CD20^+$ lymphoid neoplasia. tobias-lib.ub.uni-tuebingen.de/volltexte/2008/3316/index.html
Presta LG (2006) Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. Adv Drug Deliv Rev 5–6:640–656
Presta LG, Shields RL, Namenuk AK, Hong K, Meng YG (2002) Engineering therapeutic antibodies for improved function. Biochem Soc Trans 4:487–490
Raju TS (2008) Terminal sugars of Fc glycans influence antibody effector functions of IgGs. Curr Opin Immunol 4:471–478
Sanz L, Blanco B, Alvarez-Vallina L (2004) Antibodies and gene therapy: teaching old 'magic bullets' new tricks. Trends Immunol 2:85–91
Shields RL, Namenuk AK, Hong K, Meng YG, Rae J, Briggs J, Xie D, Lai J, Stadlen A, Li B, Fox JA, Presta LG (2001) High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. J Biol Chem 9:6591–6604
Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem 30:26733–26740
Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, Uchida K, Anazawa H, Satoh M, Yamasaki M, Hanai N, Shitara K (2003) The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type

oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem 5:3466–3473

Strome SE, Sausville EA, Mann D (2007) A mechanistic perspective of monoclonal antibodies in cancer therapy beyond target-related effects. Oncologist 9:1084–1095

Waldmann TA (2003) Immunotherapy: past, present and future. Nat Med 3:269–277

Weng WK, Levy R (2003) Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. J Clin Oncol 21:3940–3947

Weng WK, Czerwinski D, Timmerman J, Hsu FJ, Levy R (2004) Clinical outcome of lymphoma patients after idiotype vaccination is correlated with humoral immune response and immunoglobulin G Fc receptor genotype. J Clin Oncol 23:4717–4724

Chapter 50
Binding Studies with Flow Cytometry

Thomas Schirrmann

50.1 Introduction

Flow cytometry is a technique for counting, examining, and sorting microscopic particles of about 0.5–40 μm size. It allows simultaneous multiparametric analysis of physical and (bio-)chemical characteristics of thousands to millions of single cells even in large and diverse cell populations. Although only a few devices are able to recover single cells or cell populations, flow cytometry is often not correctly termed fluorescent activated cell sorting (FACS) for nonsorting applications, as well.

During flow cytometric measurement, cells are automatically separated in a fluid stream and single cells are hydrodynamically focused to pass through one or several LASER (light amplification by stimulated emission of radiation) beams. LASER light is scattered according to cell size (forward scatter, FSC) and cell granularity (sideward scatter, SSC) measured by optical detectors. LASER light also excites cellular or cell-bound fluorescent molecules to emit fluorescence light (FL) which is detected by photomultiplier detectors in combination with an optical filter system. Therefore, cells are usually stained with fluorescence-conjugated antibodies to measure antigen expression levels. Multiple antigens can be simultaneously analyzed with antibodies conjugated with different fluorochromes ("colors"). Today, flow cytometers with up to three LASERs are available that can detect more than 12 different colors in parallel. However, it is extremely difficult to translate multicolor staining into practice because the FL emitted by the fluorochromes always overlay to a certain extent into other wavelength ranges of other fluorescence detectors. Fluorescence compensation is more difficult with an increasing number of different colors. A table of fluorochromes used in antibody conjugates is given in Table 50.1.

T. Schirrmann
Technische Universität Braunschweig, Institut für Biochemie und Biotechnologie, Department of Biotechnology, Spielmannstr. 7, Braunschweig 38106, Germany
e-mail: th.schirrmann@tu-bs.de

R. Kontermann and S. Dübel (eds.), *Antibody Engineering Vol. 1*,
DOI 10.1007/978-3-642-01144-3_50,

Table 50.1 Fluorochromes used in flow cytometry

Excitation	Fluorochromes				
	Fluorescence color[a] (fluorescent light channel, FL) Fluorochrom (maximum wavelength excitation/emission)				
	Green (FL1)	Yellow (FL2)	Orange (FL3)	Red (FL4)[b]	Infra-red (FL5)[b]
Blue Argon laser (488 nm)	FITC (494/518) Alexa Fluor® 488 (495/519) Cy2® (489/506) GFP (488/508)[c] YFP (513/527)[c]	PE (565/575) TRITC (547/572)	ECD (565/615) PI (536/617)[d]	PC5 (565/670) PerCP (490/675) 7-AAD (546/647)[d]	PC7 (565/767)
Red diode laser (635 nm)				APC (650/660) APC-Cy7® (650/767) Alexa Fluor® 633 (632/647) Cy5® (649/670)	Cy7® (743/767)

7-AAD 7-aminoactinomycin D, Alexa Fluor® dyes from Invitrogen (Molecular probes); *APC* allophycocyanin, APC-Cy7, Cy® dyes from GE Healthcare; *ECD* PE-TexasRed tandem conjugate); *FITC* fluorescein isothiocyanate; *GFP* green fluorescent protein; *PC5* PE-Cy5 tandem conjugate; *PC7* PE-Cy7 tandem conjugate; *PE* R-phycoerythrin; *PerCP* peridinin chlorophyll protein; *PI* propidiumiodid; *TRITC* tetramethyl rhodamine iso-thiocyanate; *YFP* yellow fluorescent protein

[a]Typical numbering of the FL channels

[b]Not provided by all FACS machines as standard

[c]For antibody fusion proteins

[d]For staining dead cells

Flow cytometry allows the identification of cell populations within cell mixtures regarding different light scattering or by using antibodies against cell lineage specific markers. It can also quantify the number of cells and the intensity of protein expression. Since measurement comprises thousands to millions of cells, flow cytometric analysis can detect very rare cell types and allows statistical analyses which cannot be provided by fluorescence microscopy. Flow cytometry can be used for many additional applications. For example, cell numbers can be quantified if standardized fluorescent beads (e.g., Flow Count™ microspheres, Beckman Coulter) are added to the samples. Moreover, cells can be stably labeled with fluorescent dyes such as 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO, D275), the cell membrane linker PKH26 or carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE, CFSE) to analyze cell division tracking (Hasbold and Hodgkin 2000), cell proliferation (Givan et al. 2004), cytotoxicity (Johann et al. 1995), or to study the binding of different cells (Schirrmann and Pecher 2001). The very long and still expanding list of additional flow cytometry applications comprises the detection of fluorescent reporter gene products (e.g., green fluorescent protein), cell pigments (e.g., chlorophyll, phycoerythrin), DNA (Telford et al. 1992), RNA (Piwnicka et al. 1983), chromosome analysis and sorting (Bartholdi et al. 1987), as well as the detection of enzymatic activity, intracellular pH, intracellular ionized calcium (Burchiel et al. 2000), or magnesium and membrane potential (Shapiro 2000), membrane fluidity, apoptosis (Gorczyca et al. 1998), cell viability, for example, with PI or 7-AAD (Weaver 1998), multidrug resistance in cancer cells, for example, with rhodamine 123 (Seidel et al. 1995), and glutathione with monobromobimane (MBrB).

The following two protocols describe the staining of extracellular or intracellular proteins with fluorochrome-conjugated antibodies, including measurement and analysis. Methods combining intra- and extracellular staining are described elsewhere (Lecoeur et al. 1998).

50.2 Material

50.2.1 Antibodies, Chemicals, and Solutions

1. Phosphate-buffered saline (PBS): 8.0 g/L sodium chloride (NaCl), 0.2 g/L potassium chloride (KCl), 1.44 g/L di-sodiumhydrogenphosphate-di-hydrate ($Na_2HPO_4 \times 2\,H_2O$), and 0.24 g/L potassium-di-hydrogenphosphate (KH_2PO_4), pH 7.4. Note that a 10× PBS stock has a higher pH!
2. FACS buffer: PBS supplemented with 3% (v/v) fetal calf serum (FCS, e.g., PAA, Austria) or 0.5% (w/v) bovine serum albumin (BSA, fraction V, Sigma-Aldrich, Germany). To stabilize cells during staining and measurement, add freshly 0.1% (w/v) sodium azide (NaN_3) or 2 mM ethylene-diamine-tetraacetat (EDTA). Note that sodium azide (NaN_3) is very toxic and cannot be used for cell sorting application to recover vital cells.
3. Fc receptor blocking solution: Incubate rabbit normal serum (e.g., Invitrogen) for 45 min at 56°C to inactivate complement. Dilute 1:10 in PBS and perform

at least five freeze thaw cycles using a dry ice/ethanol bath or liquid nitrogen for freezing and a 37°C water bath for thawing to form immunoglobulin aggregates that efficiently block human Fc receptors. There are Fc receptor block antibody mixtures commercially available that inhibit species-specific Fc receptors CD16, CD32 and/or CD64.

4. Primary antibodies or antibody fluorochrome conjugates, including antibody fragments such as scFv or scFv-Fc fusion proteins. A list of fluorochromes used in antibody conjugates is given in Table 50.1.
5. Secondary fluorochrome-conjugated antibody for the detection of non-fluorescent labeled primary antibodies (e.g., Dianova). $F(ab')_2$ fragments from secondary antibodies can also resolve problems with unspecific Fc receptor binding. Biotinylated primary antibodies or antibody fragments can be detected by fluorochrome-conjugated anti-biotin antibodies or streptavidin.
6. Trypsin/EDTA (typically 0.025% (w/v) Trypsin and 1 mM EDTA, cell culture grade). UV irradiated trypsin/EDTA may reduce the risk for mycoplasma contamination (PAA Laboratories GmbH, Pasching, Austria).
7. 0.4% (w/v) trypan blue solution (Sigma, Munich, Germany)
8. 2× Propidium iodide (PI) solution: 2 μg/mL PI (Sigma) in PBS or FACS buffer. Alternatively, 0.005% (w/v) 7-aminoactinomycin (7-AAD) can be used instead of PI which has less crosstalk to the FL2 channel and can be better combined for staining with PE conjugates.
9. Fixation solution: 4% (v/v) formaldehyde or paraformaldehyde in PBS
10. Saponine buffer: FACS buffer containing 0.5% (w/v) saponine of Quillaja bark (Sigma). Saponine is a plant glycoside and mild nonionic detergent. It forms with membrane cholesterol ring-shaped complexes of about 8 nm diameter that can be passed through by molecules of up to 200 kDa size, including IgG antibodies. Pores are reversible and saponine must be present in all intracellular staining and washing steps. Saponine and other mild detergents, such as Tween®-20 or digitonin (Sigma), are suitable for the detection of proteins near the plasma membrane. Stronger detergents, such as 0.1% (v/v) Triton® X-100 (Sigma) or NP-40/IGEPAL (MP Biomedicals, Heidelberg, Germany), are required for nuclear antigen staining. Here, the loss of cell membrane and cytoplasm will not only result in decreased light scattering and reduced nonspecific fluorescence but also in the loss of proteins in or near the plasma membrane.
11. Solutions required for the flow cytometer device must be used according to the manufacturer's description. It is not recommended to prepare these solutions by your own because they may form precipitates which can destroy the device.

50.2.2 Disposables

1. 1.5 mL polypropylen (PP) tubes (different suppliers)
2. 96-well V-bottom PP microtiter plates (Greiner Bio-one, Frickenhausen, Germany)

3. 5–6 ml polypropylene tubes (Sarstedt, Nürnbrecht, Germany) required for measurement must exactly fit into the sample loader of the flow cytometer (e.g., 5 mL PP tube with 10 mm inner diameter and 7.5 cm length for the FC500 of Beckman Coulter). Note that the opening diameter of these tubes can vary between different suppliers.
4. For small sample volumes, 0.6–1 mL tubes can be placed into the 5 mL tubes. However, this is only recommended for manual sample loaders. Note that flow cytometers usually require about 100–150 μL initial volume until the first cells of the sample are measured. Sample volumes smaller than 200 μL are not recommended.
5. Some flow cytometers provide the measurement directly from a 96-well plate. Follow the manufacturer's recommendations.
6. Staining can be performed in standard 1.5 mL or larger PP tubes. For large sample numbers as well as for very small cell numbers ($<5 \times 10^4$ cells), it is recommended to use 96-well V-bottom PP microtiter plates (Greiner Bio-One). See also remarks in the protocols.

50.2.3 Devices

1. Centrifuge: Cooled bench top centrifuges, for example, Eppendorf 5810R (with microtiter plate adapter).
2. Flow cytometer: The two major manufacturers of flow cytometers are Becton Dickenson (Heidelberg, Germany) and Beckman Coulter (Krefeld, Germany). Two examples of flow cytometers frequently found in research labs are given:
   (a) The FC-500 (Beckman Coulter) has up to two LASERs (argon ion, 488 nm, 20 mW, and optional solid-state red diode LASER 635 nm, 25 mW) and user-interchangeable optical filters (Band Pass (BP): 525 nm, 575 nm, 675 nm, 755 nm; Dichroic Long Pass (DL): 488 nm, 550 nm, 600 nm, 710 nm; Long Pass (LP): 500 nm; Dichroic Short Pass (DSP): 615 nm; Short Pass (SP): 620 nm) and 5 photomultipliers (PMTs) as detector allowing the measurement of up to five colors in parallel.
   (b) FACS Calibur (Becton Dickenson) with up to two LASERs (argon ion, 15 mW, 488 nm, and an optional second red diode, 635 nm) and an optical filter system with bandpass filters: FL1: 530 nm (FITC), FL2: 585 nm (PE/PI), FL3: 661 nm (APC), and FL4: >650 nm (PerCP) with base unit, and FL4 (optional): >670 nm (APC).

50.2.4 Software and Tools

Flow cytometer devices are delivered with software packages for measurement and analysis tools. Subsequent analysis may require additional tools. Most cytometry analysis software packages are only commercially available and no software meets

all requirements. Newer devices measure 20–32 bit raw data in the FCS 3.0 mode which also allows fluorescence compensation after measurement but is not supported by older software. A short list of software that is not dependent on a particular flow cytometer is given.

WinMDI 2.8 (last build January 19, 2000, http://facs.scripps.edu/software.html)

Free flow cytometry analysis tool by Joe Trotter. This 16-bit Windows application does not support FCS3.0 files and is not further developed.

Flow Explorer (Version 4.2, http://software.ronhoebe.net/free/default.asp (03.02.2009))

This free software scans folders for flow cytometry Listmode-files and shows parameters, notes, histograms, bivariate dot plots, density, and contour plots.

FlowJo (Tree Star, Inc., Ashland, OR, USA, http://www.flowjo.com/)

Commercial analysis tool with many analysis options (Version 8.8 for MacOS X 10.2 and higher, Version 7.2 for Windows 2000 and higher).

WinList (Verity Software House, Topsham, ME, USA, http://www.vsh.com/)

Commercial software for flow cytometry Listmode data (Version 6 for Mac and Windows).

50.3 Protocols

50.3.1 Extracellular Staining of Cells

1. (Optional) Adherent cells are enzymatically detached by incubation with Trypsin/EDTA solution to get a single cell suspension. Keep trypsin incubation as short as possible. Add medium containing 8% (v/v) serum to inhibit trypsin and to prevent further proteolytic degradation of surface molecules.
2. Count cells using a Neubauer chamber. The vitality of the cells can be determined by trypan blue exclusion staining.
3. Centrifuge cells down for 5–10 min at 250 $\times g$ at 4°C.
4. Discard supernatant completely. Resuspend the cell pellet in an appropriate volume FACS buffer to obtain 1–5 $\times$ 10^5 cells per sample and transfer them into 1.5 mL PP tubes (or 96-well V-bottom PP microtiter plate wells. Note that instructions for staining in microtiter plates are always indicated in brackets in this protocol!).
5. Centrifuge cells for 5 min at 300 $\times g$.
6. Discard supernatant completely (Microtiter plates should be poured out immediately after centrifugation by turning the microtiter plate head-over and discard the supernatant with one (!) push. Without (!) turning the plate, the residual liquid can be further drained off by putting the plate immediately on a filter paper for a few seconds. V-bottom microtiter plates are easier to handle than U-bottom plates).
7. If necessary, Fc receptors are blocked by incubation for 15 min with 50 µL (20 µL per microtiter plate well) Fc block solution. Notice that most cell lines

do not express Fc receptors, but primary cells from the blood or lymphoid tissues contain populations with high Fc receptor expression.

8. Add more than 10 volumes of FACS buffer, that is, about 1.5 mL (230 μL in microtiter plates).
9. Spin down cells at 300 ×g for 5 min at 4°C.
10. Put tubes (or microtiter plate) on ice and resuspend cell in 50 μL (20 μL per well) in an appropriate concentration of the primary antibody. The appropriate concentration of antibody should be determined by titration. Only a saturated concentration can guarantee that the protein levels are accurately detected. However, too high antibody concentrations can lead to unspecific staining of antigen negative cells. For initial tests of 2×10^5 cells, about 0.1–1 μg bi- or multivalent antibody (IgG, IgM, and scFv-Fc) or 1–10 μg monomeric scFv should be feasible. However, low affinity antibodies may require higher concentration. Antibodies with a high off-rate usually do not work in flow cytometry.
11. Incubate at 4°C for 15 min.
12. Add more than 10 volumes of FACS buffer, that is, about 1.5 mL (or 230 μL in microtiter plates).
13. Spin down cells at 300 ×g for 5 min at 4°C.
14. Discard supernatant and repeat washing (step 12–13) once.
15. (Optional) Staining with unconjugated anti-tag antibodies for the detection of scFvs can be performed according to the primary staining (step 10–14). Anti-tag antibodies, such as 9E10 (Sigma), anti-Penta-His-Tag (Qiagen, Hilden, Germany), and anti-Hexa-His-Tag (Roche, Mannheim, Germany), usually require concentrations of 0.1–1 μg per 2×10^5 cells.
16. (Optional) Staining with a secondary antibody can be performed according to the primary staining (step 10–14). Secondary polyclonal detection reagents such as Goat anti-mouse-IgG (whole IgG-, Fab- or Fc-specific) antibody, or F $(ab')_2$ fragment (e.g., Dianova, Hamburg, Germany) usually require 0.1–1 μg per 2×10^5 cells. See also the manufacturer's recommendation.
17. To exclude "dead" cells, that is, cells with perforated plasma membrane, add PI with a final concentration of 0.5–1 μg/mL.
18. For measurement and analysis, see Sects. 50.3.3 and 50.3.4.

50.3.2 Intracellular Staining of Cells

1. (Optional) Adherent cells are enzymatically detached by incubation with Trypsin/EDTA solution to get a single cell suspension. Keep trypsin incubation as short as possible and add medium containing 8% (v/v) serum to inhibit trypsin and to prevent further proteolytic degradation of surface molecules.
2. Count the cells by using a Neubauer chamber. Vital cells can be determined by trypan blue exclusion staining.
3. Wash cells two times with 15 mL of ice-cold PBS in 15 mL PP tubes to remove serum or other proteins from the supernatant (5–10 min with 250 ×g at 4°C).

4. Discard the supernatant completely. Resuspend 1–5 $\times$ 10^6 cells in 1 mL of ice-cold PBS and add 1 mL 4% (v/v) formaldehyde/PBS. Incubate for 10–15 min at room temperature. Shake tubes a few times during fixation to prevent cell sedimentation. For nuclear staining, incubate cells with a final concentration of 0.01% (v/v) formaldehyde/PBS, followed by the addition of 1 mL ice cold methanol to each sample. Mix gently. Place at $-20°C$ for 10 min.
5. Add 13 mL of PBS, mix, and centrifuge 5 min at 400 $\times g$ at 4°C. Note! Fixed cells only form a fluffy cell pellet upon centrifugation. Therefore, the centrifugation speed should be increased to 350–400 $\times$g because fixed cells are relatively robust and tolerate higher centrifugation speeds.
6. Discard the supernatant completely, resuspend the fixed cells in 15 mL PBS and repeat the washing step twice.
7. Discard the supernatant completely, resuspend the fixed cells in an appropriate volume saponine buffer to obtain 1–5 $\times$ 10^5 cells per sample, and transfer them into 1.5 mL PP-tubes (or 96-well V-bottom PP microtiter plate wells. Note that instructions for staining in microtiter plates are always indicated in brackets in this protocol!).
8. Centrifuge cells for 5 min at 400 $\times g$.
9. Discard the supernatant completely by aspiration. (Microtiter plates should be poured out immediately after centrifugation by turning the microtiter plate head-over and discarding the supernatant with one (!) push. Without (!) turning the plate, the residual liquid can be further drained off by putting the plate immediately on filter paper for a few seconds. V-bottom microtiter plates are easier to handle than U-bottom plates.)
10. Resuspend cells in 100 µL (50 µL) of the appropriate concentration of the primary antibody diluted in saponine buffer. Buffers containing detergents such as saponine may disturb the cell pellet. Therefore, avoid foaming during pipetting. The appropriate concentration of the antibody should be determined by titration. Only a saturated concentration can guarantee that the protein levels are accurately detected. However, too high antibody concentrations can lead to unspecific staining of antigen negative cells. For initial tests of 2 $\times$ 10^5 cells, about 0.1–1 µg bi- or multivalent antibody (IgG, IgM, and scFv-Fc) or 1–10 µg monomeric scFv should be feasible. Note that most commercial primary and secondary antibodies are not tested for intracellular cell staining. Therefore, it is recommended to test and titrate all antibodies before use.
11. Incubate at RT for 30–60 min.
12. Add more than 3–10 volumes saponine buffer, that is, about 1.4 mL (or 150 µL in microtiter plates).
13. Centrifuge cells for 5 min at 400 $\times g$.
14. Discard the supernatant completely and fill up tubes (or microtiter plate wells) with saponine buffer.
15. Incubate for 30–60 min at RT to allow the release of unbound antibodies out of the cells. Mix several times during incubation to prevent cell sedimentation. The incubation time may vary depending on the individual antibody and

antibody format (For staining in microtiter plates, change the saponine buffer after 15–30 min).
16. Spin down the cells at 400 ×g for 5 min at 20°C.
17. Discard the supernatant completely and fill up tubes (or microtiter plate wells) with saponine buffer.
18. Spin down the cells at 400 ×g for 5 min at 20°C.
19. (Optional) Discard the supernatant and continue with staining using anti-tag antibodies according to the primary antibody staining (step 10–18). Anti-tag antibodies such as 9E10 (Sigma), anti-Penta-His-Tag (Qiagen), and anti-Hexa-His-Tag (Roche) usually require concentrations of 0.1–1 μg per 2×10^5 cells.
20. (Optional) Discard the supernatant and continue with staining using a secondary fluorochrome-conjugated antibody according to the primary antibody staining (step 10–18).
21. Finally, wash cells with FACS buffer to remove saponine. Centrifuge for 5 min at 400 ×*g* and resuspend in the appropriate volume FACS buffer. Do not add PI or 7-AAD)!
22. For measurement and analysis, see Sects. 50.3.3 and 50.3.4.

50.3.3 Measurement

Follow the manufacturer's protocols. Some general hints are given below.

1. Avoid cell aggregates or clumps to prevent blockage of the flow system. It can be necessary to remove aggregates through a 50–70 μm mesh filter.
2. Always include negative control samples; for example, staining with an appropriate primary antibody of the same (iso-)type that does not bind to the cells (isotype control). Mock-transfected cells, that is, transfected with the vector without the gene of interest, are the appropriate negative control for cell transfections. Set the negative reference signal between 10^0 and 10^1 in all fluorescence channels according to the negative controls.
3. Include positive controls, for example, cells that are positive for the respective antigen. Alternatively, take the sample with the highest positive signal. Adjust the detector gains that all data fit into the measurement window.
4. Do not change the parameters between measurements of samples that belong together, including the controls!!!

50.3.4 Analysis of Cells

Analysis of flow cytometry data strongly depends on the software. Some general hints are given below.

1. Analyze samples always in comparison to the appropriate control (see Sect. 50.3.3) which is used as reference. All changes such as fluorescent compensation

after measurement must be performed with the reference sample in the same way.
2. In cell mixtures, an antigen negative cell population can be used as reference.
3. Debris and cell aggregates can be excluded from the analysis in the FSC-SSC plot. Debris usually has a low FSC intensity because of smaller size, whereas cell aggregates usually have higher FSC-SSC intensities than single cells. Some blood-cell types can already be identified in the FSC-SSC plot. The small lymphocytes have relatively low FSC-SSC intensities in comparison to monocytes and dendritic cells. Granulocytes have the highest FSC-SSC intensities because of their large size and natural granularity.
4. Apoptotic cells may also be visible as a distinct population with higher SSC intensity because of intracellular apoptotic vesicles. However, it is recommended to additionally use PI (FL3) or 7-AAD (FL4) to exclude dead and apoptotic cells from analysis in the appropriate fluorescence plots.

50.4 Results

50.4.1 Extracellular Staining of Hodgkin Lymphoma Cells with αCD30 Antibody Constructs

The $CD30^-$ HD-MY-Z, and the $CD30^+$ Karpas 299 lymphoma lines were stained according to the protocol 3.1 with the CD30 specific scFv-Fc-RNase and scFv-Fc antibody constructs (Menzel et al. 2008). A fluorescein isothiocyanate (FITC)-conjugated goat anti-human-IgG (Fc specific) antibody (Dianova, 1:400) was used as a secondary antibody. Controls were stained only with the secondary antibody conjugate. The analysis of the data is shown in Fig. 50.1. Debris and cell aggregates were excluded from further analysis in the FSC-SSC density plot. Dead cells were excluded from the analysis by the positive PI staining in the FL3 channel because they lost their membrane integrity and tend to unspecific antibody binding. Finally, both gates were combined in the histogram plots. The $CD30^-$ HD-MY-Z cells were not stained by any of the antibody constructs, whereas $CD30^+$ Karpas 299 cells were stained by the αCD30-scFv-Fc, as well as by the αCD30-scFv-Fc-RNase construct. Taken together, flow cytometric data revealed a specific binding of αCD30-scFv-Fc and αCD30-scFv-Fc-RNase protein to $CD33^+$ Hodgkin lymphoma cells.

50.4.2 Intracellular Staining of CHO Cell Producing Antibody Constructs

CHO-K1 cells were transfected with the vector pCMV-αCD30scFv-Fc-RNase or pCMV-αCD30scFv-Fc encoding a CD30 specific scFv-Fc-RNase construct or its corresponding scFv-Fc antibody fragment, respectively. Stable CHO clones were selected in the presence of G418 (500 μg/ml) and subcloned several times. Before

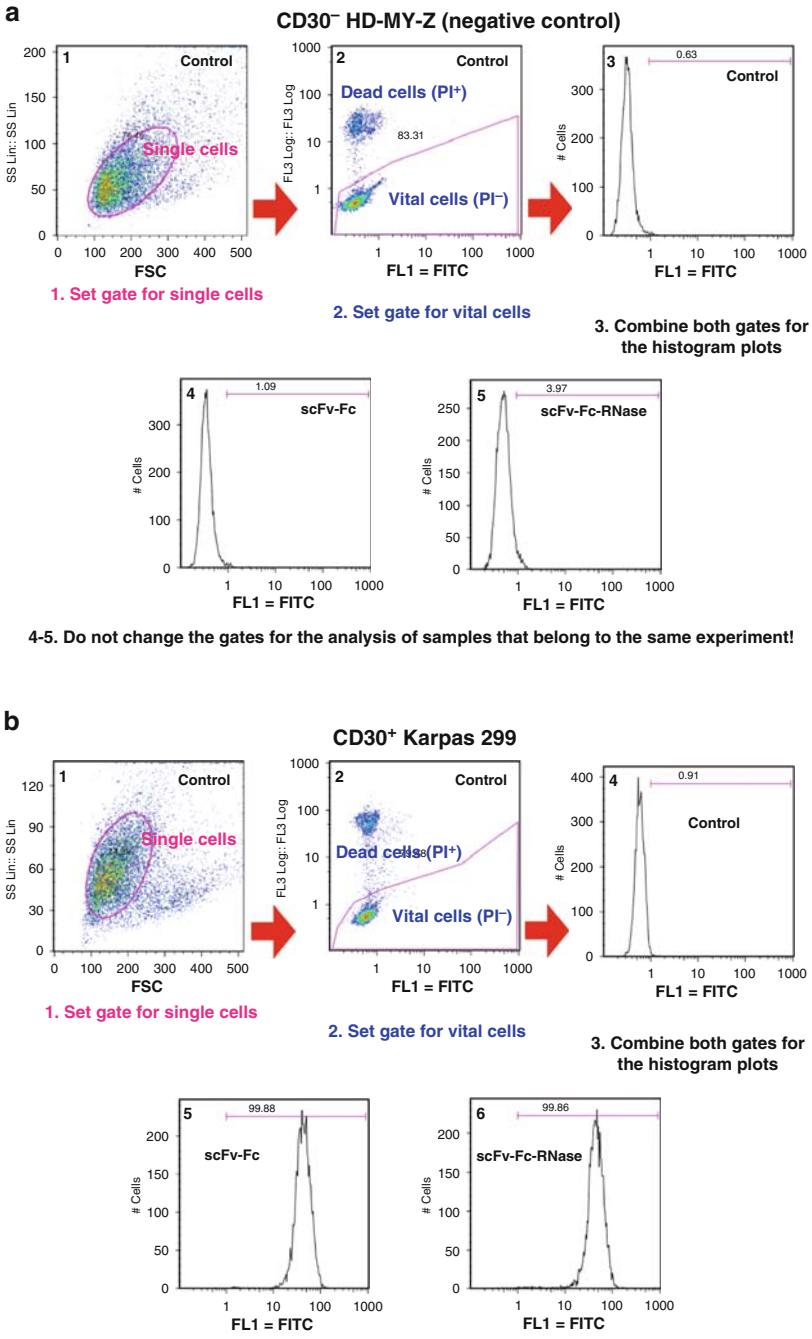


Fig. 50.1 Extracellular staining of two lymphoma cell lines with αCD30 scFv-Fc and scFv-Fc-RNase proteins. The CD30⁻ HD-MY-Z negative control cell line (a1–a5) and the CD30⁺ Karpas 299 cell line (b1–b5) were stained with an αCD30 scFv-Fc-RNase protein (a5, b5) and

staining, two clones were cultured with or without 10 μg/mL Brefeldin A that interferes with the anterograde protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus and leads to the accumulation of secretory proteins inside the ER (Nylander and Kalies 1999). After 12 h, intracellular staining was performed according to protocol 3.2 using a FITC-conjugated goat anti-human-IgG (Fc specific) antibody (Dianova, 1:400). The analysis of the data is given in Fig. 50.2. In contrast to untransfected CHO-K1 cells, both CHO-K1 clones transfected with the αCD30-scFv-Fc or the αCD30-scFv-Fc-RNase gene construct, respectively, were intracellularly stained by the goat anti-human-IgG (Fc specific) antibody. Most cells of the CHO-K1 clone transfected with αCD30-scFv-Fc showed transgene expression at high levels, whereas the CHO-K1 clone transfected with αCD30-scFv-Fc-RNase showed two populations with low and medium transgene expression levels. According to this data, the αCD30-scFv-Fc-RNase transfected CHO-K1 clone is probably not a single clone or contains a population that down-regulated the transgene expression. Incubation with Brefeldin A resulted in an accumulation of both secretory antibody constructs. Taken together, intracellular staining allowed the analysis of secretory protein expression.

50.5 Troubleshooting

Some remarks to material and methods are already given in Sects. 50.2 and 50.3. If no staining is achieved, include an appropriate positive control into your experiment, for example, a cell line that expresses the antigen or an antibody that was already successfully tested. For nonvalidated antibodies, it is recommended to include a cell line that does not express the antigen to exclude unspecific binding. Antibodies that bind well to partially or completely denatured antigens in immunoblots or ELISA often do not work in flow cytometry because they do not recognize their antigen in its native structure and natural environment." + "In addition, only antibodies with low off-rates do not dissociate during measurement in the fluid stream and and are suitable for flow cytometry. Monovalent binders (e.g., scFv, Fab) often perform only at much higher concentrations in comparison to bi- or multivalent antibody formats (IgG, IgM, and scFv-Fc). Therefore, di- or

Fig. 50.1 (continued) the corresponding αCD30 scFv-Fc antibody fragment (a4, b4). A FITC-conjugated goat anti-human-IgG (Fc specific) antibody was used as secondary antibody. Control samples were only stained with the secondary antibody conjugate (a1-3, b1-3). The analysis was performed as following: First debris and aggregates were excluded from further analysis in the FSC-SSC density plots by setting a gate around the single cell population (a1, b1). Then, PI-stained dead cells were excluded by setting an appropriate gate around the viable cell population in the FL1-FL3 density plots (a2, b2). Both gates were combined and applied to the FL1 histogram plots (a3-6, b3-6). All samples of one cell line were measured with the same instrument settings and were analyzed using the same gates

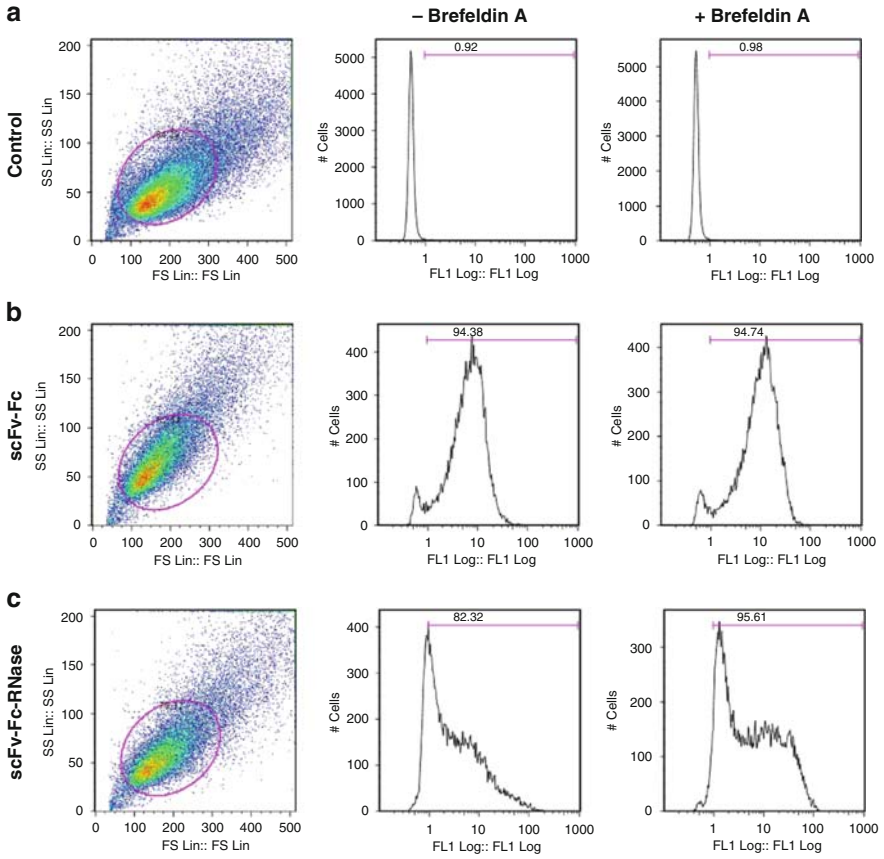


Fig. 50.2 Intracellular staining of untransfected CHO-K1 cells and two CHO-K1 clones transfected with an αCD30 scFv-Fc or an αCD30 scFv-Fc-RNase gene construct. Non-transfected CHO-K1 cells (**a**) and two CHO-K1 clones stably transfected with αCD30 scFv-Fc (**b**) or αCD30 scFv-Fc-RNase construct (**c**) were 12 h cultivated with or without Brefeldin A that blocks protein secretion and leads to an accumulation of secretory proteins in the ER. FSC-SSC density plots in the first column are used to exclude debris and cell aggregates from further analysis. This gate was used for the FL1 histogram plots showing the intracellular levels of αCD30 scFv-Fc (B) or αCD30 scFv-Fc-RNase protein (C) detected with an FITC-conjugated goat anti-human IgG (Fc specific) antibody (columns 2 and 3). All cells of the αCD30 scFv-Fc transfected CHO-K1 clone (B) showed high transgene expression level. In contrast, the CHO-K1 clone transfected with the αCD30 scFv-Fc-RNase construct (C) had two populations with low or medium transgene expression levels revealing that this cell line is not monoclonal or that a subpopulation of cells down-regulated the transgene expression. The 12 h preincubation with Brefeldin A (column 3) increased the intracellular levels of both secretory Fc fusion proteins in the transfected CHO-K1 clones

multimerization strategies should be considered for recombinant monovalent antibodies, for example, by fusion to the IgG Fc part.

Antibody aggregates or denatured antibodies can be responsible for unspecific staining. It is recommended to avoid repeated freeze-thaw cycles. After thawing,

spin antibody aggregates down at 11,000 ×g for 5 min and use supernatant for staining. Note that this procedure is not recommended for IgM antibodies. It is recommended to keep thawed antibody stocks at 4°C. If necessary, add 0.1% NaN_3 to prevent microbial contamination.

50.6 Conclusion

Flow cytometry is a powerful technology for the analysis of antigen expression in cells, but it also reveals important information about the antibodies itself. In contrast to immunoblots and ELISAs, in flow cytometry, antibodies bind the native antigen in a natural cellular context and in the presence of thousands of other antigen molecules. Accordingly, antibodies that specifically bind in flow cytometry are highly specific and often of relatively high apparent affinity. For the development of therapeutic antibodies, flow cytometry represents one of the most crucial test systems.

References

Bartholdi M, Meyne J, Albright K, Luedemann M, Campbell E, Chritton D, Deaven LL, Cram LS (1987) Chromosome sorting by flow cytometry. Methods Enzymol 151:252–267

Burchiel SW, Edwards BS, Kuckuck FW, Lauer FT, Prossnitz ER, Ransom JT, Sklar LA (2000) Analysis of free intracellular calcium by flow cytometry: multiparameter and pharmacologic applications. Methods 21:221–230

Givan AL, Fisher JL, Waugh MG, Bercovici N, Wallace PK (2004) Use of cell-tracking dyes to determine proliferation precursor frequencies of antigen-specific T cells. Methods Mol Biol 263:109–124

Gorczyca W, Melamed MR, Darzynkiewicz Z (1998) Analysis of apoptosis by flow cytometry. Methods Mol Biol 91:217–238

Hasbold J, Hodgkin PD (2000) Flow cytometric cell division tracking using nuclei. Cytometry 40:230–237

Johann S, Blumel G, Lipp M, Forster R (1995) A versatile flow cytometry-based assay for the determination of short- and long-term natural killer cell activity. J Immunol Methods 185:209–216

Lecoeur H, Ledru E, Gougeon ML (1998) A cytofluorometric method for the simultaneous detection of both intracellular and surface antigens of apoptotic peripheral lymphocytes. J Immunol Methods 217:11–26

Menzel C, Schirrmann T, Konthur Z, Jostock T, Dübel S (2008) Human antibody RNase fusion protein targeting CD30+ lymphomas. Blood 111:3830–3837

Nylander S, Kalies I (1999) Brefeldin A, but not monensin, completely blocks CD69 expression on mouse lymphocytes: efficacy of inhibitors of protein secretion in protocols for intracellular cytokine staining by flow cytometry. J Immunol Methods 224:69–76

Piwnicka M, Darzynkiewicz Z, Melamed MR (1983) RNA and DNA content of isolated cell nuclei measured by multiparameter flow cytometry. Cytometry 3:269–275

Schirrmann T, Pecher G (2001) Tumor-specific targeting of a cell line with natural killer cell activity by asialoglycoprotein receptor gene transfer. Cancer Immunol Immunother 50:549–556

Seidel A, Hasmann M, Loser R, Bunge A, Schaefer B, Herzig I, Steidtmann K, Dietel M (1995) Intracellular localization, vesicular accumulation and kinetics of daunorubicin in sensitive and multidrug-resistant gastric carcinoma EPG85-257 cells. Virchows Arch 426:249–256

Shapiro HM (2000) Membrane potential estimation by flow cytometry. Methods 21:271–279

Telford WG, King LE, Fraker PJ (1992) Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. Cytometry 13:137–143

Weaver JL (1998) Estimation of cell viability by flow cytometry. Methods Mol Biol 91:77–83

Index

C

D

E

F

G