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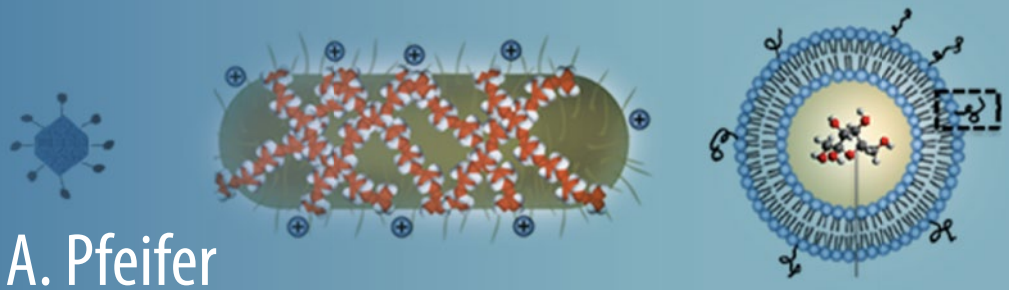

Blaine A. Pfeifer
Andrew Hill *Editors*

Vaccine Delivery Technology

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, UK

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Vaccine Delivery Technology

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Edited by

Blaine A. Pfeifer and Andrew Hill

Department of Chemical and Biological Engineering, University at Buffalo, The State University of New York, Buffalo, NY, USA

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Editors
Blaine A. Pfeifer
Department of Chemical and Biological Engineering
University at Buffalo, The State University of New York
Buffalo, NY, USA

Andrew Hill
Department of Chemical and Biological Engineering
University at Buffalo, The State University of New York
Buffalo, NY, USA

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Preface

In this book for *Methods in Molecular Biology*, titled *Vaccine Delivery Technology*, we make a holistic effort to cover the vaccine development process and the role delivery concepts contribute to a global goal of effective final health outcomes. In so doing, readers are presented with topics that span a broad array of ongoing research in vaccine production intended to be educational and useful to those both within and outside of the vaccine fields. Of note, we include chapters from both industry and academia with contributions coming from different countries, continents, and perspectives.

This diversity in content and contributions adds to the educational breadth of the book but also flavors applications by the regions of the globe contributing associated chapters. As such, the general reader will also gain appreciation for the development of vaccination research programs associated with regional disease concerns. An additional advantage to this breadth of topics is the knowledge regarding diseases that rely upon vaccine research and development to provide much needed treatment options.

In terms of content flow, the book begins with vaccine basics, including several early chapters devoted to antigen identification and selection. Chapters include computational approaches, provided by the Ellis and Daura groups, to antigen identification. These chapters build on the immense level of data generated through various next generation sequencing and associated 'omics approaches. In related chapters, the Bidmos and McKay groups discuss the identification of functional monoclonal antibodies resulting from antigen exposure.

The following and related chapters next turn to antigen preparation and established forms of antigen administration. Chapters devoted to genetic and protein antigen preparation are provided by the Lundstrom, Nichita, and Czermak groups. These preparation methods overlap with the earlier introduced chapters on monoclonal antibody capture from said antigens, particularly in the case for protein-based antigens. Included with these chapters is a contribution from the Bracewell group on rapid and high-throughput methods of antigen purification.

Subsequent chapters (contributed by the Smith, Keys, Wolff, Shukla, Micoli, Guo, and Fiebig groups) cover various formats for vaccine formulations. These include the use of viral-like particles (VLPs), whole cell vaccines, and glycoconjugate vaccines. Included in these chapters are unique methodologies for combining emerging approaches for in vivo glycoconjugation with VLP carriers. In addition, the Chakravortty group provides a chapter on attenuation methods used in particular for live whole cell vaccines.

The next several chapters, contributed by the Ramsey, Czermak, Rak, Chakravortty, Mancha-Agresti, and Pfeifer groups, focus on the central theme of this book, that is, vaccine delivery. As such, topics span viral and nonviral gene delivery technology, the use of bacterial and hybrid bacterial-biomaterial delivery devices, dual antigen delivery liposomal carriers, and needle-less noninvasive delivery technology. Intermixed with these chapters are important contributions by the Petrovsky, Zeng, and Cui groups that present methodologies associated with vaccine adjuvant selection and long-term vaccine storage preparation.

The final series of chapters are devoted to vaccine delivery effectiveness assessment. The McCluskie, Herbert, and Bou Ghanem groups provide chapters that cover confirmation of vaccine delivery carriers, common assessment methodologies (ELISpot, ELISA), methods

that profile the in vivo progression of the immune response, novel monitoring of viral infectivity quantification, and disease-specific antibody functionality. The emphasis on final vaccine effectiveness thus concludes the range of topics covered by overall book contributions.

In summary, this volume was designed broadly and generally enough to engage the nonexpert interested in the vaccine development field. Alternatively, we highlight the central theme of vaccine delivery technology through a series of chapters that cover delivery methods designed to enable and/or enhance vaccine effectiveness across specific disease applications. Combined, we expect this book to serve as a valuable resource, to those within and outside of the field, in the ongoing research pursuits to expand and improve upon the already significant impact made by vaccines.

Buffalo, NY, USA

Blaine A. Pfeifer
Andrew Hill

Contents

Contributors

Gerard Agbayani • *Human Health Therapeutics, National Research Council Canada, Ottawa, ON, Canada*

Bassel Akache • *Human Health Therapeutics, National Research Council Canada, Ottawa, ON, Canada*

Riyad F. Alzhrani • *Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA*

Vasco Azevedo • *Laboratory of Cellular and Molecular Genetics (LGCM), Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil*

Viviane Lima Batista • *Laboratory of Cellular and Molecular Genetics (LGCM), Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil*

Marie Beitelshees • *Department of Chemical and Biological Engineering, The State University of New York at Buffalo, Buffalo, NY, USA*

Monika Berger • *Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany*

Manmeet Bhalla • *Department of Microbiology and Immunology, University at Buffalo School of Medicine, Buffalo, NY, USA*

Fadil A. Bidmos • *Section of Paediatric Infectious Disease, Department of Infectious Disease, Imperial College London, London, UK*

Elsa N. Bou Ghanem • *Department of Microbiology and Immunology, University at Buffalo School of Medicine, Buffalo, NY, USA*

Daniel G. Bracewell • *Department of Biochemical Engineering, University College London, London, UK*

Norica Branza-Nichita • *Institute of Biochemistry of the Romanian Academy, Bucharest, Romania*

Insa Budde • *Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany*

Dipshikha Chakravortty • *Department of Microbiology and Cell Biology, Indian Institute of Science, Bengaluru, India; Centre for Biosystems Science and Engineering, Indian Institute of Science, Bengaluru, India*

Yanping Chen • *Center of Emphasis in Infectious Diseases, Department of Molecular and Translational Medicine, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center El Paso, El Paso, TX, USA*

Oscar Conchillo-Solé • *Institute of Biotechnology and Biomedicine, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain; Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain*

Zhengrong Cui • *Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA*

Peter Czermak • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany; Faculty of Biology and Chemistry, Justus-Liebig-University Giessen,*

Giessen, Germany; Division Bioresources, Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Giessen, Germany

Tales Fernando da Silva • *Laboratory of Cellular and Molecular Genetics (LGCM), Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil*

Akshay Datey • *Centre for Biosystems Science and Engineering, Indian Institute of Science, Bengaluru, India*

Xavier Daura • *Institute of Biotechnology and Biomedicine, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain; Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain*

Luis Cláudio Lima de Jesus • *Laboratory of Cellular and Molecular Genetics (LGCM), Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil*

Gregor Dekevic • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany*

Roberta Di Benedetto • *GSK Vaccines Institute for Global Health, Siena, Italy*

Diana Diaz-Arévalo • *Grupo Funcional de Inmunología, Fundación Instituto de Inmunología de Colombia-FIDIC, School of Medicine and Health Sciences, Universidad del Rosario, Bogotá, D.C., Colombia*

Nicole DiNardo • *Thermo Fisher Scientific Inc., Grand Island, NY, USA*

Mihaela-Olivia Dobrica • *Institute of Biochemistry of the Romanian Academy, Bucharest, Romania*

Mariana Martins Drumond • *Laboratory of Cellular and Molecular Genetics (LGCM), Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil; Department of Biological Sciences, Federal Center for Technological Education of Minas Gerais (CEFET/MG), Belo Horizonte, Minas Gerais, Brazil*

Kashyap Kumar Dubey • *Department of Biotechnology, Central University of Haryana, Mahendergarh, Haryana, India*

Renu Dudani • *Human Health Therapeutics, National Research Council Canada, Ottawa, ON, Canada*

Friederike Eilts • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany*

John T. Ellis • *School of Life Sciences, University of Technology Sydney (UTS), Ultimo, NSW, Australia*

Timm Fiebig • *Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany*

Yasmine Gabal • *School of Chemical Engineering, Oklahoma State University, Stillwater, OK, USA*

Carlo Giannelli • *GSK Vaccines Institute for Global Health, Siena, Italy*

Camilla A. Gladstone • *Section of Paediatric Infectious Disease, Department of Infectious Disease, Imperial College London, London, UK*

Stephen J. Goodswen • *School of Life Sciences, University of Technology Sydney (UTS), Ultimo, NSW, Australia*

Jagadeesh Gopalan • *Centre for Biosystems Science & Engineering, Indian Institute of Science, Bangalore, Karnataka, India; Department of Aerospace Engineering, Indian Institute of Science, Bangalore, Karnataka, India*
Anna Góra-Sochacka • *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland*
Yanping Guo • *Flow Cytometry Core Facility, National Heart and Lung Institute, Imperial College London, London, UK*
Zhongwu Guo • *Department of Chemistry, University of Florida, Gainesville, FL, USA*
Anna-Barbara Hachmann • *Thermo Fisher Scientific Inc., Grand Island, NY, USA*
Dipasree Hajra • *Department of Microbiology and Cell Biology, Indian Institute of Science, Bengaluru, India*
Julie Harnischfeger • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany*
Colin G. Hebert • *LumaCyte, LLC, Charlottesville, VA, USA*
Andrew Hill • *Department of Chemical and Biological Engineering, University at Buffalo, The State University of New York, Buffalo, NY, USA*
Yoshikazu Honda-Okubo • *Vaxine Pty Ltd, Warradale, SA, Australia; College of Medicine and Public Health, Flinders University, Adelaide, SA, Australia*
Shaleem I. Jacob • *Department of Biochemical Engineering, University College London, London, UK*
Yimei Jia • *Human Health Therapeutics, National Research Council Canada, Ottawa, ON, Canada*
Lukas KÄßer • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany*
Paul J. Kennedy • *School of Computer Science, Faculty of Engineering and Information Technology and the Centre for Artificial Intelligence, University of Technology Sydney (UTS), Ultimo, NSW, Australia*
Timothy G. Keys • *Institute of Microbiology, ETH Zurich, Zurich, Switzerland*
Tom A. Kloter • *Institute of Microbiology, ETH Zurich, Zurich, Switzerland*
Spyridon Konstantinidis • *Department of Biochemical Engineering, University College London, London, UK*
Atichat Kuadkitkan • *Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand*
Punit Kumar • *Department of Biotechnology, University Institute of Engineering and Technology, Maharshi Dayanand University Rohtak, Rohtak, Haryana, India; Department of Clinical Immunology, Allergology and Microbiology, Karaganda Medical University, Karaganda, Kazakhstan*
Paul R. Langford • *Section of Paediatric Infectious Disease, Department of Infectious Disease, Imperial College London, London, UK*
Catalin Lazar • *Institute of Biochemistry of the Romanian Academy, Bucharest, Romania*
Lei Li • *Vaxine Pty Ltd, Warradale, SA, Australia; College of Medicine and Public Health, Flinders University, Adelaide, SA, Australia*
Christa Litschko • *Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany*

Daniel Loewe • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany*
Keven Lothert • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany*
Kenneth Lundstrom • *PanTherapeutics, Lutry, Switzerland*
Zbigniew Madeja • *Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland*
Pamela Mancha-Agresti • *Laboratory of Cellular and Molecular Genetics (LGCM), Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil; Biomedical Science Institute (ICBM), Catholic of Cuyo University, San Juan, CP, Argentina*
Michael J. McCluskie • *Human Health Therapeutics, National Research Council Canada, Ottawa, ON, Canada*
Paul F. McKay • *Section of Immunology of Infection, Department of Infectious Disease, Imperial College London, London, UK*
Francesca Micoli • *GSK Vaccines Institute for Global Health, Siena, Italy*
Chaeho Moon • *Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA*
Roozbeh Nayerhoda • *Department of Biomedical Engineering, University at Buffalo, The State University of New York at Buffalo, Buffalo, NY, USA*
Kathryn K. Oi • *Institute of Microbiology, ETH Zurich, Zurich, Switzerland*
Nikolai Petrovsky • *Vaxine Pty Ltd, Warradale, SA, Australia; College of Medicine and Public Health, Flinders University, Adelaide, SA, Australia*
Blaine A. Pfeifer • *Department of Chemical and Biological Engineering, University at Buffalo, The State University of New York, Buffalo, NY, USA*
Christopher L. Pinder • *Section of Immunology of Infection, Department of Infectious Disease, Imperial College London, London, UK*
Ruiquan Qi • *Department of Chemical and Biological Engineering, University at Buffalo, The State University of New York at Buffalo, Buffalo, NY, USA*
Hui Qian • *Nanotechnology Research Center, National Research Council Canada, Edmonton, AB, Canada*
Monika Rak • *Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland*
Suwipa Ramphan • *Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand*
Joshua D. Ramsey • *School of Chemical Engineering, Oklahoma State University, Stillwater, OK, USA*
Keya L. Rodrigues • *LumaCyte, LLC, Charlottesville, VA, USA*
Denise Salzig • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany*
Robin J. Shattock • *Section of Immunology of Infection, Department of Infectious Disease, Imperial College London, London, UK*
Pratyoosh Shukla • *Enzyme Technology and Protein Bioinformatics Laboratory, Department of Microbiology, Maharshi Dayanand University Rohtak, Rohtak, Haryana, India*

Shaunna R. Simmons • *Department of Microbiology and Immunology, University at Buffalo School of Medicine, Buffalo, NY, USA*
Sara Siris • *Section of Paediatric Infectious Disease, Department of Infectious Disease, Imperial College London, London, UK*
Brittany R. Smith • *Department of Chemistry, University of Florida, Gainesville, FL, USA*
Duncan R. Smith • *Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand*
Wannapa Sornjai • *Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand*
Felicity C. Stark • *Human Health Therapeutics, National Research Council Canada, Ottawa, ON, Canada*
Laura J. Suggs • *Department of Biomedical Engineering, Cockrell School of Engineering, The University of Texas at Austin, Austin, TX, USA*
Sunita • *Enzyme Technology and Protein Bioinformatics Laboratory, Department of Microbiology, Maharshi Dayanand University Rohtak, Rohtak, Haryana, India*
Ana Paula Tapia-Costa • *Biomedical Science Institute (ICBM), Catholic of Cuyo University, San Juan, CP, Argentina*
Essi Y. I. Tchalla • *Department of Microbiology and Immunology, University at Buffalo School of Medicine, Buffalo, NY, USA*
Nitwara Wikan • *Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand*
Robert O. Williams III • *Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA*
Michael W. Wolff • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany*
Suchin Worawichawong • *Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand*
Haiyue Xu • *Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA*
Daniel Yero • *Institute of Biotechnology and Biomedicine, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain; Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain*
Mingtao Zeng • *Center of Emphasis in Infectious Diseases, Department of Molecular and Translational Medicine, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center El Paso, El Paso, TX, USA*
Jan Zitzmann • *Institute of Bioprocess Engineering and Pharmaceutical Technology (IBPT), Technische Hochschule Mittelhessen (THM) - University of Applied Sciences, Giessen, Germany*

Chapter 1

Vaccine Delivery and Immune Response Basics

Andrew Hill, Marie Beitelshees, and Blaine A. Pfeifer

Abstract

In this opening chapter, we outline the basics of vaccine delivery and subsequent immune reactivity. Vaccine delivery is an augmentation to immunization more generally in that a delivery reagent is harnessed to improve administration of the key ingredient (i.e., the antigen) needed to provoke an immune response. In this chapter, we discuss the evolution of vaccine design and how such efforts evolved into targeted administration/delivery of key antigens. We then provide overview descriptions of vaccine immune responses and methods for assessment. More generally, the chapter sets the tone for the remainder of this book, which will focus upon each step of the vaccine process with a special emphasis on how vaccine delivery contributes to overall health outcomes.

Key words Vaccine, Antigen, Adjuvant, Antigen-presenting cell, Humoral response, Cellular response

1 Introduction to Vaccination

Vaccines represent one of the greatest medical achievements in the history of mankind due to the role they have played in preventing disease. Since their introduction, vaccines have significantly curtailed various diseases such as small pox, diphtheria, polio, measles, pneumococcal disease, and so on in countries such as the USA (*see* Table 1) [1]. The reduction of many of these disease cases have been achieved through the implementation of pediatric regimens against such pathogens [1]. Furthermore, vaccines are useful within the broader population to reduce the burden associated with diseases caused by pathogens such as the influenza virus, shingles, and the human papillomavirus (HPV). In addition, vaccines against diseases such as yellow fever are available to provide protection to individuals during travel. The primary benefit that is realized from vaccination is that it provides a prophylaxis against potentially deadly diseases, rather than treating the diseases as they occur. These benefits can be enhanced through the achievement of a phenomena known as herd immunity, in which vaccination of a

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Table 1
Disease reduction associated vaccine implementation in the USA [16]

Disease	Type of vaccine	Prevaccine annual cases	Year vaccine introduced	Postvaccine annual cases[a]
Smallpox	Similar virus	29,000	1798	0 (100%)
Diphtheria	Toxoid	21,053	1923	0 (100%)
Pertussis	Inactivated	200,752	1926	15,632 (92.2%)
Tetanus	Toxoid	580	1927	41 (92.9%)
Polio	Inactivated	36,110	1955	0 (100%)
Measles	Attenuated	530,217	1963	56 (99.9%)
Mumps	Attenuated	162,344	1967	6584 (95.9%)
Rubella	Attenuated	47,897	1969	12 (99.9%)
Hepatitis B	Subunit	66,232	1981	13,169 (80.1%)
Hib[b]	Glycoconjugate	20,000	1987	50 (99.8%)
Hepatitis A	Inactivated	117,333	1995	15,298 (87.0%)
Varicella	Attenuated	4,085,120	1995	612,768 (85.0%)
Pneumococcal	Glycoconjugate	63,067	2000	41,550 (34.1%)

[a]Percentages refer to percentage of disease reduction observed in 2006 following the introduction of the vaccine
[b]*Haemophilus influenzae type b*

portion of the population is able to extend protection to the population that is unvaccinated. This occurs because the pathogens do not have available hosts through which they can transmit themselves to infect the vulnerable population. The result enables vaccines to provide substantial economic benefits that are typically presented as quality-adjusted life years (QALYs) or disability-adjusted life years (DALYs) saved compared to nonvaccination [2, 3].

The isolation of pathogens subsequently provided an opportunity for the development of vaccines targeting specific targets. Early vaccine development involved the isolation of various human pathogens and subjecting them to various procedures to weaken their virulence. One of the initial studies to achieve this was the development of a vaccine against anthrax in animals using bacteria that had been attenuated using carbolic acid [4]. A similar approach utilized formaldehyde to attenuate the rabies virus that had been isolated from cerebral tissue [5]. In subsequent vaccines, additional strategies for the attenuation or inactivation of pathogens were developed, which include heat, chemical methods (e.g., hydroxylamine, β-propiolactone, and methylene blue), and irradiation (e.g., ultraviolet) [6]. These studies have facilitated the development and manufacturing of whole-pathogen vaccines (WPVs). This laid the

framework for future work which has provided a demonstration that immunization with a weakened or inactivated pathogen could be used to provide immunity towards the live version.

Despite the utility of various WPVs, advanced fermentation and culturing techniques for growing the pathogens were not initially developed. As such technology became more advanced, vaccines that targeted toxic factors produced by the pathogens were increasingly developed. This approach facilitated the development of vaccines against the bacteria that cause diphtheria and tetanus. As the appreciation for using components of pathogens in vaccines in place of the whole pathogen grew, subunit vaccines containing polysaccharides (e.g., Pneumovax 23®), proteins (e.g., Flublok®), and glycoconjugates (e.g., Prevnar) became increasingly developed. However, one distinct disadvantage associated with subunit vaccines is that they invoke a considerably weaker immune response compared to killed or attenuated vaccines [7]. This observation led to the development of vaccine components known as adjuvants, which boost the immunogenicity of subunit vaccines. Although aluminum salt (alum-based) adjuvants are the most commonly used adjuvants, alternatives containing attenuated endotoxin (i.e., monophosphoryl lipid A) [8] and an emulsion-based system [9] have been developed.

As increasingly diverse systems were developed for the assembly of vaccines, increasing the scientific understanding of the immune system became important for optimizing vaccine efficacy. For example, understanding the mechanism by which various portions of bacteria or viruses (i.e., antigens) illicit an immune response is an important factor in vaccine design. Protein antigens are generally recognized as thymus cell (T cell)-dependent antigens, whereas polysaccharides are T cell-independent antigens [10]. Consequently, solutions of bacterial polysaccharides are poorly immunogenic in infants and do not create a long-lasting immunological memory [10]. This led to the development of glycoconjugate vaccines, which conjugate bacterial polysaccharides to carrier proteins to promote a T cell-dependent response against the polysaccharides and provide potent immunity in infants [10]. Such vaccines have been utilized for vaccination of infants against *Streptococcus pneumoniae* to provide serotype-specific immunization that has reduced the incidence of invasive pneumococcal disease by over 90% [11, 12]. Taken together, these examples have motivated a higher understanding of how the immune response vaccines illicit translates into clinical efficacy.

2 Overview of Immune Response

Following the injection of the vaccine into a patient, the antigens contained within the vaccines are processed to develop an immune response. Upon initial introduction of the antigens, the body must recognize the antigens introduced by the vaccine and mount an immune response toward them. Although the body's innate immune response can mount an initial response to the antigens, the goal of a vaccine is to develop a longer-lasting response from the body's adaptive immune system.

The initial stages of this process occur when the cells of the body's immune system recognize epitopes or moieties associated with the vaccine antigen. Antigen-presenting cells (APCs) such as dendritic cells will recognize and phagocytose the vaccine particles and process them internally. As a result of this process, fragments of the digested antigen are displayed on the APC's surface in association with the major histocompatibility (MHC) II protein (Fig. 1). The APC can then travel to the body's lymph nodes to help develop a stronger and longer-lasting humoral immune response against the vaccine antigen. Alternative B cells can recognize antigens that have not been processed by an APC and mount an immune response. These antigens, also known as T cell-independent antigens, include molecules such as bacterial capsular polysaccharides. When this type of immune response is generated, it is typically immunoglobulin (Ig)M-biased and demonstrates a weaker and shorter-lasting immune response compared to that obtained through the involvement of T cells.

Once the APC has reached the lymph nodes, it is capable of participating in T cell-dependent maturation of B cells. Both of these cells travel to lymph nodes to play a role in the adaptive immune response. During the initial stage of the process, receptors on the surface of CD4+ Helper T cells interact with the MHCII receptor of the APC and the processed vaccine antigen that is associated with it. This interaction activates the CD4+ T cell and can enable it to stimulate reproduction and activation of B cells. When the activated T cell interacts with a B cell displaying the appropriate antigen, it releases cytokines that promote proliferation of the B cell population and their subsequent maturation into antibody (typically IgG)-secreting plasma cells. In addition, this interaction also promotes a process known as somatic hypermutation in which a great degree of mutation of the variable region of the B cell antibody is achieved. As a consequence of this process, antibody variants processing greater degrees of affinity for the antigen can be generated to develop a highly potent and specific immune response. Once these immune cells have been generated, they can either disseminate to become effector cells or remain in lymph nodes as memory cells to enable the body to mount a rapid response if a similar infection occurs.

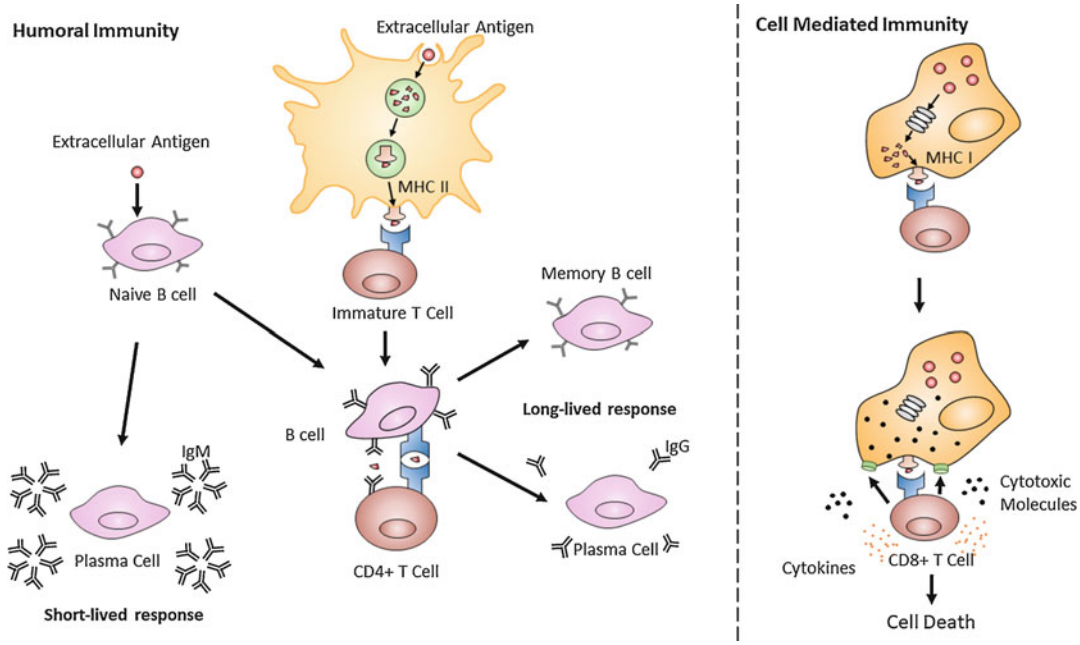

Fig. 1 Overview of humoral and cellular immunity. In the humoral immune response (shown on left), an APC such as a dendritic cell phagocytoses a pathogen or vaccine antigen and processes it internally. After processing, an antigen epitope is displayed on the cell's surface attached to the MHC II receptor. When the APC is a B cell, it can become a plasma cell and secrete IgM antibodies. When a CD4+ T cell interacts with the APC, it becomes activated and promotes proliferation and somatic hypermutation within B cells. These B cells can then either be stored as memory cells or disseminate into the blood as plasma cells secreting IgG antibodies. In the cellular immune response (shown on the right), a virus-infected or tumor cell containing a nucleus processes an intracellular protein and presents an epitope on its surface attached to the MHC I receptor. A CD8+ T cell can then bind to the MHC I receptor and become activated. Upon activation, the CD8+ T cell secretes various cytokines as well as cytotoxic chemicals that form pores within the bound cell and promote apoptosis

To describe an example of this process, we will highlight recent work to formulate a vaccine against the bacterial pathogen *Streptococcus pneumoniae*. This vaccine was comprised of 20–24 pneumococcal capsular polysaccharides encapsulated within a liposome [13, 14]. The liposome was also surface-decorated with pneumococcal virulence factors using either a His-tag or streptavidin-biotin system [13]. Through this approach, the vaccine was able to generate a CD4+ T cell-dependent immune response resulting in significant IgG production against both the capsular polysaccharide and protein antigens [13, 14]. Moreover, this work also demonstrated that encapsulation of the polysaccharides, normally a T cell-independent antigen, within a protein decorated liposome was able to generate a T cell-dependent response. This was driven by the observation of IgM to IgG class switching that is a hallmark of the T cell-dependent response. Interestingly, this provided evidence that physical colocalization of the polysaccharides with a T cell-

dependent antigen, the surface protein, was able to provide an immune response similar to that observed for direct chemical conjugation of polysaccharides to carrier proteins (i.e., glycoconjugate vaccines). Furthermore, the researchers demonstrated that the resulting immune response provides protection against pneumococcal disease using various animal models and an in vitro correlate assay [13, 14]. This work highlights how a vaccine was designed to invoke a specific immune response to provide protection against pneumococcal infections.

As an alternative to the aforementioned immune response, the immune system can direct a response towards infected bodily cells. This occurs through the action of CD8+ T cells that recognize peptides (processed antigen fragments produced intracellularly) attached to the surface of cells via the MHC I receptor (Fig. 1). Unlike MHC II, which is present on APCs, MHC I is expressed on the surface of nucleated bodily cells. If a cell becomes infected with a virus or becomes a tumor, intracellular proteins can be processed for presentation on the cellular surface via attachment to MHC I and then be recognized by CD8+ T cells. When the T cells recognize and bind to the epitope-bound MHC I receptor, the T cell becomes activated and can initiate a cellular immune response. In this response, the T cell secretes cytokines, such as tumor necrosis factor alpha (TNFα) or interferon gamma (IFN-γ), that demonstrate antitumor and antiviral effects. The T cells also secrete cytotoxic chemicals that serve to create pores within the membrane of the bound cells through which proteases can enter. Once the proteases have entered the targeted cell, they are able to degrade any viral proteins and promote cellular apoptosis. Once this has been achieved, the CD8+ T cell is able to continue its response as needed against additional infected cells.

3 Development of Assays to Evaluate Vaccine Efficacy

In order to determine whether a vaccine has the potential to provide protective immunity within a patient, various assays have been developed to evaluate the immune response and determine whether it is sufficiently protective. One significant challenge facing various vaccines is the fact that the incidence rate of disease is frequently very low. As shown in Table 1, the incidence and fatality rates of bacterial pathogens frequently occurs on the rate of a few cases per hundred thousand people. Consequently, performance of clinical trials to demonstrate efficacy of vaccines against these organisms represents a substantial challenge since a massive number of patients would be necessary. For example, if a clinical trial is seeking to demonstrate the efficacy of the vaccine through the reduction of disease incidence or associated death, a sufficient number of patients would be required to capture differences of

groups when cases of disease occurs around 1 in 10,000 people. Furthermore, multiple trial arms would be required to cover a placebo control as well as other potential groups that would be necessary depending on the vaccine being evaluated. This is further complicated for vaccines in which a current standard of care exists and cannot be ethically denied to patients. Consequently, the tested vaccine needs a mechanism for demonstrating both superiority to the current vaccine in certain tests while demonstrating non-inferiority in areas of overlap. This situation is made more complex by bacteria for which there are multiple strains or serotypes (e.g., *Streptococcus pneumoniae*) in which vaccines provide protection against only a subset of bacterial diversity. If reduction of disease were the only mechanism for evaluating efficacy, then not only would new vaccines need to include sufficient patients to statistically evaluate reduction of disease, this number would need follow-up assays to demonstrate the vaccine's efficacy in a serotype-dependent manner.

Thus, numerous assays have been developed to link in vitro assay results to clinical efficacy of vaccines. The quantitative power of these assays, called "correlates of protection" (COPs), have been demonstrated in a clinical setting to predict clinical utility of a vaccine through in vitro testing. As a result, a vaccine's efficacy can be potentially determined using a number of patients that is far lower than required using reduction in disease incidence.

One such COP for protein antigens against bacterial pathogens is the development of assays that measure the production of antibodies against the bacteria. These may include enzyme-linked immunosorbent assays (ELISAs) or neutralization assays such as opsonophagocytic activity (OPA) assays in which antibody titers and their ability to promote opsonization of bacteria is determined. For example, through clinical trials, such assays have been developed for pneumococcal disease that include ELISAs which can show IgG concentrations >0.35 μg/mL and OPA assays showing $\geq 50\%$ bacteriocidal activity when dilution is less than 1 in 8 [15]. Since each of these values have been associated with the efficacy of pneumococcal glycoconjugate vaccines, subsequent vaccines against the bacteria are able to demonstrate efficacy by achieving each of these titers for both previous and new serotypes included in vaccines. Similar assays have also been developed against various other bacterial pathogens to measure vaccine efficacy without requiring additional massive clinical trials.

References

1. Centers for Disease Control and Prevention (CDC) (1999) Impact of vaccines universally recommended for children--United States, 1990-1998. MMWR Morb Mortal Wkly Rep 48(12):243
2. Whitney CG, Zhou F, Singleton J, Schuchat A (2014) Benefits from immunization during the vaccines for children program era-United States, 1994-2013. MMWR Morb Mortal Wkly Rep 63(16):352–355
3. Szucs T (2000) Cost-benefits of vaccination programmes. Vaccine 18(Suppl 1):S49–S51. https://doi.org/10.1016/S0264-410X(99)00464-8
4. Scorpio A, Blank TE, Day WA, Chabot DJ (2006) Anthrax vaccines: pasteur to the present. Cell Mol 63(19-20):2237–2248. https://doi.org/10.1007/s00018-006-6312-3
5. Smith KA (2012) Louis Pasteur, the father of immunology? Front Immunol 3:68. https://doi.org/10.3389/fimmu.2012.00068
6. Turner GS, Squires EJ, Murray HGS (1970) Inactivated smallpox vaccine. A comparison of inactivation methods. J Hyg 68(2):197–210. https://doi.org/10.1017/s0022172400028679
7. Moyle PM, Toth I (2013) Modern subunit vaccines: development, components, and research opportunities. ChemMedChem 8(3):360–376. https://doi.org/10.1002/cmdc.201200487
8. Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, Kielland A, Vosters O, Vanderheyde N, Schiavetti F, Larocque D, Van Mechelen M, Garcon N (2009) AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol 183(10):6186–6197. https://doi.org/10.4049/jimmunol.0901474
9. Heijmans S, De Meulemeester M, Reynders P, Giet D, Demanet E, Devresse P-Y, Icardi G, Dramé M, Roman F, Gillard P (2011) Immunogenicity profile of a 3.75-μg hemagglutinin pandemic rH5N1 split virion AS03(A)-adjuvanted vaccine in elderly persons: a randomized trial. J Infect Dis 203(8):1054–1062. https://doi.org/10.1093/infdis/jiq174
10. Weintraub A (2003) Immunology of bacterial polysaccharide antigens. Carbohydr Res 338(23):2539–2547. https://doi.org/10.1016/j.carres.2003.07.008
11. CDC (2000) Active bacterial core surveillance report, emerging infections program network, *Streptococcus pneumoniae*, 1999. Centers for Disease Control and Prevention, Paris
12. CDC (2015) Active bacterial core surveillance report, emerging infections program network, *Streptococcus pneumoniae*, 2015. Centers for Disease Control and Prevention, Paris
13. Hill AB, Beitelshees M, Nayerhoda R, Pfeifer BA, Jones CH (2018) Engineering a next-generation glycoconjugate-like *streptococcus pneumoniae* vaccine. ACS Infect Dis 4:1553. https://doi.org/10.1021/acsinfecdis.8b00100
14. Jones CH, Zhang G, Nayerhoda R, Beitelshees M, Hill A, Rostami P, Li Y, Davidson BA, Knight P, Pfeifer BA (2017) Comprehensive vaccine design for commensal disease progression. Sci Adv 3(10):e1701797. https://doi.org/10.1126/sciadv.1701797
15. Andrews NJ, Waight PA, Burbidge P, Pearce E, Roalfe L, Zancolli M, Slack M, Ladhani SN, Miller E, Goldblatt D (2014) Serotype-specific effectiveness and correlates of protection for the 13-valent pneumococcal conjugate vaccine: a postlicensure indirect cohort study. Lancet Infect Dis 14(9):839–846. https://doi.org/10.1016/S1473-3099(14)70822-9
16. Roush SW, Murphy TV (2007) Historical comparisons of morbidity and mortality for vaccine-preventable diseases in the United States. JAMA 298(18):2155–2163. https://doi.org/10.1001/jama.298.18.2155

Chapter 2

Isolating Pathogen-Specific Human Monoclonal Antibodies (hmAbs) Using Bacterial Whole Cells as Molecular Probes

Sara Siris, Camilla A. Gladstone, Yanping Guo, Christopher L. Pinder, Robin J. Shattock, Paul F. McKay, Paul R. Langford, and Fadil A. Bidmos

Abstract

The immunoglobulin capture assay (ICA) enables the enrichment for pathogen-specific plasmablasts from individuals with a confirmed adaptive immune response to vaccination or disseminated infection. Only single recombinant antigens have been used previously as probes in this ICA and it was unclear whether the method was applicable to complex probes such as whole bacterial cells. Here, we describe the enrichment of plasmablasts specific for polysaccharide and protein antigens of both *Streptococcus pneumoniae* and *Neisseria meningitidis* using whole formalin-fixed bacterial cells as probes. The modified ICA protocol described here allowed for a pathogen-specific hmAb cloning efficiency of >80%.

Key words Immunoglobulin capture assay, Pathogen-specific plasmablasts, Bacteria, Whole cells, Vaccine antigen discovery

1 Introduction

Methods for analysis of the human adaptive immune response to vaccination or disseminated infection include the cloning and in vitro expression of human monoclonal antibodies (hmAbs) from antibody-producing cells (APCs: memory B-cells, plasmablasts and plasma cells). Targeting plasmablasts, especially, for hmAb cloning is useful as rapid expansion of a plasmablast population occurs immediately following antigen encounter—this expansion is characterized by differentiation leading to increased specificity for the presenting antigen [1]. Expression cloning of hmAbs from plasmablasts has been achieved by sorting of individual cells into multiwell plates followed by a sequential PCR that generates amplicons of the variable regions of heavy (VH) and light

Sara Siris and Camilla Gladstone contributed equally to this work.
Paul Langford and Fadil Bidmos contributed equally to this work.

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(VL = κ or λ) chains of individual hmAbs. These VH and VL amplicons are subsequently incorporated into expression plasmids using molecular cloning strategies (restriction endonuclease or ligation-independent) [2]. In vitro production of individual recombinant hmAbs occurs in human embryonic kidney cells (HEK-293) transfected with cognate VH and VL plasmid pairs; hmAbs secreted into culture supernatants are subsequently screened for pathogen/antigen specificity and functional activity.

Low hmAb cloning efficiencies are obtained in studies where rare antigens are sought, blood sample volume is low or when the magnitude/timing of the plasmablast component of the immune response to the pathogen/antigen of interest has not been sufficiently quantified. To counter low hmAb cloning efficiencies, enrichment for plasmablasts of interest is performed prior to fluorescence-activated cell sorting (FACS). In vivo enrichment of human plasmablasts has been achieved by transplantation of irradiated SCID/beige mice with human peripheral blood mononuclear cells (PBMCs) premixed with antigens of interest [3]. In vitro enrichment has also been achieved by separation of individual cells into droplets, analyses of the secretome of each cell within the droplet, and immediate sorting of cells producing the desired antibodies [4]. However, the use of these enrichment methods has not been widely reported in the literature for a variety of reasons including the complexity of the techniques and unavailability of specialist equipment in standard research laboratories.

Recently, a simplified enrichment protocol, known as the immunoglobulin capture assay (ICA), was described for the in vitro identification of plasmablasts of interest. In the ICA, a streptavidin anti-CD45 and biotin anti-human IgG scaffold is assembled on the surface of plasmablasts to prevent diffusion of secreted IgG away from the secreting plasmablast. Interactions between these "captured" IgG molecules and antigens of interest are subsequently analyzed during FACS. Positive interaction events, that is, plasmablasts whose IgG have bound to the antigen of interest, are sorted, while "nonbinders" (non-IgG and nonspecific IgG) are excluded [5]. Production of hmAbs from individual plasmablasts is subsequently performed as described above. Cloning into expression vectors and expression of hmAbs have been comprehensively described in published protocols [2, 6].

Only single recombinant vaccine antigens have been utilized in this assay previously. Here, we demonstrate that a complex probe such as whole bacterial cells can be efficiently utilized for the enrichment of pathogen-specific plasmablasts (Fig. 1). Using formalin-fixed cells representing four capsular variants (6A, 7F, 14, and 19F) of the Gram-positive pneumococcus, we were able to achieve a hmAb cloning efficiency of ~82%. Our panel of cloned hmAbs targeted either the 6A, 7F, or 14 capsules—no cross-reactivity between structurally dissimilar capsules was discerned. The modified ICA protocol was also readily applicable to

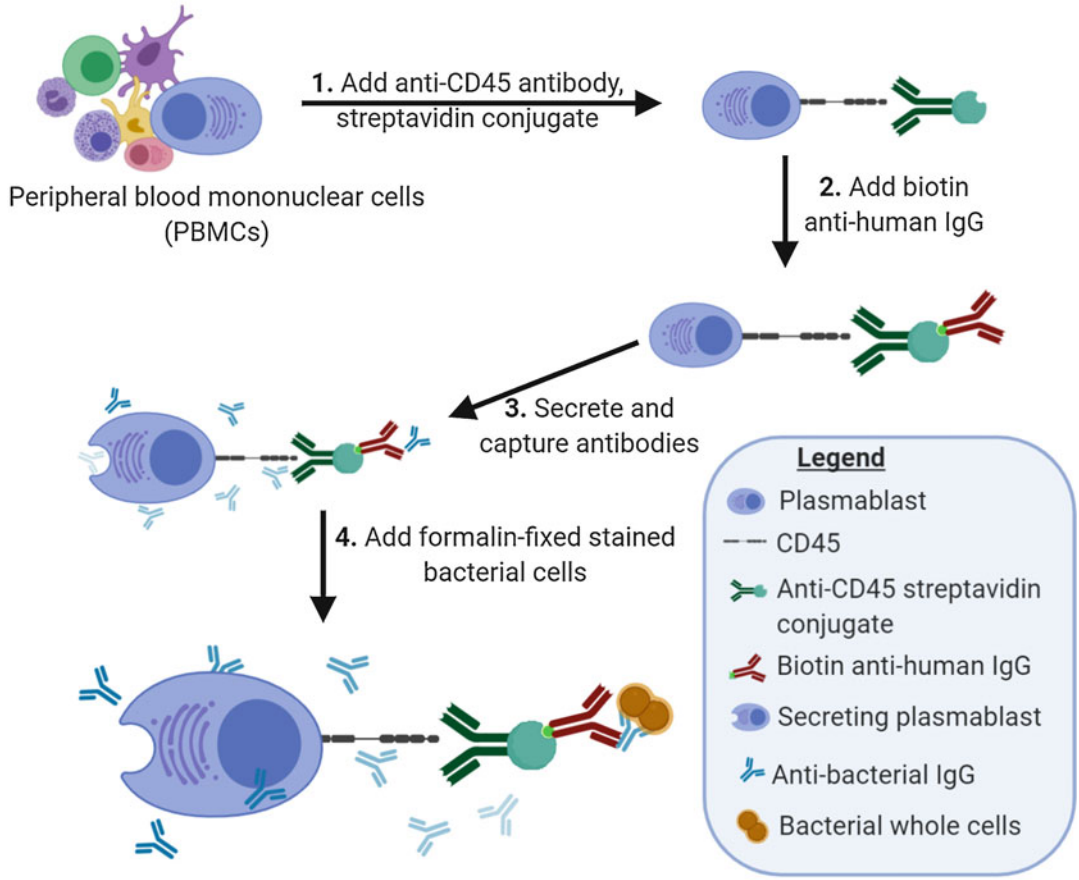

Fig. 1 Schematic illustration of the experimental procedure. Quasi-presentation of secreted IgG is achieved by exploitation of the surface expression of CD45 on plasmablasts. An anti-CD45 streptavidin conjugate antibody is then used as a link between the CD45-expressing plasmablast and the anti-human IgG (conjugated to biotin) that will capture IgG secreted into the immediate milieu of the plasmablast. This captured IgG can then bind to a cognate antigen, if present, on the bacterial surface

Gram-negative bacteria yielding similar results following cloning of capsular and protein vaccine-induced antimeningococcal hmAbs from ICA-enriched plasmablasts.

2 Materials

Refer to storage instructions for all reagents. Ensure safety guidelines and proper waste disposal procedures are followed.

2.1 Bacterial Growth, Fixing, and Staining

1. Frozen stocks of pneumococcal and meningococcal strains.
2. Brain-Heart Infusion (BHI) broth supplemented with 0.5% yeast extract (BHI-YE): Suspend 14.8 g of BHI powder and 2 g of yeast extract in 383.2 mL of deionized water. Sterilize at

121 °C for 15 min. Allow to cool to room temperature (RT) before use. Prepare fresh each time.

3. Blood agar plates.
4. Cell culture flask with vented caps.
5. 10 μL sterile disposable inoculation loops.
6. Research CO_2 incubator.
7. Benchtop centrifuge.
8. Dulbecco's phosphate buffered saline (DPBS).
9. Bacterial fixing solution: 0.5% formaldehyde solution in DPBS.
10. 0.1 M sodium hydrogen carbonate (0.1 M $NaHCO_3$): Dissolve 3.36 g of $NaHCO_3$ crystals in 396.64 mL of deionized water. Sterilize at 121 °C for 15 min. Allow to cool to room temperature (RT) before use.
11. Spectrophotometer.
12. Cell staining solution: Dissolve 100 mg of 5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester (FAM SE) in 10 mL of dimethyl sulfoxide (DMSO) to give a final concentration of 10 mg/mL. Protect from light by wrapping in aluminum foil.

2.2 ICA

1. Frozen stocks of PBMCs.
2. 5 mL round bottom polystyrene tubes.
3. Water bath.
4. Cell counter, slides, and trypan blue dye.
5. Single-color compensation beads.
6. RPMI-1640 medium.
7. R10 medium: RPMI-1640, 10% fetal bovine serum.
8. ICA buffer (DPBS, 1% bovine serum albumin, 50 U/mL benzonase nuclease): Dissolve 5 g of bovine serum albumin (BSA) flakes in 495 mL DPBS. Add 100 μL benzonase nuclease to give 50 U/mL final concentration (*see* **Note 1**). Prepare on day of experiment.
9. Zombie NIR™ Fixable Viability Kit: Follow manufacturer instructions for preparation of the dye.
10. Anti-human CD45 streptavidin conjugate: Prepare using a conjugation kit. Follow the manufacturer's instructions (*see* **Note 2**).
11. Biotin-SP-AffiniPure F(ab′)2 fragment goat anti-human IgG, Fcγ fragment specific (biotin anti-human IgG).
12. Fluorescent antibodies:
    (a) APC anti-human CD3 antibody (APC).

(b) APC anti-human CD14 antibody (APC).

(c) Brilliant Violet 421™ (BV421) anti-human CD19 antibody.

(d) Brilliant Violet 605™ (BV605) anti-human CD20 antibody.

(e) PE anti-human CD27 antibody (PE).

(f) PerCP/Cyanine5.5 anti-human CD38 antibody (PerCP/Cy5.5).

(g) Alexa Fluor® 594 AffiniPure goat anti-human serum IgA, α chain specific (AF594).

(h) Brilliant Violet 650™ anti-human IgM antibody (BV650).

(i) Brilliant Violet 785™ anti-human IgD antibody (BV785).

13. Catch buffer: Nuclease- and protease-free water, 10 mM Tris–HCL 8.0, 1 U RNAsin.

3 Methods

3.1 Pneumococcal Growth, Fixing, and Staining

1. Streak a loopful of pneumococcal frozen stocks on blood agar. Incubate for 14–16 h at 37 °C, 5% CO_2.
2. From the blood agar plates, suspend 10–20 colonies of pneumococcal growth in 10 mL of BHI-YE culture medium in a 50 mL centrifuge tube.
3. Transfer the pneumococcal suspension to a cell culture flask with vented cap (*see* **Note 3**).
4. Incubate the flask overnight for 14–16 h at 37 °C, 5% CO_2 without shaking (*see* **Note 4**).
5. Pellet pneumococci from the overnight culture at 3000 × *g* for 5 min, RT.
6. Resuspend pneumococcal cells in 10 mL bacterial fixing solution. Incubate for ≥4 h at RT.
7. Wash pneumococcal cells thrice in 10 mL 0.1 M $NaHCO_3$. Resuspend cells to OD_{600} of 0.2 (10^8 cells/mL).
8. Resuspend each aliquot of 10^8 cells in 495 μL of 0.1 M $NaHCO_3$. Add 5 μL of cell staining solution. Incubate for 1 h at 37 °C.
9. Wash stained pneumococcal cells five times in 500 μL of 0.1 M $NaHCO_3$.
10. Resuspend stained pneumococcal cells in ICA buffer. Stained cells can be stored overnight at 4 °C, protected from light.

3.2 Meningococcal Growth, Fixing, and Staining

1. Streak a loopful of meningococcal frozen stocks on blood agar. Incubate for 14–16 h at 37 °C, 5% CO_2.
2. Harvest cells from the overnight growth into 10 mL bacterial fixing solution using a sterile 10 μL disposable loop.
3. Incubate for ≥4 h at RT.
4. Wash meningococcal cells thrice in 10 mL 0.1 M $NaHCO_3$. Resuspend cells to OD_{600} of 0.1 (~10^7 cells/mL).
5. Resuspend each aliquot of 10^7 cells in 495 μL of 0.1 M $NaHCO_3$. Add 5 μL of cell staining solution. Incubate for 1 h at 37 °C.
6. Wash stained meningococcal cells at 800 × *g* for 10 min in 500 μL of 0.1 M $NaHCO_3$.
7. Repeat **step 6** above four more times.
8. Resuspend stained meningococcal cells in ICA buffer. Stained cells can be stored overnight at 4 °C, protected from light.

3.3 ICA

3.3.1 PBMC Thawing

1. Warm the RPMI-1640 and R10 to RT. Transfer 9 mL of RPMI-1640 per five million live PBMCs into a 50 mL centrifuge tube.
2. Transfer PBMC vials from −80 °C to a 37 °C water bath. Check the vials periodically and remove following complete thawing.
3. Immediately after complete thawing, add the PBMC sample dropwise into the RPMI-1640 medium.
4. Pellet the PBMCs at 500 × *g*, RT for 8 min. Discard the supernatant.
5. Resuspend the PBMCs in 10 mL of ICA buffer.
6. Add 10 μL of the sample to 10 μL of trypan blue dye. Count the number of cells in this mixture using an automated cell counter.
7. Pellet the PBMCs (from **step 5** above) at 500 × *g*, RT for 8 min. Discard the supernatant.
8. Resuspend the PBMCs at a concentration of 3–4 million live PBMCs per 100 μL in ICA buffer. Store on ice.

3.3.2 IgG Secretion and Capture

1. Label 2 mL microcentrifuge tubes as follows:
   (a) "C" for unstained PBMCs.
   (b) "CS" for stained PBMCs (no ICA).
   (c) "CF-APC" for fluorescence minus APC control.
   (d) "CF-BV421" for fluorescence minus BV421 control.
   (e) "CF-BV605" for fluorescence minus BV605 control.
   (f) "CF-PE" for fluorescence minus PE control.

(g) "CF-PerCP/Cy5.5" for fluorescence minus PerCP/Cy5.5 control.

(h) "CF-AF594" for fluorescence minus AF594 control.

(i) "CF-BV785" for fluorescence minus BV785 control.

(j) "CF-BV650" for fluorescence minus BV650 control.

(k) "CF-Zombie" for fluorescence minus Zombie NIR control.

(l) "CS-ICA" for stained PBMCs (ICA).

2. Transfer 100 μL of PBMCs to each tube. Store tubes (**a**)–(**k**) above on ice (*see* **Note 5**).
3. Add 1 μL of Zombie NIR live/dead dye to PBMCs in tube "CS-ICA". Incubate at RT for 20 min, in dark.
4. Wash PBMCs in tube "CS-ICA" with 2 mL ICA buffer.
5. Pellet PBMCs in tube "CS-ICA" at 500 × *g*, RT for 8 min. Discard the supernatant.
6. Resuspend PBMCs in tube "CS-ICA" in 100 μL ICA buffer.
7. Add 1 μg of the anti-human CD45 Streptavidin conjugate to PBMCs in tube "CS-ICA." Mix contents of the tube by gentle tapping. Incubate on ice for 20 min.
8. Repeat **steps 4–6**.
9. Add 1 μL of the Biotin anti-human IgG antibody PBMCs in tube "CS-ICA." Mix contents of the tube by gentle tapping. Incubate on ice for 20 min.
10. Repeat **steps 4** and **5**.
11. Resuspend PBMCs in tube "CS-ICA" in 1 mL of R10. Transfer contents of this tube to a fresh 2 mL microcentrifuge tube. Label the new tube as "CS-ICA."
12. For secretion and capture of IgG, cap the tube tightly and incubate at 37 °C for 1 h on a tube rotator.

During the 1 h secretion/capture step, prepare the single stain and fluorescence minus one controls:

1. Vortex the single-color compensation beads. Transfer 1 drop each (~50 μL) into separate labeled tubes, one tube for each fluorescent antibody.
2. Add each antibody to its corresponding tube, as follows:
   (a) Anti-CD3 APC: 2 μL.
   (b) Anti-CD14 APC: 1 μL.
   (c) Anti-CD19 BV 421: 2 μL.
   (d) Anti-CD20 BV 605: 3 μL.
   (e) Anti-CD27 PE: 1 μL.

(f) Anti-CD38 PerCP/Cy5.5: 2 μL.
(g) Anti-human IgA Alexa Fluor 594: 5 μL.
(h) Anti-human IgD BV 786: 5 μL.
(i) Anti-human IgM BV 650: 5 μL.
(j) Zombie NIR Live/Dead: 1 μL.

3. Incubate the single-stain control tubes on ice for 20 min.
4. For the fluorescence minus one controls, add all antibodies except the APC antibodies to tube "CF-APC" (*see* **step 1** of Subheading 3.3.2). Repeat this procedure for other tubes excluding the relevant antibody for each control. For gating purposes, add all antibodies to tube "CS." Incubate all control tubes on ice for 20 min.
5. Wash all bead and PBMC control tubes with 2 mL ICA buffer.
6. Pellet stained beads and PBMCs at 500 × *g*, RT for 8 min. Discard the supernatant.
7. Resuspend stained beads and PBMCs in 750 μL of ICA buffer. Transfer these into fresh, labeled 5 mL polystyrene tubes. Store on ice, in dark, until flow cytometry processing.

After IgG secretion and capture, transfer the sample into a fresh, labeled ("CS-ICA") tube and proceed with the following steps:

1. Pellet PBMCs in tube "CS-ICA" at 500 × *g*, RT for 8 min. Discard the supernatant.
2. Resuspend PBMCs in tube "CS-ICA" in 100 μL ICA buffer.
3. Add FAM SE-stained bacteria (at a ratio of 16 bacterial cells to 1 PBMC—*see* **Note 6**) to CS-ICA. Incubate at RT, in the dark, for 20 min.
4. Wash PBMC–bacteria complexes in tube "CS-ICA" with 2 mL ICA buffer.
5. Pellet PBMC–bacteria complexes at 500 × *g*, RT for 8 min. Discard the supernatant.
6. Resuspend PBMC–bacteria complexes in 74 μL ICA buffer.
7. Prepare a cocktail of all fluorescent antibodies as follows:
   (a) Anti-CD3 APC: 2 μL.
   (b) Anti-CD14 APC: 1 μL.
   (c) Anti-CD19 BV 421: 2 μL.
   (d) Anti-CD20 BV 605: 3 μL.
   (e) Anti-CD27 PE: 1 μL.
   (f) Anti-CD38 PerCP/Cy5.5: 2 μL.
   (g) Anti-human IgA Alexa Fluor 594: 5 μL.
   (h) Anti-human IgD BV 786: 5 μL.

(i) Anti-human IgM BV 650: 5 μL.

(j) Add the cocktail of antibodies to tube CS-ICA (*see* **Note 7**).

8. Incubate the "CS-ICA" tube on ice for 20 min, in the dark.
9. Wash stained PBMC–bacteria complexes with 2 mL ICA buffer.
10. Pellet stained PBMC–bacteria complexes at 500 × *g*, RT for 8 min. Discard the supernatant.
11. Resuspend PBMC–bacteria complexes in 750 μL of ICA buffer. Transfer these into a fresh, labeled 5 mL polystyrene tube.
12. Pass PBMC controls and experiment samples through strainers into fresh, labeled polystyrene tubes to avoid clogging the sorter.
13. Gate for live, pathogen-specific plasmablasts as follows:
    (a) CD3 negative, CD14 negative.
    (b) CD19 positive, CD 20 low/negative.
    (c) CD27 high, CD38 high.
    (d) IgD negative, IgA negative.
    (e) IgM negative, FAM SE positive.
14. Sort pathogen-specific plasmablasts singly into rows A–G of a 96-well plate containing 10 μL of catch buffer. Row H will serve as negative control in downstream PCRs.
15. Refer to previously published protocols for VH/VL amplification and cloning from individual plasmablasts and hmAb expression [2, 6].

4 Notes

1. It is expected that some cell lysis will occur during PBMC thawing and/or during the assay. Released nucleic acid from lysed cells leads to cell clumping, which significantly reduces recovery of single cells available for FACS. We found that including Benzonase Nuclease (Fisher Scientific, #10725899) in the assay buffer prevented cell clumping, ensured easy resuspension of cells following the wash steps and allowed for full PBMC recovery at the end of the assay.
2. We used the Lightning-Link® streptavidin antibody labeling kit (Expedeon, #708-0030) and an anti-human CD45 antibody (BioLegend, #304002).

3. Growth of pneumococcal strains is variable. Depending on how many cells are required in downstream applications, multiple flasks per strain may be required.
4. We find that pneumococcal growth is better with the larger surface area provided by flasks. Vented caps allow for better aeration of culture. Satisfactory growth of cultures was obtained without shaking of flasks.
5. It is important that antibodies are titrated for number of PBMCs stained, especially if there are differences in PBMC numbers between control and experiment samples. Significant gating issues may arise including inability to visualize certain populations if the right antibody amounts are not used.
6. We tested four different bacteria-to-PBMC ratios—1:1, 4:1, 16:1, and 64:1. No significant difference was observed between the 16:1 and 64:1 ratios; both, however, yielded isolation of significantly higher pathogen-specific plasmablasts than the 1:1 and 4:1 ratios, as determined by FACS.
7. When analyzing more than one sample, a mastermix of the antibody cocktail can be prepared.

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References

1. Fink K (2012) Origin and function of circulating plasmablasts during acute viral infections. Front Immunol 3:78
2. Smith K, Garman L, Wrammert J et al (2009) Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. Nat Protoc 4(3):372
3. Lin Z, Chiang NY, Chai N et al (2014) In vivo antigen-driven plasmablast enrichment in combination with antigen-specific cell sorting to facilitate the isolation of rare monoclonal antibodies from human B cells. Nat Protoc 9 (7):1563
4. Mazutis L, Gilbert J, Ung WL et al (2013) Single-cell analysis and sorting using droplet-based microfluidics. Nat Protoc 8(5):870
5. Pinder CL, Kratochvil S, Cizmeci D et al (2017) Isolation and characterization of antigen-specific plasmablasts using a novel flow cytometry–based Ig capture assay. J Immunol 199 (12):4180–4188
6. Ho IY, Bunker JJ, Erickson SA et al (2016) Refined protocol for generating monoclonal antibodies from single human and murine B cells. J Immunol Methods 438:67–70

Chapter 3

Use of Chlamydial Elementary Bodies as Probes to Isolate Pathogen-Specific Human Monoclonal Antibodies

Christopher L. Pinder, Paul F. McKay, and Robin J. Shattock

Abstract

Chlamydia trachomatis is one of the most prevalent sexually transmitted infectious agents in the world and the leading cause of infectious blindness. The role of antibodies in the prevention and clearance of infection is still not fully understood, but the analysis of the immunoglobulin response to novel vaccine candidates is an important part of many of these studies. In this chapter, we describe a novel method to identify and isolate *Chlamydia*-specific memory B cells by fluorescence-activated cell sorting (FACS) using fluorescently labeled whole bacteria from cryopreserved human PBMC samples. This method allows for live single cells to be sorted for cell culture, in vitro assays, single-cell RNA sequencing, and cloning of paired heavy and light chains for recombinant monoclonal antibody production.

Key words Antigen-specific, B cell, Antibody, Chlamydia, Elementary body, Immunoglobulin, IgG

1 Introduction

Chlamydia trachomatis is a Gram-negative intracellular bacterial pathogen, responsible for over 100 million sexually transmitted infections a year [1] and nearly 40 million active cases of trachoma, an ocular chlamydial infection and the leading cause of infectious blindness [2]. While protective immunity can be developed in the host by infection, damage caused by the inflammatory response to the bacteria can cause scarring in infected tissues, resulting in infertility or blindness [3].

There has therefore been substantial effort to develop a vaccine to *C. trachomatis* that would prevent infection and clear the bacteria before damage can occur. Initial work focused on live or attenuated whole-organism vaccination, and while capable of inducing a protective immune response, this protection was often short-lived and highly serovar-specific [4, 5]. More recent vaccine designs have used purified or engineered protein antigens, primarily the immunodominant major outer membrane protein (MOMP), which

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accounts for over 60% of the surface of the elementary body (EB), the extracellular, infectious form of *Chlamydia* [6–9].

The immune response to *Chlamydia* has been equally well studied, mainly using the related *C. muridarum* in mouse models. It has been shown that primary immunity to *Chlamydia* is reliant on CD4 T lymphocytes [10, 11], data which is supported by the observation that CD4-deficient HIV patients are more likely to develop *Chlamydia* infection and be affected by long-term sequelae of infection [12]. The role of antibodies and B lymphocytes in immunity to *Chlamydia* is less well defined, although it has been shown that the presence of specific antibodies at the site of exposure can prevent infection to the same degree as CD4 T cells [13, 14].

For these reasons, analyzing the antibody response to *Chlamydia*, either from infection with the bacteria or immunization with a novel vaccine candidate, is of crucial importance. Immunoglobulin repertoire analysis has been significantly advanced in the last two decades with new developments in single-cell sorting and sequencing techniques. It is now possible to analyze the antibody response on a monoclonal basis, identifying individual antibodies against a specific immunogen that can be useful for research, diagnostics, or design of new and more immunogenic vaccines (Fig. 1). Fluorescent antigen probes have been of particular use in pre-screening and enriching for antigen-specific B cells during the cell sorting process [15–20].

This chapter will outline the use of whole fluorescently labeled elementary bodies to isolate human memory B cells specific to *C. trachomatis* serovars, derived from exposure to both natural infection with trachoma and to a novel MOMP-based vaccine antigen.

2 Materials

2.1 PBMC Isolation and Cryopreservation

1. Phosphate-buffered saline (PBS).
2. Histopaque-1077 [Sigma-Aldrich].
3. PBS containing 1% bovine serum albumin (PBS/BSA).
4. Pasteur pipettes.
5. Freezing medium: 90% fetal bovine serum (FBS), 10% dimethyl sulfoxide (DMSO).
6. Cell freezing chamber.

2.2 EB Staining

1. SPG buffer: 200 mM sucrose, 20 mM sodium phosphate, 5 mM glutamic acid.
2. Fixation buffer: 3.7% formaldehyde in sterile purified water.
3. Vybrant™ DiO Cell-Labeling solution [Thermo Fisher Scientific].

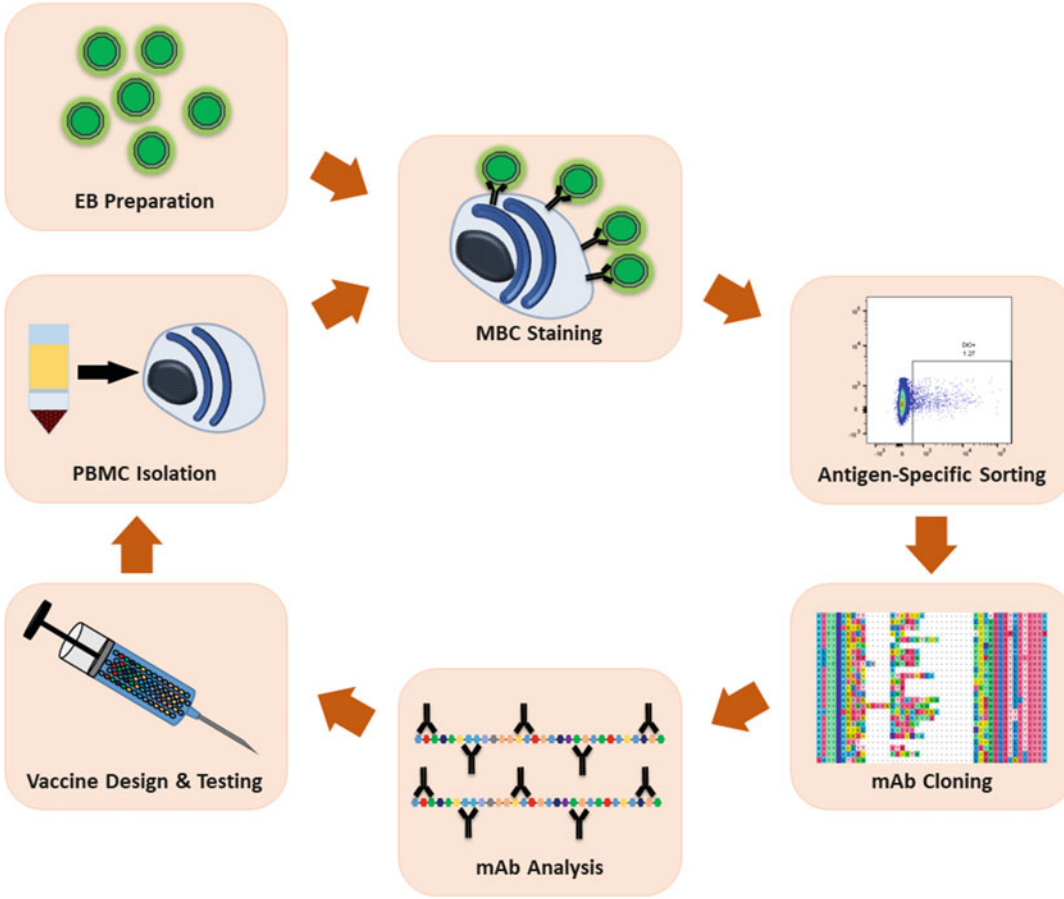

Fig. 1 Schematic showing process of antibody isolation and vaccine development. Following vaccination with a novel immunogen, PBMCs are isolated from blood taken from the vaccine recipients and are stained using fluorescent elementary bodies (EBs) that are prepared concurrently with the PBMCs. This staining allows for antigen-specific B cells to be sorted and the immunoglobulin genes cloned and produced recombinantly, where they can be analyzed for affinity, specificity, and neutralization potential. This data can then be used to design a new immunogen better able to push the humoral immune response toward epitopes that were shown to be beneficial

4. 1 mL syringe with 27 G needle.
5. *C. trachomatis* elementary bodies, stored at −80 °C in SPG buffer.

2.3 Cell Sorting

1. RPMI medium (R0).
2. Amine-reactive viability dye [e.g., Zombie Fixable Viability Dye from BioLegend].
3. Fluorescent antibodies against CD3, CD14, CD19, CD27, CD38, IgG, IgM, IgD, and IgA.
4. 5 mL Polystyrene tubes for FACS.

5. 35 μm nylon mesh.
6. Compensation beads for antibodies [e.g., CompBeads from BD Biosciences].
7. Compensation beads for viability dye [e.g., ArC Amine Reactive Compensation Bead Kit from Thermo Fisher Scientific].
8. Cell sorter with suitable number of channels, using a 70 μm nozzle [e.g., FACSAria III from BD Biosciences].

3 Methods

3.1 PBMC Isolation and Cryopreservation

Due to the nature of human studies, the collection of blood samples is subject to the availability of the individuals to be sampled and trained phlebotomy personnel. Blood sampling may also take place a considerable distance from the researchers. Therefore, blood samples are often processed by isolation and cryopreservation of peripheral blood mononuclear cells (PBMCs), which can then be thawed for downstream analysis. This protocol was designed using cryopreserved PBMC samples ranging in storage time from several months to over 10 years but could easily be adapted for use on freshly isolated PBMCs.

1. Transfer blood samples from collection tubes to 50 mL centrifuge tubes and dilute with an equal amount of PBS (*see* **Note 1**).
2. Pipette 15 mL of room temperature Histopaque into a 50 mL centrifuge tube. Carefully layer no more than 30 mL of the diluted blood on to the Histopaque layer. Repeat for as many tubes as needed for the complete diluted blood sample.
3. Centrifuge the layered blood for 20 min at 800 × *g*, ensuring that the brake is disabled.
4. Carefully remove the tubes from the centrifuge. Using a serological pipette, remove as much of the top layer of plasma as possible without disturbing the thin band of PBMCs below. The plasma can be discarded or stored for further analysis.
5. Using a Pasteur pipette, transfer the thin PBMC layer below the plasma to a new 50 mL centrifuge tube.
6. Top up the tube to 45 mL with 1% PBS/BSA and centrifuge for 10 min at 200 × *g* at room temperature, with the brake turned on.
7. Remove the tube from the centrifuge and pour off the supernatant. Resuspend the cell pellet in 45 mL of 1% PBS/BSA and centrifuge for 10 min at 200 × *g* at room temperature, with the brake turned on.

8. Pour off the supernatant and resuspend the cells in 1 mL of 1% PBS/BSA. Count the cells using any preferred method.
9. Centrifuge the cells for 5 min at 350 × *g* at room temperature. Remove the supernatant and resuspend in freezing medium to a concentration of 1×10^7 cells/mL.
10. Aliquot the cell suspension into cryovials at a volume of 1 mL per tube. Place the tubes in a cell freezing chamber and transfer the chamber to a −80 °C freezer. After 24 h, the tubes can be moved to a −150 °C freezer or a liquid nitrogen storage vessel. This allows samples to be stored and shipped prior to downstream analysis.

3.2 Preparation of Labeled EBs

Elementary bodies (EBs) of *C. trachomatis* should be purified and quantified (*see* **Note 2**) using a preferred in-house method, which will not be covered here. For the purpose of this protocol, aliquots containing 10^7 purified EBs in SPG buffer should be prepared from a stock and stored at −80 °C. Labeled EBs should be prepared fresh from these aliquots and used for cell staining on the same day.

1. Thaw an aliquot of 10^7 EBs on wet ice for 5–10 min or until liquid.
2. Add 1 mL of PBS to the EB sample and transfer to a 1.5 mL microcentrifuge tube. Centrifuge for 20 min at >20,000 × *g* at 4 °C (*see* **Notes 3** and **4**).
3. Carefully aspirate the supernatant using a 200 µL pipette without disturbing the EB pellet area.
4. Add 500 µL of prepared fixation buffer to the tube and vortex to resuspend the EBs. Incubate for 20 min at room temperature.
5. Add 500 µL of PBS to the tube and vortex. Centrifuge for 20 min at >20,000 × *g* at 4 °C.
6. Carefully aspirate the supernatant using a 200 µL pipette without disturbing the EB pellet.
7. Add 500 µL of PBS containing 1 µL of Vybrant DiO staining solution to the EBs. Vortex to resuspend them and incubate for 20 min at 37 °C protected from light.
8. Add 500 µL of 1% PBS/BSA and transfer the EB suspension to a new 1.5 mL microcentrifuge tube (*see* **Note 5**).
9. Centrifuge for 20 min at >20,000 × *g* at 4 °C. Carefully aspirate the supernatant using a 200 µL pipette without disturbing the EB pellet.
10. Add 100 µL of 1% PBS/BSA to the tube and vortex to resuspend the EBs. Keep this suspension on ice protected from light.

11. Immediately prior to use, pass the EB suspension through a 27 G needle 5–10 times to break up any large aggregates.

3.3 Staining of Memory B Cells

1. Thaw cryopreserved PBMCs by placing the frozen tube directly into a 37 °C water bath until almost all of the sample has thawed.
2. Transfer the entire sample to a 50 mL centrifuge tube and top up to 30 mL with room temperature R0 media. Add the media dropwise to the cell suspension using a serological pipette while gently rocking the tube to mix the contents.
3. Centrifuge the cell suspension for 5 min at 500 × *g* at 4 °C.
4. Pour off the supernatant and resuspend the cell pellet in 45 mL of R0. Centrifuge the cell suspension for 5 min at 500 × *g* at 4 °C.
5. Pour off the supernatant and resuspend the cell pellet in 1 mL of PBS. Count the cells using any preferred method.
6. Top up the cell suspension to 45 mL with PBS. Centrifuge the cell suspension for 5 min at 500 × *g* at 4 °C.
7. Aspirate the supernatant and resuspend the pellet in 100 μL of PBS. Transfer the cell suspension to a 5 mL FACS tube.
8. Add the recommended volume of viability dye based on the cell count from **step 5**. Incubate the cells at room temperature for 20 min in the dark.
9. Add 1 mL of 1% PBS/BSA to the cells. Centrifuge for 5 min at 350 × *g* at 4 °C and pour off the supernatant.
10. Resuspend the cell pellet in the 100 μL of labeled EB suspension from Subheading 3.2. Incubate the cells at room temperature for 20 min in the dark.
11. Add 1 mL of 1% PBS/BSA to the cells and centrifuge for 5 min at 350 × *g* at 4 °C.
12. Prepare a master mix containing titrated volumes of fluorescent antibodies against CD3, CD14, CD19, CD27, CD38, IgM, IgD, IgG, and IgA, to a total volume of 100 μL, in 1% PBS/BSA (*see* **Notes 6** and **7**).
13. Pour off the supernatant from the cell pellet and resuspend in 100 μL of the antibody master mix. Incubate for 20 min on wet ice.
14. Add 1 mL of 1% PBS/BSA to the cells. Centrifuge for 5 min at 350 × *g* at 4 °C and pour off the supernatant.
15. Resuspend in 500 μL of 1% PBS/BSA. Pass the cell suspension through a 35 μm mesh to remove cell aggregates prior to sorting. Keep the cells on ice and in the dark until loading into the sorter.

3.4 Sorting of Chlamydia-Specific Cells

1. The sorter should be fitted with a 70 μm nozzle for optimal sorting and viability of human immune cells.
2. Set the compensation values for the sort using single-stained compensation beads for each of the fluorescent antibodies and the viability dye.
3. Use a fluorescence minus one (FMO) control stained with viability dye and fluorescent antibodies but lacking the DiO-labeled EBs to set the gates for the sort. Acquire at least 10^6 events on the cell sorter from the FMO sample (*see* **Note 8**).
4. To gate for the cells of interest (*see* Fig. 2), first create a plot examining FSC-A and SSC-A. Adjust the threshold value of FSC-A to remove small debris from the analysis, and set a gate around the lymphocyte population.
5. From the lymphocytes, create a plot with FSC-A and FSC-H in order to define singlets and remove aggregates from the analysis.
6. From the singlet gate, create a plot of CD3/14 and CD19. Set a gate around the $CD19^+$ $CD3/14^-$ population to identify the B cells.
7. Within the B cell population, create a plot of CD27 and CD38. Set a gate around the $CD27^+$ $CD38^-$ population to identify memory B cells (MBCs).
8. Within the MBC population, create a plot of IgG and IgA. Set a gate around the IgG^+ IgA^- population (*see* **Note 9**).
9. To remove any mistakenly-gated unswitched memory B cells, create a plot within the IgG^+ IgA^- population of IgM and IgD. Set a gate around the IgM^- IgD^- population to identify the IgG^+ MBCs.
10. Within the IgG^+ MBC population, create a plot to show DiO. Set the DiO^+ gate immediately above the negative population shown in the FMO control.
11. Using these gates, sort the DiO^+ IgG^+ MBC population into a tube or plate containing a buffer dependent on downstream analysis, such as culture media or RNA preservation buffer (*see* **Note 10**).

4 Notes

1. Dilution of blood is needed to reduce the density of the sample so as to not "overload" the Histopaque layer and reduce PBMC recovery. If the blood sample has been kept for several hours before PBMC isolation, it may be necessary to increase the dilution with PBS to 2:1 to counter potential aggregation.

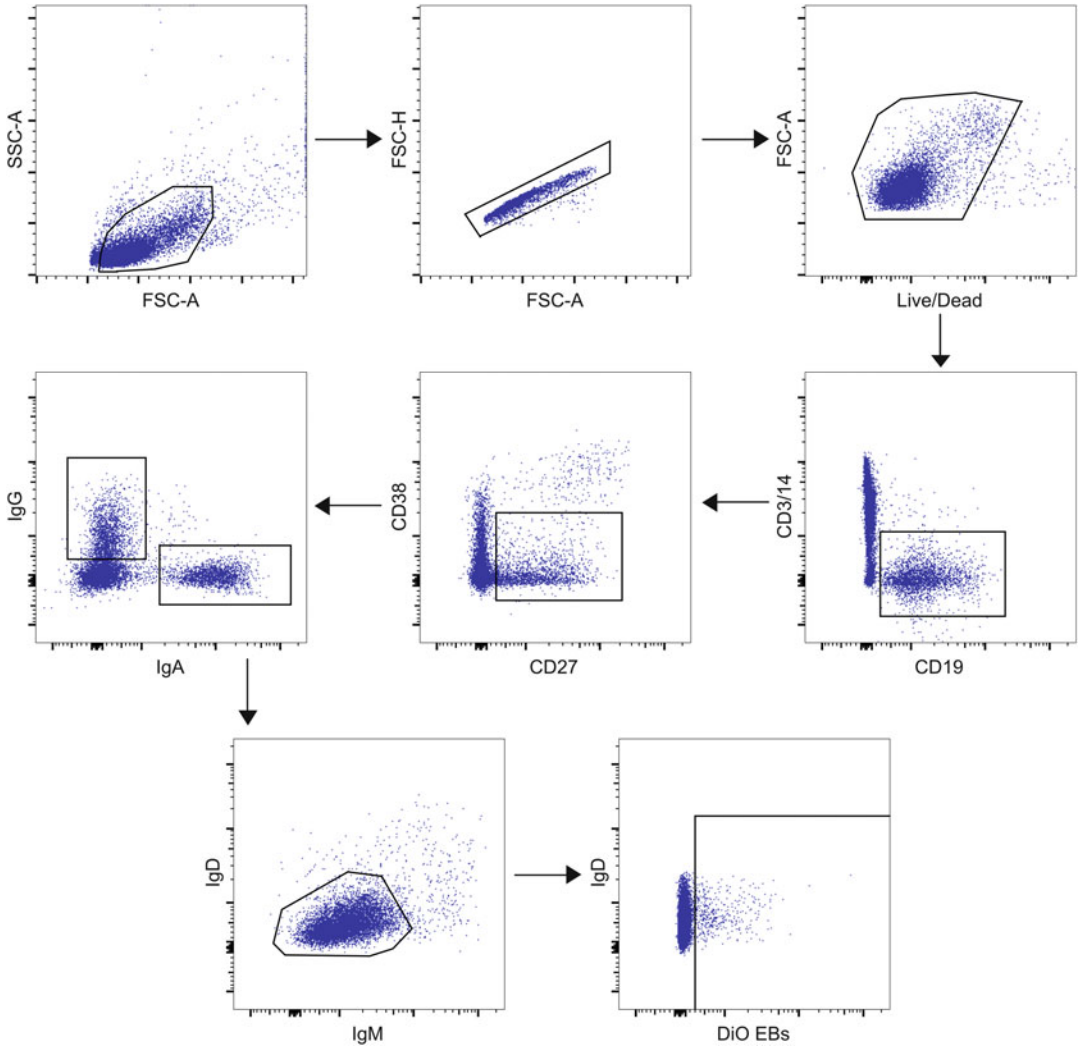

Fig. 2 Gating strategy for identification of EB-specific IgG memory B cells. Lymphocytes are first gated on an FSC-A vs. SSC-A plot, followed by gating of single cells on an FSC-A vs. FSC-H plot. Live cells are then gated followed by B cells, identified as $CD19^+$ $CD3/14^-$. Within the B cell population, memory B cells are gated as $CD27^+$ $CD38^-$. Memory B cells are then selected by Ig isotype of interest, in this case IgG or IgA. IgM/D^+ memory B cells are then excluded before gating of the EB-DiO^+ cells

2. Purified EB samples are commonly quantified in inclusion forming units (IFU) by in vitro assays [21]. However this method does not count the many noninfectious EBs that will still be stained by this protocol and will bind to and label cells. Therefore it is advised to quantify the EBs prior to this protocol using a method to measure absolute particle number or total protein.
3. When pelleting EBs during the staining procedure, a microcentrifuge with a maximum speed of >20,000 × *g* should be

used. However a higher speed rotor with a maximum speed of $>30{,}000 \times g$ can also be used for a higher recovery of EBs.

4. Due to the small size of the EBs, a pellet will not be visible with the numbers used here. Before loading the tube into the centrifuge, use a marker pen to indicate the side of the tube that will be facing the outside edge of the rotor where the EBs will pellet.
5. DiO is a hydrophobic dye and requires strong vortexing to mix into an aqueous solution. It will also adhere to the walls of microcentrifuge tubes, so after incubation of the EBs with DiO it is necessary to transfer the EB suspension to a fresh tube to limit contamination of the labeled EBs with free DiO.
6. Both CD3 and CD14 are used to identify abundant non-B cells in the PBMC sample and as such can be conjugated to the same fluorochrome in a "dump" channel.
7. An IgG antibody should be identified that does not bind to the antigen-binding portion of the IgG molecule and would inhibit binding of the EB to the B cell.
8. The FMO should be an identically-processed PBMC sample, thawed and stained concurrently with the experimental sample (s). However this sample does not need to contain B cells reactive against *C. trachomatis* EBs and thus can be isolated from a control sample, removing the need to use a valuable experimental sample.
9. *C. trachomatis*–reactive MBCs can also be identified and sorted from the IgA^+ population using a similar gating strategy that includes removal of IgM^+ and IgD^+ MBCs.
10. If *C. trachomatis*–reactive B cells are to be sorted for the purpose of cloning and/or sequencing of the Ig mRNA, the sorting procedure should take place under RNA-sterile conditions.

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References

1. Newman L, Rowley J, Hoorn Vander S et al (2015) Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. PLoS One 10:1–17

2. Satpathy G, Behera H, Ahmed N (2017) Chlamydial eye infections: current perspectives. Indian J Ophthalmol 65:97
3. Lijek RS, Helble JD, Olive AJ et al (2018) Pathology after Chlamydia trachomatis infection is driven by nonprotective immune cells that are distinct from protective populations. Proc Natl Acad Sci 115:2216
4. Nicolle C, Cuenod A, Baizot L (1913) Etude experimentale du trachome. Les Arch l'Institut Pasteur Tunis 4:157–182
5. Collier LH, Blyth WA (1966) Immunogenicity of experimental trachoma vaccines in baboons. II. Experiments with adjuvants, and tests of cross-protection. J Hyg (Lond) 64:529–544
6. Olsen AW, Follmann F, Erneholm K et al (2015) Protection against chlamydia trachomatis infection and upper genital tract pathological changes by vaccine-promoted neutralizing antibodies directed to the VD4 of the major outer membrane protein. J Infect Dis 212:978–989
7. Batteiger BE, Rank RG, Bavoil PM et al (1993) Partial protection against genital reinfection by immunization of guinea-pigs with isolated outer-membrane proteins of the chlamydial agent of guinea-pig inclusion conjunctivitis. J Gen Microbiol 139:2965–2972
8. Badamchi-Zadeh A, McKay PF, Korber BT et al (2016) A multi-component prime-boost vaccination regimen with a consensus MOMP antigen enhances chlamydia trachomatis Clearance. Front Immunol 7:162
9. Pal S, Peterson EM, de la Maza LM (2005) Vaccination with the chlamydia trachomatis major outer membrane protein can elicit an immune response as protective as that resulting from inoculation with live bacteria. Infect Immun 73:8153–8160
10. Morrison SG, Su H, Caldwell HD et al (2000) Immunity to murine Chlamydia trachomatis genital tract reinfection involves B cells and CD4+ T cells but not CD8+ T cells. Infect Immun 68:6979–6987
11. Morrison RP, Feilzer K, Tumas DB (1995) Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in *Chlamydia trachomatis* genital tract infection. Infect Immun 63:4661–4668
12. Plummer FA, Simonsen JN, Cameron DW et al (1991) Cofactors in male-female sexual transmission of human immunodeficiency virus type 1. J Infect Dis 163:233–239
13. Morrison SG, Morrison RP (2001) Resolution of secondary *Chlamydia trachomatis* genital tract infection in immune mice with depletion of both CD4+ and CD8+ T cells. Infect Immun 69:2643–2649
14. Morrison SG, Morrison RP (2005) A predominant role for antibody in acquired immunity to chlamydial genital tract reinfection. J Immunol 175:7536–7542
15. Scheid JF, Mouquet H, Feldhahn N et al (2009) A method for identification of HIV gp140 binding memory B cells in human blood. J Immunol Methods 343:65–67
16. Hayakawa K, Ishii R, Yamasaki K et al (1987) Isolation of high-affinity memory B cells: phycoerythrin as a probe for antigen-binding cells. Proc Natl Acad Sci U S A 84:1379–1383
17. Doucett VP, Gerhard W, Owler K et al (2005) Enumeration and characterization of virus-specific B cells by multicolor flow cytometry. J Immunol Methods 303:40–52
18. Woda M, Friberg H, Currier JR et al (2016) Dynamics of dengue virus (DENV)–specific B cells in the response to DENV serotype 1 infections, using flow cytometry with labeled virions. J Infect Dis 214:1001–1009
19. Degauque N, Ngono AE, Akl A et al (2013) Characterization of antigen-specific B cells using nominal antigen-coated flow-beads. PLoS One 8:e84273
20. Lugaajju A, Reddy SB, Rönnberg C et al (2015) Novel flow cytometry technique for detection of Plasmodium falciparum specific B-cells in humans: increased levels of specific B-cells in ongoing infection. Malar J 14:370
21. Frohlich K, Hua Z, Wang J et al (2012) Isolation of Chlamydia trachomatis and membrane vesicles derived from host and bacteria. J Microbiol Methods 91:222–230

Chapter 4

Computational Antigen Discovery for Eukaryotic Pathogens Using *Vacceed*

Stephen J. Goodswen, Paul J. Kennedy, and John T. Ellis

Abstract

Bioinformatics programs have been developed that exploit informative signals encoded within protein sequences to predict protein characteristics. Unfortunately, there is no program as yet that can predict whether a protein will induce a protective immune response to a pathogen. Nonetheless, predicting those pathogen proteins most likely from those least likely to induce an immune response is feasible when collectively using predicted protein characteristics. *Vacceed* is a computational pipeline that manages different standalone bioinformatics programs to predict various protein characteristics, which offer supporting evidence on whether a protein is secreted or membrane -associated. A set of machine learning algorithms predicts the most likely pathogen proteins to induce an immune response given the supporting evidence. This chapter provides step by step descriptions of how to configure and operate *Vacceed* for a eukaryotic pathogen of the user's choice.

Key words *Vacceed*, Machine learning, In silico vaccine discovery, Computational antigen discovery, Eukaryotic pathogen

1 Introduction

Protein sequences are not random assemblies of amino acids. There is a precise biological reason why one particular amino acid is connected to another, which ultimately contributes to a protein's distinctive characteristics [1]. Researchers, over the last two decades, have developed bioinformatics programs that exploit informative signals or patterns encoded within these amino acid sequences to predict protein characteristics. Examples of these characteristics are subcellular localization [2], presence and location of signal peptide cleavage sites [3], and transmembrane topology [4]. With respect to discovering protein vaccine candidates, no signal has yet been detected that helps predict a characteristic signifying a protein's contributing capacity to a protective immune response in a host. Consequently, the current computational antigen discovery aspiration is to distinguish those pathogen proteins

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most likely (referred to henceforth as positives) from those least likely (referred to henceforth as negatives) to induce an immune response.

Vacceed is the collective name for a configurable pipeline of linked bioinformatics programs, Perl scripts, R functions, and Linux shell scripts [5]. It was inspired by the principles of reverse vaccinology [6], whereby antigen discovery starts in silico using the pathogen genome rather than the traditional culture-based method of cultivating and dissecting the pathogen itself. *Vacceed* has been designed to facilitate an automated, high-throughput computational approach to predict vaccine candidates against eukaryotic pathogens given protein sequences [7]. The pipeline uses various standalone bioinformatics programs to predict various protein characteristics. *Vacceed* is grounded on the underlying premise that there is an expected difference between the set of characteristics defining positives to those of negatives. These differences are typically not apparent to an observer and hence applying a rule-based approach to distinguish proteins is not feasible. Conversely, machine learning (ML) has the capacity to detect obscure differences. *Vacceed* uses a set of ML algorithms trained on protein characteristics of known positives and negatives to distinguish if a yet to be classified protein is a positive or negative [8]. So far, *Vacceed* has been used in studies to predict vaccine candidates for *Neospora caninum* [9] and *Cystoisospora suis* [10].

This chapter provides step by step descriptions of how to configure and operate *Vacceed* for a eukaryotic pathogen of the user's choice. A prerequisite for pathogen choice, nonetheless, is a substantial representation of the pathogen's proteome in the form of quality protein sequences.

2 *Vacceed* Core Background Information

Vacceed can be downloaded from https://github.com/goodswen/vacceed/releases. The download package includes a comprehensive *Vacceed* User Guide and sample data. Note that *Vacceed* has been designed for a Linux operating system and has only been tested on Red Hat Enterprise Linux 7.5 but is expected to work on most Linux distributions.

Each data processing stage in the *Vacceed* pipeline is an independent resource, which is built from a central Linux shell script encapsulating all programs needed to perform specific but related tasks. Typical tasks include predicting a particular protein characteristic. By default, *Vacceed* uses seven bioinformatics programs to predict protein characteristics: SignalP 5.0 [11] (predicts presence and location of signal peptide cleavage sites using deep neural networks); WoLF PSORT 0.2 [12] and TargetP 2.0 [2] (predict subcellular localization); TMHMM 2.0 [4] (predicts

Fig. 1 Extract from a species configuration file

transmembrane domains in proteins); Phobius 1.01 [13] (predicts transmembrane topology and signal peptides); DeepLoc 1.0 [14] (predicts eukaryotic protein subcellular localization using deep learning); and IEDB peptide–MHC binding predictors (MHCI version 2.17 and MHCII version 2.16.3) [15] (*see* **Note 1**). Observe that each of the seven programs have specific version numbers on which *Vacceed* has been tested. There is no assurance older or newer program versions will work.

The most pertinent file from a user's perspective is a species configuration file in a header-key format (*see* Fig. 1). For example, [Resources] is the header, and "name" is the key. Text following the "=" sign is configurable. A suggested convention is to have one configuration file for each target pathogen. *Vacceed* is started by entering only one command in a Linux Shell (or terminal) (e.g., perl startup *xx*, where *xx* is a user specified code that links *Vacceed* to the target pathogen configuration file). No other commands are required.

Once *Vacceed* is started, each resource listed after the "name" key is consecutively executed. Resource names can be in any order or even excluded with the exception of VALIDATE (*see* **Note 2**) and EVIDENCE (*see* **Note 3**), which must always be the first and last in the list, respectively. Any key in the configuration file can be used as a variable replacement in the rest of the configuration file. That is, a "$" character preceding a word denotes a variable; for example, $work_dir is replaced by "$HOME/vacceed" throughout the configuration file on execution.

Typical *Vacceed* run times are dependent on various factors including numbers of proteins to process, programs to execute (resources), computer processors (cores), and the amount of memory. For example, a test with 500 proteins processed through all resources completed in 3 h, 21 min, and 17 s using Red Hat Enterprise Linux Workstation release 7.5, 64 bit kernel, and 32 MB memory with 8 cores; however, the same test without the resources MHCI and MHCII completed in 23 min and 54 s. *Vacceed* takes advantage of multicore processors. By default, the proteins to process are split into subsets by the number of cores and then each subset is processed in parallel.

3 Methods

3.1 Running Vacceed with Sample Data

The *Vacceed* installation provides sample data comprising a small collection of *Toxoplasma gondii* proteins as input. The purpose of this section is to test the *Vacceed* installation.

1. Install *Vacceed* (*see* **Note 4**).
2. Edit the species configuration file "toxoplasma.ini" located in the directory <*install_dir*>/vacceed/start/config_dir (where <*install_dir*> is the directory in which *Vacceed* was installed). Under the [Resources] header, remove MHCI and MHCII (*see* **Note 5**).
3. Under the [Main] header, change the current path assigned to work_dir to *install_dir*/vacceed/.
4. Under the [Main] header, assign an appropriate e-mail address to email_url.
5. In a command-line terminal, change directory to *install_dir*/vacceed/start.
6. Enter the command: **perl startup tg**.
7. An e-mail is automatically sent either when the pipeline is successfully completed or immediately when an error occurs. A log file is attached to the e-mail providing details of success or failure (*see* **Note 6**).

```
#ID,ada,knn,nb,nn,rf,svm,average_ML_score
BBOV_IV006420,1.000,1.000,1.000,1.000,1.000,0.994,0.999
BBOV_II001970,1.000,1.000,1.000,1.000,1.000,0.983,0.997
BBOV_III005590,0.984,0.667,0.883,0.800,0.604,0.872,0.801
BBOV_I004490,0.403,0.333,0.980,0.200,0.391,0.202,0.418
BBOV_IV002290,0.427,0.667,0.000,0.000,0.463,0.141,0.283
BBOV_III001400,0.015,0.333,0.764,0.200,0.255,0.121,0.281
BBOV_III011900,0.000,0.000,0.000,0.000,0.001,0.007,0.001
```

Fig. 2 Extract from main *Vacceed* output file "vaccine_candidates". Where ID = protein identifier, ada = adaptive boosting, knn = *k*-nearest neighbor classifier, nb = Naive Bayes classifier, nn = neural network, rf = random forest, and svm = support vector machines. vaccine_candidates is a comma-delimited file containing an ordered list of all machine learning (ML) algorithm scores for each protein processed (seven in this instance). Each ML algorithm generates probabilities that the YES and NO classifications are correct, but only YES probabilities are displayed in the output. The "average ML score" for each protein is the average probabilities of the YES classifications. The list order is descending based on "average ML score" value. An appropriate threshold value (e.g., 0.5) can be compared to the average ML score to determine the relevant class, positive or negative

8. If successful, the main output file called "vaccine_candidates" is created in the directory *install_dir*/vacceed/toxoplasma/proteome. This file contains a list of all processed proteins ranked on average ML scores (*see* Fig. 2 and **Note 7**).

3.2 Running Vacceed with User Provided Data

Once the *Vacceed* installation has been successfully tested, *Vacceed* can be configured and operated for a eukaryotic pathogen of the user's choice. *Neospora caninum* is used here for demonstration purposes.

1. Collect *all* known protein sequences of the target pathogen into one file (*see* **Note 8**). The sequences must be in a FASTA format with a sequence identifier in the following layout: >*xx*|protein Identifier (ID)| text (optional), where *xx* can be any characters (e.g., "tr" or "sp" as per UniProt identifiers).
2. Copy the entire template_species directory to a user-named directory (e.g., neospora).
3. Copy file from **step 1** into *install_dir*/vacceed/neospora/proteome.
4. Copy the species configuration file "toxoplasma.ini" located in the directory *install_dir*/vacceed/start/config_dir to "neospora.ini".
5. Add a new line to startup.ini located in *install_dir*/vacceed/start/:

```
nc< Neospora caninum <pipeline<neospora.ini< install_dir/
vacceed/start/config_dir
```

6. Edit neospora.ini to match the following:

```
work_dir="install_dir/vacceed"
species_dir="neospora"
email_url="your_email@address" (user e-mail address)
proteome_fasta="proteome.fasta" (protein sequence file as per
step 1)
prot_id_prefix="xx" (needs to match the sequence identifier as
per step 1)
```

7. Modify the [Resources] in neospora.ini, if required. That is, remove any resource names between VALIDATE and EVIDENCE that are not required (e.g., MHCI and MHCII).
8. Change directory to *install_dir*/vacceed/start in a command-line terminal.
9. Enter the command: **perl startup nc** (where "nc" is as per **step 5**).
10. Check results in "vaccine_candidates" in *install_dir*/vacceed/neospora/proteome.

3.3 Creating Pathogen Specific Training Data

Training data here is essentially the collection of predicted evidence (referred to henceforth as evidence profiles) from the seven bioinformatics programs for those proteins known to be positive or negative. A training data file called "train_profiles" is provided with the *Vacceed* package as part of the *T. gondii* sample data (*see* **Note 9**). A previous study [8] tested *Vacceed* with different evidence profiles compiled from different eukaryotic species. It concluded that there is no fundamental difference in evidence profile patterns; for example, a model trained on one species can be used to classify proteins from another. This is because the bioinformatics programs are designed or ML trained for eukaryotes in general. Therefore, the creation of a pathogen specific training dataset is not a mandatory step. However, an ideal training dataset is one that contains the greatest variety of evidence profiles (*see* **Note 10**) irrespective of the source species; for example, quality and variety are indisputably the most important factors that impact the accuracy of ML algorithms [8]. A new or amended training file is recommended under any of the following circumstances: a bioinformatics program is upgraded, that is, it has improved accuracy; experimentally proved immunogenic proteins become available; and a new prediction program is added (*see* Subheading 3.5).

1. Collect as many proteins as possible for the target species that are known to induce an immune response in the relevant host. The proteins will represent the "positives" for the training file (*see* **Note 11**).

2. Collect proteins that *do not* induce an immune response. These proteins will represent the "negatives" (*see* **Note 12**).
3. Create a file (e.g., positives.fasta) containing the positive sequences in a FASTA format.
4. Create a file (e.g., negatives.fasta) containing the negative sequences in a FASTA format.
5. Copy the entire template_species directory to a user-named directory (e.g., training).
6. Copy FASTA files from **steps 3** and **4** into *install_dir*/vacceed/training/proteome.
7. Copy "toxoplasma.ini" to "train.ini".
8. Add a new line to startup.ini located in *install_dir*/vacceed/start/:

```
train< Neospora caninum <pipeline<train.ini< install_dir/vacceed/start/config_dir
```

9. Edit train.ini to match the following:

```
work_dir="install_dir/vacceed"
species_dir="training"
email_url="your_email@address" (user e-mail address)
proteome_fasta="positives.fasta" (as per step 3)
prot_id_prefix="xx" (needs to match the sequence identifier)
```

10. Modify [Resources] in train.ini if required; for example, remove any resource not installed or required.
11. Change directory to *install_dir*/vacceed/start in a command-line terminal.
12. Enter the command: **perl startup train** (where "train" is as per **step 8**).
13. Copy the file "evidence_profiles" from *install_dir*/vacceed/training/pipeline/evidence/output to *install_dir*/vacceed/training/pipeline/evidence/training_files.
14. Rename evidence_profiles to a user-defined name (e.g., neospora_profiles).
15. Add ",YES" to the end of each row in the new training file (exclude the first row). The "YES" is the required target label for the positives.
16. Edit train.ini to match the following:

```
proteome_fasta="negatives.fasta" (as per step 4)
```

17. Change directory to *install_dir*/vacceed/start in a command-line terminal.
18. Enter the command: **perl startup train**.

19. Add ",NO" (i.e., the required target label for the negatives) to the end of each row in evidence_profiles in *install_dir*/vacceed/training/pipeline/evidence/output.
20. Append the entire contents of the amended evidence_profiles (except first row) to the new training file (e.g., neospora_profiles).
21. Copy new training file to *install_dir*/vacceed/<new species>/evidence/training_files where <new species> is the directory created for the target species (e.g., neospora).
22. Edit the species configuration file (e.g., neospora.ini) and change the value of the train_file key under header [EVIDENCE] to the new training file (e.g., neospora_profiles) (*see* **Note 13**).
23. The new training data should be evaluated with techniques such as *k*-fold cross validation (*see* **Note 14**) and the ML algorithm parameters tweaked to improve performance (*see* **Note 15**).

3.4 Creating MHCI and MHCII Training Data

This section is only applicable when using resources MHCI and/or MHCII ***and*** the target pathogen host is **not** human. By default, *Vacceed* uses human alleles (e.g., HLA-A*01:01) for peptide–MHC binding predictions. The following describes steps required to setup MHCI for a host other than human (e.g., mouse).

1. Follow **steps 1–4** from Subheading 3.3.
2. Create a file (e.g., mouse_mchI_alleles) in a comma-delimited format containing all required mouse alleles and peptide lengths (e.g., H-2-IAb,8 where each "allele,length" is on a separate line) (*see* **Note 16**).
3. Copy the entire template_species directory to a user-named directory (e.g., mouse), and then copy FASTA files from **steps 3** and **4** to *install_dir*/vacceed/mouse/proteome.
4. Copy mouse_mchI_alleles to *install_dir*/vacceed/mouse/pipeline/mhci/alleles.
5. Copy "toxoplasma.ini" to "mouse.ini".
6. Add a new line to startup.ini located in *install_dir*/vacceed/start/:

```
m< mouse <pipeline<mouse.ini< install_dir/vacceed/start/config_dir
```

7. Edit mouse.ini to match the following:

```
work_dir="install_dir/vacceed"
species_dir="mouse"
email_url="your_email@address" (user e-mail address)
```

```
proteome_fasta="positives.fasta"
prot_id_prefix="xx" (needs to match the sequence identifier)
allele_file="mouse_mchI_alleles" (located under the resource
[MHCI_files])
```

8. Modify [Resources] in mouse.ini to "name=VALIDATE, MHCI".
9. Change directory to *install_dir*/vacceed/start in a command-line terminal.
10. Enter the command: **perl startup m** (where "m" is as per **step 6**).
11. Copy mhci_ml.txt from *install_dir*/vacceed/mouse/pipeline/mhci/output to *install_dir*/vacceed/mouse/pipeline/mhci/training_files.
12. Rename mhci_ml.txt to a user-defined name (e.g., mouse_mhci_ml.txt).
13. Add ",YES" to the end of each row in the new training file (exclude the first row).
14. Edit mouse.ini to match the following:

```
proteome_fasta="negatives.fasta"
```

15. Change directory to *install_dir*/vacceed/start in a command-line terminal.
16. Enter the command: **perl startup m**.
17. Add ",NO" to the end of each row in mhci_ml.txt in *install_dir*/vacceed/mouse/pipeline/mhci/output.
18. Append the entire contents of the amended mhci_ml.txt (except first row) to the new training file (e.g., mouse_mhci_ml.txt).
19. Copy new training file to *install_dir*/vacceed/<new species>/mhci/training_files where <new species> is the directory created for the target species (e.g., new_mouse).
20. Edit the species configuration file (e.g., new_mouse.ini) and change the value of the train_file key under header [MHCI_files] to the new training file (e.g., mouse_mhci_ml.txt).
21. Repeat the steps above to create an MHCII training file, but change mhci to mhcii (*see* **Note 17**).

3.5 Add a New Resource

New programs to predict protein characteristics will inevitably be developed in the future. This section describes how to incorporate a new program into *Vacceed*, which essentially is adding a new resource with the goal of extracting relevant evidence from the new program output to append to evidence profiles.

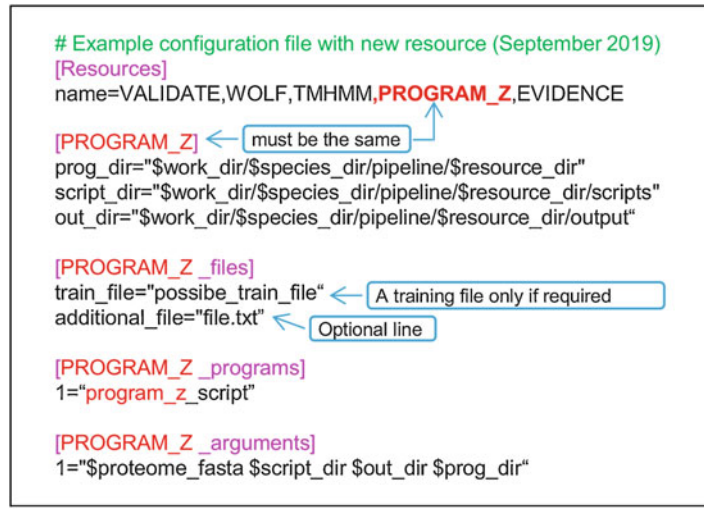

Fig. 3 Example of new resource added to species configuration file

1. Install and test new program with sample data to ensure it runs successfully from any directory (*see* **Note 18**).
2. Determine the input requirements and the output format of new program.
3. Add a new resource name (e.g., program_Z) in an appropriate configuration file:

```
[Resources]
name=VALIDATE,WOLF,TMHMM,PROGRAM_Z,EVIDENCE
```

4. Add a new section to the same configuration file. The easiest way to do this is to copy an existing resource and amend accordingly (*see* Fig. 3). The texts highlighted in red are the only parts expected to be changed.
5. Create a new directory in *install_dir*/vacceed/*new_species*/pipeline using the same name as the new resource (but in lowercase; e.g., program_z).
6. Create two directories called "output" and "scripts" in the program_z directory.
7. Copy "template_resource_script" from *install_dir*/vacceed/*new_species*/pipeline/common_programs to *install_dir*/vacceed/*new_species*/pipeline/program_z.
8. Rename "template_resource_script" to a user-named file (e.g., program_z_script) (*see* **Note 19**).

9. Amend program_z_script where it states << Add new programs here >>. Example:

```
echo "script_step=\">> executing program_z\"" >> $script_dir/
script$chr_no
echo "program_z $required_input $out_dir" >> $script_dir/
script$chr_no || error_exit
```

where $required_input is the input as determined in **step 2**.

10. A generic Perl script called "get_evidence.pl" (located in *install_dir*/vacceed/*new_species*/pipeline/common_programs) can be amended accordingly to extract the relevant evidence from the program_z output file (*see* **Note 20**). Alternatively, any programming language can be used to write a program to extract evidence. In such a case, the program name would need to replace "get_evidence.pl" in program_z_script. Regardless of the extraction program, evidence needs to be saved in a user-named file with the suffix "_evd" (e.g., programz_evd in the directory *install_dir*/vacceed/*new_species*/pipeline/evidence/output).

4 Notes

1. The bioinformatics programs are third-party and are not part of the *Vacceed* package. Furthermore, installation steps for the third-party programs are not described in this chapter. Most of the programs provide a **ReadMe** file with instructions. Even so, these installations are still a challenging aspect to preparing *Vacceed* ready for use. It is highly recommended to seek the help of an administrator or an experienced Linux user.
2. *Vacceed* checks to see if a protein sequence contains invalid letters (e.g., characters other than [ACDEFGHIKLMNPQRSTVWY]).
3. *Vacceed* collates relevant, predicted protein characteristics (typically in the form of numerical values) into one file called evidence_profiles, that is, contents of all files with the extension "_evd" in the evidence/output directory are combined as columns into evidence_profiles.
4. Ensure that each third-party program runs successfully before testing *Vacceed*.
5. The computation takes less than a few minutes depending on the computer environment to run the test when both MHCI and MHCII are removed. Furthermore, MHCI and MHCII predictions on the whole are not accurate [16] (particularly MHCII [17]) and only marginally contributed to the *Vacceed* end result when tested with the *T. gondii* sample data [8].

6. If *Vacceed* fails with the test data then it will inevitably fail with any other data. The expected reason for the failure is installation issues of one or more of the third-party programs (*see* **Note 4**). The log file may give clues as to which third-party program(s) is the culprit.
7. The ML algorithms used are listed in the configuration file under the header [Evidence] and the key "algorithms."
8. It is recommended that *all* known pathogen proteins are processed irrespective of protein name or expected function. This allows for an unbiased approach.
9. This training file contains 475 positives of mainly T. gondii proteins (nine are *N. caninum*). A small selection of these proteins are known to induce an immune response, but most are proteins predicted to be membrane-associated or secreted, that is, proteins exposed to the immune system. There are 501 *T. gondii* proteins representing negatives, which were defined by the protein's predicted subcellular location, that is, neither membrane-associated nor secreted.
10. Variety, in this instance from a ML perspective, is having a generalized selection of proteins in the training file that are representative of all conceivable types of positive and negative proteins. For example, with a limited selection, a ML algorithm may not generalize to evidence profiles not seen when it was learning (i.e., poorly predicts when given new data).
11. Finding training proteins for most species is not a trivial task. The expectation is that a thorough search of the literature will be required. Even then, there may still be an inadequate number of examples to create a training file. A suggested compromise is to use positive proteins from a closely related organism or proteins "expected" to induce or not induce an immune response. For instance, use proteins known to be exposed to the immune system (e.g., membrane associated or secreted proteins) for positives and nonexposed proteins (e.g., proteins normally located in the interior of the organism) as negatives.
12. A drawback for collecting negative examples is that a protein cannot definitively be defined a negative unless it has been explicitly tested in a laboratory.
13. The same proteins should never be used for training and evaluation. This would introduce biased results. Typically, the proteins are randomly divided into two sets: one set containing the majority of data (e.g., 80%) for training and the other set (e.g., 20%) used to evaluate the trained model's performance.
14. *k*-Fold cross-validation is a resampling statistical method used to estimate the performance of ML models. The "*k*" refers to the number of groups that a given data sample is to be split; for

example, tenfold cross-validation indicates the sample data is split into ten groups. One group in turn is used as a test dataset and the remaining groups used for training. The average of the *k* evaluation scores provides an indication of how the model is expected to perform when used to make predictions on data not used during model training.

15. The distributed version of *Vacceed* is configured to run ML algorithms via R functions contained in packages. The algorithms are executed using Rscript. There are three R functions in *install_dir*/vacceed/<new species>/evidence that encapsulate the relevant command for each algorithm: <al>_wrapper.R, <al>_runPred.R, and <al>_makePred.R, where <al> is the algorithm abbreviation. Parameters to fine-tune the algorithms can be modified in <al>_makePred.R (e.g., parameters "ntree" and/or "mtry" in rf_makePred.R, where rf = random forest, ntree = number of decision trees, and "mtry" = number of variables to try at each split in the decision tree).
16. Run the following command to see available class I alleles: `./src/predict_binding.py IEDB_recommended mhc` (only listed alleles can be used).
17. Run the following command to see available class II alleles:

```
python mhc_II_binding.py allele (only listed alleles can be used).
```

18. May need to append new program location to the PATH variable.
19. This is a template script only and will need to be edited appropriately to suit the new program. There are user comments denoted by a "#" symbol, but a familiarity with Linux scripting is expected.
20. Amending get_evidence.pl requires experience in writing Perl scripts. Reading step 8 under the section "Adding a new resource" in the Vacceed User Guide may prove useful when amending get_evidence.pl.

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References

1. Lee D, Redfern O, Orengo C (2007) Predicting protein function from sequence and structure. Nat Rev Mol Cell Biol 8(12):995–1005. https://doi.org/10.1038/nrm2281
2. Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2(4):953–971. https://doi.org/10.1038/nprot.2007.131
3. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8(10):785–786. https://doi.org/10.1038/nmeth.1701
4. Krogh A, Larsson B, von Heijne G, Sonnhammer ELL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305(3):567–580. https://doi.org/10.1006/jmbi.2000.4315
5. Goodswen SJ, Kennedy PJ, Ellis JT (2014) Vacceed: a high-throughput in silico vaccine candidate discovery pipeline for eukaryotic pathogens based on reverse vaccinology. Bioinformatics 30(16):2381–2383. https://doi.org/10.1093/bioinformatics/btu300
6. Rappuoli R (2000) Reverse vaccinology. Curr Opin Microbiol 3(5):445–450. https://doi.org/10.1016/s1369-5274(00)00119-3
7. Goodswen SJ, Kennedy PJ, Ellis JT (2013) A guide to in silico vaccine discovery for eukaryotic pathogens. Brief Bioinform 14(6):753–774. https://doi.org/10.1093/bib/bbs066
8. Goodswen SJ, Kennedy PJ, Ellis JT (2013) A novel strategy for classifying the output from an in silico vaccine discovery pipeline for eukaryotic pathogens using machine learning algorithms. BMC Bioinformatics 14:315. https://doi.org/10.1186/1471-2105-14-315
9. Goodswen SJ, Kennedy PJ, Ellis JT (2017) On the application of reverse vaccinology to parasitic diseases: a perspective on feature selection and ranking of vaccine candidates. Int J Parasitol 47(12):779–790. https://doi.org/10.1016/j.ijpara.2017.08.004
10. Palmieri N, Shrestha A, Ruttkowski B, Beck T, Vogl C, Tomley F, Blake DP, Joachim A (2017) The genome of the protozoan parasite Cystoisospora suis and a reverse vaccinology approach to identify vaccine candidates. Int J Parasitol 47(4):189–202. https://doi.org/10.1016/j.ijpara.2016.11.007
11. Armenteros JJA, Tsirigos KD, Sonderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H (2019) SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 37(4):420. https://doi.org/10.1038/s41587-019-0036-z
12. Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K (2007) WoLF PSORT: protein localization predictor. Nucleic Acids Res 35:W585–W587. https://doi.org/10.1093/nar/gkm259
13. Kall L, Krogh A, Sonnhammer ELL (2004) A combined transmembrane topology and signal peptide prediction method. J Mol Biol 338(5):1027–1036. https://doi.org/10.1016/j.jmb.2004.03.016
14. Armenteros JJA, Sonderby CK, Sonderby SK, Nielsen H, Winther O (2017) DeepLoc: prediction of protein subcellular localization using deep learning. Bioinformatics 33(21):3387–3395. https://doi.org/10.1093/bioinformatics/btx431
15. Vita R, Zarebski L, Greenbaum JA, Emami H, Hoof I, Salimi N, Damle R, Sette A, Peters B (2010) The immune epitope database 2.0. Nucleic Acids Res 38:D854–D862. https://doi.org/10.1093/nar/gkp1004
16. Bui HH, Sidney J, Peters B, Sathiamurthy M, Sinichi A, Purton KA, Mothe BR, Chisari FV, Watkins DI, Sette A (2005) Automated generation and evaluation of specific MHC binding predictive tools: ARB matrix applications. Immunogenetics 57(5):304–314. https://doi.org/10.1007/s00251-005-0798-y
17. Wang P, Sidney J, Dow C, Mothe B, Sette A, Peters B (2008) A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. PLoS Comput Biol 4(4). https://doi.org/10.1371/journal.pcbi.1000048

Chapter 5

Antigen Discovery in Bacterial Panproteomes

Daniel Yero, Oscar Conchillo-Solé, and Xavier Daura

Abstract

There is still a lack of vaccines for many bacterial infections for which the best treatment option would be a prophylactic one. On the other hand, effectiveness has been questioned for some existing vaccines, prompting new developments. Therapeutic vaccines are also becoming a treatment option in specific cases where antibiotics tend to fail. In this scenario, refinement and extension of the classical reverse vaccinology approach is allowing scientists to find new and more effective antigens. In this chapter, we describe an in silico methodology that integrates pangenomic, immunoinformatic, structural, and evolutionary approaches for the screening of potential antigens in a given bacterial species. The strategy focuses on targeting relatively conserved epitopes in core proteins to design broadly cross-protective vaccines and avoid allele-specific immunity. The proposed methodological steps and computational tools can be easily implemented in a reverse vaccinology approach not only to identify new leads with strong immune response but also to develop diagnostic assays.

Key words Computational antigen discovery, Immunoinformatics, Bacteria, Vaccines

1 Introduction

Pathogen antigens are molecules or molecular complexes of a pathogenic organism that are directly recognized by specific immune system receptors (e.g., T-cell and B-cell receptors) or secreted immunoglobulins and that may elicit a host immune response. Not all antigens are competent immunogens or induce a protective adaptive response. In fact some pathogens display antigens whose objective is to misdirect or even suppress the host immune response. The search for vaccine candidates is therefore not a "simple" search for antigens but a search for antigens capable of eliciting a protective memory immune response [1].

The availability of whole genome sequences and corresponding proteomes for a diversity of strains of each of the main pathogenic bacterial species promises to accelerate the identification of vaccine candidates [2]. Thus, the discovery of protein antigens by computational methods is progressively more reliant on an integrated analysis of the ever-increasing genomic and proteomic data. At

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the time of writing, the NCBI database provides more than 200,000 entries for prokaryote genomic sequence data at different assembly levels, covering almost 7000 species and including multiple strains of nearly all important pathogens (https://www.ncbi.nlm.nih.gov/genome/microbes/). A clear example of that is the causative agent of tuberculosis *Mycobacterium tuberculosis*, for which more than 6000 assemblies can be accessed through public databases, representing a significant global collection of circulating strains. This flood of freely available genomic information provides a powerful tool for large-scale genome analyses and pangenome approaches to identify conserved vaccine antigens [2]. The term pangenome refers to the entire genomic repertoire of a given species or higher taxa and it includes both the genes shared by the genomes of all strains (core genome) and the genes present only in some strains of a species (accessory genome) [3, 4]. Consequently, the panproteome is the full set of proteins that might be expressed by a group of related organisms.

The process of conceiving vaccines starting from a pathogen's sequenced genome is largely known as reverse vaccinology [5]. Nearly 20 years after the first reverse vaccinology study (*Neisseria meningitidis* serogroup B [6]), this approach has evolved with a pangenome perspective toward the discovery of universal antigens [2]. In pangenomic reverse vaccinology, high-throughput in silico analyses of multiple genomic or proteomic data sets are performed to identify features that might be predictive of conserved vaccine candidates [7, 8]. A similar approach may also be applied to identify vaccine candidates discriminating pathogenic and nonpathogenic members of a same species or of related species, by performing a subtractive proteomic analysis on the two groups [7, 9, 10]. The panproteome of the pathogenic bacteria can also be compared to the human proteome to exclude antigens that could lead to an autoimmune cross-reaction [11]. Subtractive genomics and proteomics analyses along with reverse vaccinology approaches not only enable the identification of pathogen-specific antigens but may also support drug-target discovery projects [12]. The design and production of vaccines based on reverse vaccinology has been validated by the introduction of a successful vaccine against Meningococcus B strains [13]. This multicomponent vaccine contains three main surface-associated protein antigens discovered by mining the genome of *N. meningitidis* with a classical reverse vaccinology approach. The antigens were initially selected in silico based on their subcellular localization and validated in vivo for their ability to elicit bactericidal antibodies and induce protection in an animal model [6, 14].

With the progress of immunoinformatics [15], the shortlisting of proteins on the basis of cellular localization and conservation has been complemented with their screening for B- and/or T-cell epitopes by various prediction algorithms. Many reverse

vaccinology studies have thus incorporated the use of web-based tools or open-source programs for the identification of epitopes in the process of selection of potential vaccine candidates [16–23]. These tools have been integrated to subtractive and reductionist analyses to identify antigenic and immunogenic MHC class I, MHC class II and/or B-cell epitopes present in preselected candidates, potentially eliciting T- and/or B-cell mediated immune responses. Data-driven and structure-based methods have been developed for the identification of T-cell antigenic regions or epitopes in pathogen proteins, in most cases relying on the prediction of peptide-MHC binding as a surrogate of TCR recognition [24, 25]. Data-driven methods for peptide-MHC binding prediction use experimental binding data to infer peptide-sequence motifs or profiles or to train advanced machine-learning algorithms. There are also in silico tools for prediction of humoral immunity [24]; however, the identification of B-cell epitopes is still complex since they can be conformational in nature and discontinuous. The immune epitope database (IEDB) is a freely available, manually curated resource that contains epitope-specific experimental assays and hosts methodologically diverse tools for the prediction and analysis of both B- and T-cell epitopes [26, 27].

Combination of the genome-based and immunoinformatic methodology with structural and systems-biology approaches has consolidated further the standing of reverse vaccinology as a rational vaccine design process. The final objective of structural vaccinology, beyond its contribution to antigen and epitope identification, is the rational, structure-based optimization of vaccine candidates using three-dimensional data on the antigens, epitopes, and their interactions with immune-system components, as well as on potential scaffolds for antigen engineering [16, 28–31]. On the other hand, systems vaccinology exploits datasets retrieved from systems-based studies to facilitate rational design of safe and efficacious vaccines [32, 33]. This approach combines results from functional omics (transcriptomics, proteomics, metabolomics, etc.) with data from preclinical and clinical studies and bioinformatic analyses of large-scale protein–protein interactions or evolutionary dynamics of antigens.

What makes an antigen a good vaccine candidate is, however, a matter of fine balances. For example, although conservation is a desirable property to ensure population coverage, highly conserved proteins tend to be poor immunogens [34, 35]. In contrast, highly immunogenic, immunodominant epitopes tend to be highly variable [36]. This is not unexpected, since pathogens are naturally under strong selection within the host and proteins under positive selection are therefore more likely to be involved in immunogenicity [37]. Currently available sequencing data and bioinformatics tools enable the assessment of genetic variation of candidate antigens and their epitopes among large amounts of closely related

strains and species. In this regard, the pattern of synonymous and nonsynonymous mutations in protein coding sequences can be exploited as an additional characteristic to identify potential vaccine candidates. All this said, neither immunogenicity nor immunodominance predict protective immunity [38]. In fact, immunodominance can be exploited by the pathogen as an immune-evasion mechanism [39], whereas subdominant epitopes are often more conserved and may elicit immune responses that, although weaker, can promote host resistance [40]. As a conclusion, the properties one should be looking for in trying to identify new antigens for vaccine design depend greatly on the available knowledge on the pathogen and its interaction with the immune system.

Here, we present a general methodology for in silico vaccine candidate discovery in bacterial pathogens (Fig. 1). Ideally, the in silico candidate selection process should identify antigens presenting low failure risks at later, more expensive, vaccine development stages. Thus, an effective computational pipeline should identify putative antigens together with their immunogenic potential, including type(s) of elicited response(s) and their protective potential or risk of involvement in immune-evasion strategies. While

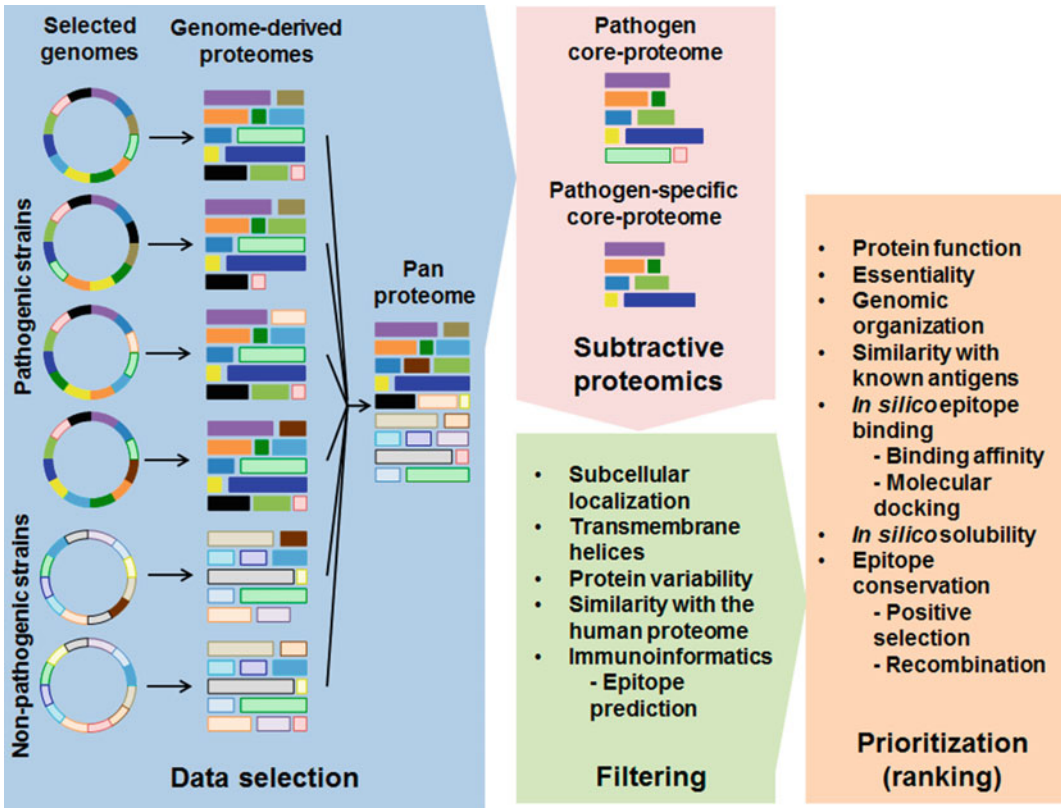

Fig. 1 Schematic workflow of antigen discovery from bacterial panproteomes

many of these aspects cannot be properly addressed yet, we describe a number of steps and related computational tools that when properly combined in a pipeline may support the discovery of new, effective antigens in a bacterial group of interest.

2 Materials

Biological databases mentioned below, widely used and integrated in reverse vaccinology pipelines, are provided in Table 1. This table also lists free and open-source tools and software packages that can be used along with the in silico antigen discovery process. The utility of some of these tools in specific stages of the process is detailed in corresponding sections of the methodology, together with hints for their proper use. A note is also given on whether these tools can process datasets in a high-throughput manner and whether they can be implemented in a computational pipeline.

3 Methods

The strategy we propose here is divided into four main stages (Fig. 1): data acquisition, a subtractive proteomic approach and the steps of filtering and prioritizing candidates. The strategy starts with the selection of representative genome sequences for an organism (species) or a group of organisms and their subsequent analysis. Subtractive proteomics along with classical reverse vaccinology is applied for screening core protein antigens conserved across circulating strains in pathogenic species. To refine the set of selected candidates, we finally look at proteins that are more likely to be effective antigens by combining a set of criteria.

3.1 Selection of Species and Strains and Acquisition of Complete Genome Nucleotide/Protein Sequences

This is one of the most important steps in the process. The strains selected should be representative for the global pathogen population. Missing a part of this population may result in a vaccine ineffective against some pathogen variants. When the genome of the pathogen is very similar to that of a nonpathogenic organism (e.g., *Burkholderia pseudomallei* vs. *Burkholderia thailandensis*, *N. meningitidis* vs. *Neisseria lactamica*, Enterohemorrhagic *Escherichia coli* strains vs. nonpathogenic *E. coli* strains) different organisms or organism subgroups can be included in the analysis in order to identify antigens specific for the pathogenic set. In some cases, these antigens will be pathogenicity or virulence factors themselves, and their immunoblocking (e.g., by a therapeutic vaccine) can be a valid therapeutic strategy [70].

Genome sequences and annotation data for bacterial species are available in multiple statuses according to the quality of the assembly: Complete (all chromosomes are present and gapless and there

Table 1
Bioinformatics tools, web servers, and databases relevant to the antigen discovery pipeline described herein

Tool [reference]	URL (http or ftp)	Description	CLI run[a]
3.1. Selection of species and strains and acquisition of genomic sequences			
NCBI genomes [41]	https://www.ncbi.nlm.nih.gov/genome	NCBI complete genomes database	Yes
ENA assembly search portal [42]	https://www.ebi.ac.uk/ena/data/warehouse/search?portal=assembly	ENA complete genomes database accession portal	Yes
PATRIC [43]	https://www.patricbrc.org	Pathosystems Resource Integration Center, provides integrated data and analysis tools to support biomedical research on bacterial infectious diseases	Yes
3.2. Comparative analysis			
CD-HIT [44]	http://weizhongli-lab.org/cd-hit/	Sequence clustering by similarity, based on all vs. all sequence comparison and an arbitrary cutoff	Yes
OrthoMCL [45]	https://orthomcl.org/orthomcl/	MCL clustering of blast searches	Yes
OMA [46]	http://omabrowser.org/standalone	Score-based clique selection from graphs constructed by reciprocal best hit of all vs. all genome/proteome comparisons	Yes
Complete Reciprocal Best Hit [47]		Selection of complete graph constructed by reciprocal best hit of all vs. all genome/proteome comparisons	Yes
3.3.1. Annotations: Protein function			
UniProt [48]	https://www.uniprot.org	Main site for protein sequence and functional information	Yes
NCBI gb file [41, 49]	https://www.ncbi.nlm.nih.gov/genome	Complete genome annotated file used as source of all required data	Yes
3.3.2. Annotations: Subcellular localization			
PSORTb [50]	https://www.psort.org/psortb/	Bacterial cellular localization prediction tool	Yes
3.3.3. Annotations: Protein solubility related data			
TMHMM [51]	http://www.cbs.dtu.dk/services/TMHMM/	Prediction of transmembrane helices	Yes
FoldIndex [52]	https://fold.weizmann.ac.il/fldbin/findex	Prediction of unfolded/unstructured regions	Yes
Aggrescan [53]	http://bioinf.uab.es/aggrescan/	Prediction of aggregation hot spots	Yes

(continued)

Table 1
(continued)

Tool [reference]	URL (http or ftp)	Description	CLI run[a]
SSpro/ACCpro [54]	http://scratch.proteomics.ics.uci.edu	Surface accessibility prediction	Yes
3.4.1. Epitope prediction: MHC binding affinity			
IEDB [55]	http://tools.iedb.org/main/tcell/	Multiple methods for Epitope prediction	Yes
NetMHCpan [56]	http://www.cbs.dtu.dk/services/NetMHCpan/	Peptide-MHC Class I interaction predictions integrating eluted ligand and peptide binding affinity data	Yes
NetMHCIIpan [57]	http://www.cbs.dtu.dk/services/NetMHCIIpan/	Peptide-MHC class II binding prediction	Yes
NetMHCcons [58]	http://www.cbs.dtu.dk/services/NetMHCcons/	A consensus method for MHC class I binding predictions	Yes
3.4.2. Epitope prediction: Structure-based antibody-binding regions			
BEPPE [59]	http://bioinf.uab.es/BEPPE/	Prediction of antigenic B- and T-cell epitopes via Energy Decomposition Analysis	No
EDP [60]		Prediction of protein-protein interaction sites using electrostatic desolvation profiles	No
3.5. Similarity with human proteins			
NCBI Blast vs. human [11]	https://blast.ncbi.nlm.nih.gov/	Sequence similarity search vs. human genome	Yes
3.6. Antigen variability and epitope conservation among different genomes			
Distmat (EMBOSS) [61]	http://www.bioinformatics.nl/cgi-bin/emboss/distmat	Distance methods	Yes
MEGAX [62]	https://www.megasoftware.net/	Distance methods	Yes
PVS (Protein Variability Server) [63]	http://imed.med.ucm.es/PVS/	Site-by-site amino acid diversity	No
DnaSP [64]	http://www.ub.edu/dnasp/	Distance methods	No
PAML package [65]	http://abacus.gene.ucl.ac.uk/software/paml.html	Site-to-site variation in synonymous substitution rate (Codeml)	Yes
HyPhy package [66]	https://hyphy.org/	Site-to-site variation in synonymous substitution rate (REL) and recombination detection (GARD)	Yes

(continued)

Table 1
(continued)

Tool [reference]	URL (http or ftp)	Description	CLI run[a]
Datamonkey 2.0 [67]	https://www.datamonkey.org/	A web interface to run HyPhy standard analyses	No
PHYLIP package [68]	http://evolution.genetics.washington.edu/phylip.html	Phylogenies (evolutionary trees) by the parsimony algorithm	Yes
PhyML [69]	http://www.atgc-montpellier.fr/phyml/	Phylogenies (evolutionary trees) based on the maximum-likelihood principle	Yes

[a]The software can be run through a Command Line Interface (CLI) and can be therefore integrated in a computational pipeline for the automation of the entire process

are no unplaced scaffolds); Chromosome (a chromosome is present but can have gaps, unplaced scaffolds or contigs); Scaffold (some contigs are joined); and Contigs (assembled sequences are unconnected and unlocalized). Thus, the number of genes and coding sequences (CDSs) of different genomes of the same species can show artifactual differences, making the selection of proteins present in all strains of the species (core proteome) or specific for a selected group practically impossible. To circumvent this problem, the strains of a species with CDS number and genome size deviating largely from those of strains with "Complete" status need to be removed from the initial panel of strains. At the same time, a maximum allowed number of contigs and a minimum N50 should be used for strains with "Contigs" or "Scaffold" status, to ensure a minimum quality of the genomes used.

How this step is completed will highly depend on the source (s) for the genome sequences. Although many databases exist, in Table 1 we list the three main ones. We recommend NCBI genomes [41] since it usually contains the largest number of species/strains and one can retrieve data in an unattended manner (with scripts) following the same steps that one would perform interactively through a web browser, thus allowing the combination of both retrieval options.

3.2 Comparative Analysis

In the previous step all selected genomes were downloaded, preferably in a full gene bank format. Corresponding files will contain the full genome sequence with all annotated genes and their associated features, in a way that is easy to manually visualize and automatically extract for later processing (i.e., gene and protein sequences and annotated data such as protein function or their database links).

Comparative analysis is then done at the CDS level, determining the protein orthologous groups with sizes ranging from the

total number of organisms used (core-proteome) to one (genome-specific protein). This can be done with several programs, of which we propose three in Table 1 that are representative for different methods used in available software [44–46]. We recommend a complete-graph-based reciprocal-best-hit method (e.g., [47]) since, in addition to determining the orthologous groups, it will facilitate the removal of paralogs (the presence of paralogs is hinted by the generation of noncomplete graphs) and the identification of proteins truly specific of a subgroup of genomes (see below).

3.2.1 Subtractive Pangenome Analysis

When the objective is to select orthologous groups made of proteins present only in a defined subgroup of genomes (e.g., pathogens), only complete graph–based methods are recommended. Due to the way each method determines the orthologous groups, neither arbitrary similarity thresholds nor Markov clustering (MCL) nor similarity-based clique methods, where similar proteins may end up in different clusters, will guarantee the "non-presence" of similar proteins in other organisms. When using any of the latter three methods, one may have the temptation to calculate the core proteome for the whole panel of strains, then calculate the core proteome for a selected subgroup and finally subtract the first result from the second. Note that when doing this, all proteins in the resulting list will be in all members of the selected subgroup and will not be in all members of the full panel (as intended) but may still be present in some members of the full panel that do not belong to the selected subgroup.

3.3 Annotations

All available data for each protein has to be taken into account. This data will be crucial when the final selection is made, not only to decide if the protein is a good vaccine candidate but also to determine if, with ease, it can be produced in vitro and purified.

3.3.1 Protein Function

It may be extracted from different sources, but since UniProt [48] contains almost all available data for each protein, it is our primary source for annotations. From here, Gene Ontology (GO) codes [71], Pfam domains [72], and Interpro data [73] can be assigned to each orthologous group. Lately, many proteins have been removed from UniProt due to redundancy issues, but it is highly probable that at least one protein per orthologous group remains in the database, and its annotated data may then be made extensive to all other members of the orthologous group. If this were not the case, some functional data can be extracted from the initially downloaded gene bank file.

3.3.2 Subcellular Localization

The analysis of subcellular localization has been central to reverse vaccinology since its conception [6]. It is important to select proteins that are accessible to antibodies on the pathogen's surface if one is looking for humoral immunity. Although modern vaccine

research considers all potential actors in an immune response, the pharmaceutical industry has traditionally considered the ability of a vaccine preparation to induce complement-mediated in vitro killing of the targeted bacteria to be an essential condition, as this tends to correlate with vaccine efficacy in humans [5, 74]. Subcellular localization can be predicted, for example, with PsortB [50], which stands as one of the most reliable applications for this matter.

3.3.3 Protein Solubility

Apart from the annotated data it is important to have information on structural features and physical-chemical properties. Selection of a protein that is not soluble or that will give problems in the production and purification process should be avoided. Likewise, selection of an epitope that is highly hydrophobic or has a propensity to aggregate for peptide production should be also avoided.

These issues can be addressed by prediction of transmembrane helices [51], aggregation propensity [53], surface accessibility [54], and unstructured regions [52]. The information provided by these analyses can be very valuable for the prioritization of predicted antigens and epitopes.

3.4 Epitope Prediction

In general, we identify a protein as a potential antigen through the prediction of epitopes. Currently, we may predict protein fragments binding to MHC class I or MHC class II molecules as well as protein regions physicochemically amenable to the binding of antibodies. The accuracy of predictors may in some cases exceed 80% (particularly for MHC class I), but one should rather count on an average performance of 70%, and even that may be very optimistic depending on the alleles involved. Peptide processing predictions may be used to enhance the accuracy of MHC class I binding predictions [75], although when dealing with bacterial pathogens one will be more often interested in MHC class II binding, which predictions are per se less reliable. The prediction of TCR recognition is in its infancy and no reliable prediction tools exist yet.

3.4.1 MHC Binding Affinity Analysis

There exist several methods and programs for this purpose. The IEDB database offers a tool implementing several of these methods for both MHC class I and II peptide binding prediction [55]. We recommend this tool, as it ranks amongst the best in terms of accuracy and provides a common interface for class I and II input and output, allowing the test of different methods using the same input data and reading the results in the same format. If a local run is required, we usually use standalone programs implementing NetMHCpan methods for both MHC class I and II [56, 57]. For MHC class I, the software NetMHCcons implements multiple prediction methods and presents a consensus as a result [58]. However it is slower, more complex to install and uses an older version of the NetMHCpan method.

3.4.2 HLA Selection and Population Analysis

The repertoire of MHC molecules and their frequencies varies for different human population groups; therefore, an adequate selection of the relevant HLA alleles should be made if a specific population is targeted. The Allele Frequency Net Database [76] can be used to select the appropriate alleles in such cases. Otherwise, IEDB provides two lists for MHC class I and II allele frequencies and reference sets with maximal population coverage [77–79].

3.4.3 Structure-Based Identification of Antibody Binding Regions

B-cell epitopes are not necessarily lineal and continuous but may include amino acids far from each other in the protein sequence. There are no good data-driven predictors of B-cell epitopes, and one generally needs to resort to structure-based methods predicting protein regions susceptible of binding to other proteins, for example an antibody. Here, we propose two structure-based methods BEPPE [59] and EDP [60] that have been successfully used to predict antibody epitopes in a series of studies on *B. pseudomallei* antigens [80–83]. These methods use relatively inexpensive approximations (compared to, for example, molecular dynamics simulations [84]) to evaluate the internal energy distribution and topology of the protein or its surface desolvation free energy and predict from these quantities the regions more likely to bind an antibody (or other protein). Since they require the structure of the protein and a nonnegligible computation time, they cannot be used in a high-throughput manner. However, they have proven very helpful to validate potential candidates and eventually assist antigen redesign.

3.5 Similarity with Human Proteins and Human Microbiome Proteins

It has been suggested that linear B-cell epitopes found in pathogen proteins do not share in general any sequence identity with human proteins, and when they do it is always with proteins known to be autoantigens [11]. In any case, clearly, one should avoid the selection of antigens including epitopes with any significant similarity to a human sequence. Every potential antigen candidate should be therefore screened against the whole human proteome and any matching region annotated for later evaluation.

To avoid the selection of candidates that may lead to an immune cross-reaction against bacteria of the human microbiome, one may include in the initial subtractive analysis (*see* Subheading 3.2.1) at least one genome of those species (or serotypes within a species) known to be phylogenetically close to the target species (or serotype) and part of the human microbiome. For example, when targeting Enteropathogenic *E. coli* one should include in the analysis a number of genomes representative of commensal *E. coli* strains and preselect proteins not common to the two groups.

3.6 Antigen Variability and Epitope Conservation Among Different Genomes

As already mentioned, an ideal vaccine would be based on conserved antigens eliciting a protective memory immune response. In this regard, antigen variability resulting from bacterial genetic diversity is a major obstacle to vaccine broad-spectrum efficacy [85]. The availability of multiple genomic sequences from different strains of the same pathogenic bacterial species greatly facilitates the analysis of sequence variability in vaccine candidate encoding genes. Sequence variability in multiple alignments of ortholog protein sequences may be calculated as a simple percentage of the number of variable positions in the alignment or, better, as the average amino acid identity (overall uncorrected *p*-distance). However, when variation among sequences is large and the alignment algorithm inserts gaps, these simple methods will inaccurately estimate antigen diversity in a bacterial population. To identify conserved epitopes, the gaps are often removed and the ungapped multiple alignments are used for further analysis. Distance methods for the analysis of multiple sequence alignments can be found in many open source software and freely available packages (*see* Table 1). The analysis of amino acid diversity can be also done site-by-site by estimating the absolute site variability within a multiple sequence alignment. PVS (Protein Variability Server) is a web-based tool that provides absolute sequence variability estimates *per site* in multiple sequence alignments in terms of Shannon entropy, Simpson diversity index and Wu-Kabat variability coefficient [63]. Shannon entropy has been used to assess the sequence variation of viral proteomes in reverse vaccinology approaches [86].

The study of antigenic variability can be also approached from phylogenetics, since the large diversity found in bacterial pathogens has partly emerged as an evolutionary strategy to evade host immunity. The ability of bacteria to constantly and rapidly evolve by natural selection via recombination and mutation has shaped the high diversity found in protein antigenic regions [87]. Genome-wide evidence for positive selection and recombination in bacterial genomes has demonstrated that genes coding for putative antigens and virulence factors are prone to natural selective pressure [88–91]. Nonsynonymous mutations are translated into differences at protein level and can directly affect protein function and their recognition by the immune system receptors. Therefore, the proportion of synonymous and nonsynonymous differences can be used to test the action of natural selection on protein-coding genes and it has been widely used as an indicator of positive (Darwinian) selection. DnaSP (DNA Sequence Polymorphism) and MEGA (Molecular Evolutionary Genetics Analysis) are freely available software packages for the analysis of sequence polymorphisms using multiple sequence alignments [62, 64]. They can estimate sequence diversity and several measures of DNA sequence variation within populations in synonymous or nonsynonymous sites.

The proportion of sites under selection in a sequence alignment is a sign of how the evolutionary forces are acting on the sequence; however, for a given gene only a small fraction of codon positions will be subject to strong positive selection pressure. This may happen, for instance, in pathogen epitopes where certain positively selected codons may lead to escape variants able to evade the immune system [30, 92, 93]. The most widely used methods for the identification of sites under positive selection estimate the rates of fixation of nonsynonymous and synonymous mutations at each codon site based on maximum-likelihood substitution models. Site-to-site variation in selection pressure across coding regions can be analyzed with the popular software HyPhy [66] and PAML [94]. The Codeml program of PAML and REL methods in HyPhy constitute very similar approaches for site-to-site selection analysis [95]. These methods take phylogenies into account; therefore, in order to conduct the analysis one needs a multiple sequence alignment and a corresponding phylogenetic tree. Accurate phylogenies can be constructed using the DNAPARS program (parsimony algorithm) in the PHYLIP package or the PhyML program (maximum likelihood) [69]. However several other algorithms can be employed to do the same. Prior to the selection analysis, recombination signals between sequences in the alignment of orthologous sequences should be detected, because recombination events have a profound effect on evolutionary inferences and on the identification of codon sites under positive selection [96]. The GARD algorithm implemented in the HyPhy package uses a genetic algorithm to screen multiple sequence alignments for recombination and identify putative recombination breakpoints [97]. Once recombination has been identified, it is necessary to split the alignment into nonrecombinant sequence fragments and the selection analyses can be run separately for each fragment [95]. Figure 2 provides as an example a sequence conservation analysis for two candidate proteins predicted as pathogen-specific antigens in *B. pseudomallei* [16], pathogenic gram-negative bacteria responsible for melioidosis. This analysis showed that the protein BPSL1626 is highly conserved in this species [16], but the protein BPSS1727 suffers extensive variability because it is subject to recombination and selection pressure. The figure also shows a pipeline proposal for genome-wide identification of positive selection and recombination in bacterial pangenomes.

3.7 Scripting and Automatization

Most of the programs used in the antigen-identification protocol presented here have a command line interface or are accessible as web services and can be run in a noninteractive mode, facilitating their inclusion in a pipeline for the automation of the entire process. It is thus possible to script the whole sequence of tasks so that after the selection of species/strains no additional user intervention is required. The output of such a pipeline will be a final list of

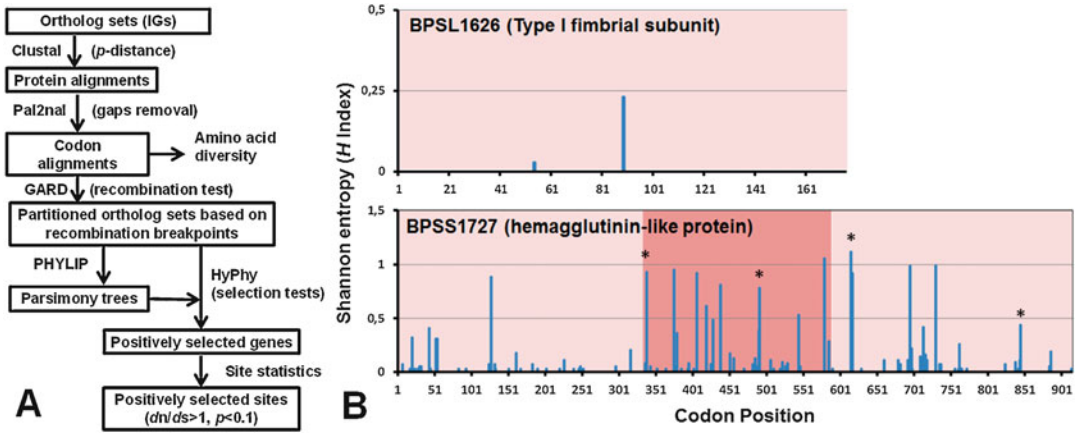

Fig. 2 Proposed stages for genome-wide identification of positive selection and recombination in bacterial pangenomes (**a**). Sequence conservation analysis for two *Burkholderia pseudomallei* candidate proteins (BPSL1626 and BPSS1727) in 344 strains (**b**). Asterisks indicate positively selected sites. Same color in the graphic background indicates same recombination fragments

candidate proteins with their properties for the user to evaluate. The fact that all programs are independent and a large number of proteins may be examined prompts for straightforward parallelization of proteins/tasks so that if a computer cluster is available the entire process can be completed in a very acceptable time.

4 Conclusions and Key Challenges

The classical in silico reverse vaccinology strategy has been widely used for the identification of surface-exposed and secreted extracellular proteins as potential candidates for vaccines seeking to promote a dominantly humoral immune response. This simple analysis may be further complicated by the incorporation of computational methods to identify B-cell epitopes, often conformational and discontinuous in nature, in candidate proteins. Most methods for the prediction of B-cell epitopes require the three-dimensional structure of the antigen [25]. The recognition of specific protein fragments as T-cell epitopes (both HLA class I and HLA class II restricted) has been also successfully integrated into antigen discovery pipelines. Although HLA (particularly HLA class I) epitope prediction is more advanced and reliable than that of B-cell epitope prediction, HLA presentation does not guarantee TCR recognition. This results in a systematic overprediction of T-cell epitopes (false positive epitopes), in addition to the usual limitations of predictors based on far from perfect experimental datasets [25]. Therefore, we recommend combining different HLA epitope prediction algorithms and selecting those epitopes showing a relative consensus. As mentioned, for HLA class I preliminary

predictions of antigen processing may be performed. In addition, depending on the specific pathogen, its immunology and the target community, one needs to consider the set of relevant HLA alleles and the accuracy of corresponding predictors, which go from reasonably good to very poor depending on the amount of experimental data available for their training. The accuracy of the predictor for every particular allele should be documented in the program.

When dealing with multiple strains in a pangenome approach, the selection of genomes for subsequent analysis should pay careful attention to data quality and the representativeness of the sample. Strain selection should represent as much as possible the global pathogen population. If strain selection is biased towards a particular lineage or genotype, this will lead to an overestimation of the size of the core genome and an underestimation of antigen variability. The quality of the genome sequences is also critical. Low quality genome assembly and annotation can lead to data loss during comparative genomics analysis and a decrease in the proportion of core genes. To avoid the loss of an orthologous group due to incomplete genome sequences in the sample, one option is to make the core genome search less restrictive. For example, by applying a cutoff value for gene prevalence in the sample close to 100% or simply defining core genes as those genes that are present in all strains except one or two (soft core proteome). Recently, a strategy based on the prevalence of essential genes to remove genome sequences with poor or low coverage has been reported [8]. The presence of paralogous gene families in the sample also complicates the computational determination of the core genome and hinders an accurate estimation of sequence variability and the proportion of sites under selection. In addition, paralog genes encoding proteins appear to play a role in DNA recombination and antigenic variation [98–100]. All things considered, it is reasonable to filter out any cluster containing paralogous genes from the list of candidate antigens.

Most antigen discovery strategies are aimed at the selection of conserved epitopes in pathogen proteins. Theoretically, an ideal vaccine should provide a broad coverage. From an evolutionary perspective, conserved antigens are often assumed to be less immunogenic than highly variable ones [101]. In other words, immunodominant antigens are frequently also the most variable. Studies of vaccine antigen diversity in natural populations have revealed regions of proteins under selective pressure as immune-system targets [14, 102, 103]. Conversely, other studies have identified and validated highly conserved and protective bacterial vaccine antigens [8, 104]. In light of this, sequence variability (as many other properties) cannot be straightforwardly used as a criterion for antigen prioritization but must be evaluated as per case. Nevertheless, extensive variability in a protein alignment should be a sufficient sign to filter out a protein during the selection process.

Finally, the selection of candidates to pass on to experimental evaluation will depend on knowledge (the more the better) on the pathogen, its interaction with the host immune system and the nature of the desired immune response(s), which in turn will depend on the purpose of the vaccine (prophylactic or therapeutic). Often, it is a good idea to rank the list of candidates according to different sets of criteria, including apparently more mundane issues such as everything around ease of production, solubility, stability, and other properties of the proteins or peptides that might be relevant to manufacturing and even dispensing the vaccine. The prioritization of a given protein or set of proteins over others in the initial in silico steps of reverse vaccinology remains challenging. Even so, the reductionist nature of the approach results in a relatively manageable number of rationally selected potential vaccine candidates amenable to experimental evaluation.

References

1. Levine MM, Dougan G, Good MF, Liu MA, Nabel GJ, Nataro JP, Rappuoli R (2017) New generation vaccines, 4th edn. Taylor & Francis Group, Boca Raton, FL
2. Bidmos FA, Siris S, Gladstone CA, Langford PR (2018) Bacterial vaccine antigen discovery in the reverse vaccinology 2.0 era: progress and challenges. Front Immunol 9:2315. https://doi.org/10.3389/fimmu.2018.02315
3. Tettelin H, Masignani V, Cieslewicz MJ et al (2005) Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome.". PNAS 102:13950–13955
4. Vernikos G, Medini D, Riley DR, Tettelin H (2015) Ten years of pan-genome analyses. Curr Opin Microbiol 23:148–154
5. Rappuoli R (2000) Reverse vaccinology. Curr Opin Microbiol 3:445–450
6. Pizza M, Scarlato V, Masignani V et al (2000) Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science 287:1816–1820
7. Moriel DG, Scarselli M, Serino L, Mora M, Rappuoli R, Masignani V (2008) Genome-based vaccine development: a short cut for the future. Hum Vaccin 4:184–188
8. Moriel DG, Tan L, Goh KGK, Phan M-D, Ipe DS, Lo AW, Peters KM, Ulett GC, Beatson SA, Schembri MA (2016) A novel protective vaccine antigen from the core *Escherichia coli* genome. mSphere 1(6). https://doi.org/10.1128/mSphere.00326-16
9. Moriel DG, Bertoldi I, Spagnuolo A et al (2010) Identification of protective and broadly conserved vaccine antigens from the genome of extraintestinal pathogenic *Escherichia coli*. PNAS 107:9072–9077
10. Ariel N, Zvi A, Makarova KS, Chitlaru T, Elhanany E, Velan B, Cohen S, Friedlander AM, Shafferman A (2003) Genome-based bioinformatic selection of chromosomal *Bacillus anthracis* putative vaccine candidates coupled with proteomic identification of surface-associated antigens. Infect Immun 71:4563–4579
11. Amela I, Cedano J, Querol E (2007) Pathogen proteins eliciting antibodies do not share epitopes with host proteins: a bioinformatics approach. PLoS One 2:e512
12. Barh D, Tiwari S, Jain N, Ali A, Santos AR, Misra AN, Azevedo V, Kumar A (2011) In silico subtractive genomics for target identification in human bacterial pathogens. Drug Dev Res 72:162–177
13. Rappuoli R, Pizza M, Masignani V, Vadivelu K (2018) Meningococcal B vaccine (4CMenB): the journey from research to real world experience. Exp Rev Vaccin 17:1111–1121
14. Giuliani MM, Adu-Bobie J, Comanducci M et al (2006) A universal vaccine for serogroup B meningococcus. Proc Natl Acad Sci U S A 103:10834–10839
15. Tomar N, De RK (2014) Immunoinformatics: a brief review. Methods Mol Biol 1184:23–55

16. Capelli R, Peri C, Villa R et al (2018) BPSL1626: reverse and structural vaccinology reveal a novel candidate for vaccine design against *Burkholderia pseudomallei*. Antibodies 7:26
17. Pajon R, Yero D, Niebla O et al (2009) Identification of new meningococcal serogroup B surface antigens through a systematic analysis of neisserial genomes. Vaccine 28:532–541
18. He Y, Xiang Z, Mobley HLT (2010) Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development. J Biomed Biotechnol 2010:297505. https://doi.org/10.1155/2010/297505
19. Naz K, Naz A, Ashraf ST, Rizwan M, Ahmad J, Baumbach J, Ali A (2019) PanRV: pangenome-reverse vaccinology approach for identifications of potential vaccine candidates in microbial pangenome. BMC Bioinformatics 20:123. https://doi.org/10.1186/s12859-019-2713-9
20. Solanki V, Tiwari V (2018) Subtractive proteomics to identify novel drug targets and reverse vaccinology for the development of chimeric vaccine against *Acinetobacter baumannii*. Sci Rep 8:9044
21. Jaiswal V, Chanumolu SK, Gupta A, Chauhan RS, Rout C (2013) Jenner-predict server: prediction of protein vaccine candidates (PVCs) in bacteria based on host-pathogen interactions. BMC Bioinformatics 14:211
22. Zvi A, Rotem S, Bar-Haim E, Cohen O, Shafferman A (2011) Whole-genome immunoinformatic analysis of F. tularensis: predicted CTL epitopes clustered in hotspots are prone to elicit a T-cell response. PLoS One 6: e20050
23. Solanki V, Tiwari M, Tiwari V (2019) Prioritization of potential vaccine targets using comparative proteomics and designing of the chimeric multi-epitope vaccine against *Pseudomonas aeruginosa*. Sci Rep 9:1. https://doi.org/10.1038/s41598-019-41496-4
24. Dhanda SK, Usmani SS, Agrawal P, Nagpal G, Gautam A, Raghava GPS (2017) Novel in silico tools for designing peptide-based subunit vaccines and immunotherapeutics. Brief Bioinform 18:467–478
25. Sanchez-Trincado JL, Gomez-Perosanz M, Reche PA (2017) Fundamentals and methods for T- and B-cell epitope prediction. J Immunol Res 2017:2680160. https://doi.org/10.1155/2017/2680160
26. Fleri W, Paul S, Dhanda SK, Mahajan S, Xu X, Peters B, Sette A (2017) The immune epitope database and analysis resource in epitope discovery and synthetic vaccine design. Front Immunol 8:278. https://doi.org/10.3389/fimmu.2017.00278
27. Vita R, Mahajan S, Overton JA, Dhanda SK, Martini S, Cantrell JR, Wheeler DK, Sette A, Peters B (2019) The immune epitope database (IEDB): 2018 update. Nucleic Acids Res 47:D339–D343
28. Cozzi R, Scarselli M, Ferlenghi I (2013) Structural vaccinology: a three-dimensional view for vaccine development. Curr Top Med Chem 13:2629–2637
29. Anasir MI, Poh CL (2019) Structural vaccinology for viral vaccine design. Front Microbiol 10:738. https://doi.org/10.3389/fmicb.2019.00738
30. Nuccitelli A, Rinaudo CD, Brogioni B et al (2013) Understanding the molecular determinants driving the immunological specificity of the protective pilus 2a backbone protein of group B streptococcus. PLoS Comput Biol 9: e1003115
31. Capelli R, Marchetti F, Tiana G, Colombo G (2017) SAGE: a fast computational tool for linear epitope grafting onto a foreign protein scaffold. J Chem Inf Model 57:6–10
32. Raeven RHM, van Riet E, Meiring HD, Metz B, Kersten GFA (2019) Systems vaccinology and big data in the vaccine development chain. Immunology 156:33–46
33. Oberg AL, Kennedy RB, Li P, Ovsyannikova IG, Poland GA (2011) Systems biology approaches to new vaccine development. Curr Opin Immunol 23:436–443
34. De Groot AS, Ardito M, Moise L, Gustafson EA, Spero D, Tejada G, Martin W (2011) Immunogenic consensus sequence T helper epitopes for a Pan-*Burkholderia* biodefense vaccine. Immunome Res 7(2). https://doi.org/10.4172/1745-7580.1000043
35. Goodswen SJ, Kennedy PJ, Ellis JT (2014) Enhancing in silico protein-based vaccine discovery for eukaryotic pathogens using predicted peptide-MHC binding and peptide conservation scores. PLoS One 9:e115745. https://doi.org/10.1371/journal.pone.0115745
36. Seib KL, Zhao X, Rappuoli R (2012) Developing vaccines in the era of genomics: a decade of reverse vaccinology. Clin Microbiol Infect 18(Suppl 5):109–116
37. Goodswen SJ, Kennedy PJ, Ellis JT (2018) A gene-based positive selection detection approach to identify vaccine candidates using *Toxoplasma gondii* as a test case protozoan pathogen. Front Genet 9:332. https://doi.org/10.3389/fgene.2018.00332

38. Gilchuk P, Hill TM, Wilson JT, Joyce S (2015) Discovering protective CD8 T cell epitopes—no single immunologic property predicts it! Curr Opin Immunol 34:43–51
39. Akram A, Inman RD (2012) Immunodominance: a pivotal principle in host response to viral infections. Clin Immunol 143:99–115
40. Rodrigues MM, Ersching J (2015) Neglected tropical diseases, bioinformatics, and vaccines. J Infect Dis 211:175–177
41. Haft DH, DiCuccio M, Badretdin A et al (2018) RefSeq: an update on prokaryotic genome annotation and curation. Nucleic Acids Res 46:D851–D860
42. Harrison PW, Alako B, Amid C et al (2019) The european nucleotide archive in 2018. Nucleic Acids Res 47:D84–D88
43. Wattam AR, Davis JJ, Assaf R et al (2017) Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. Nucleic Acids Res 45: D535–D542
44. Fu L, Niu B, Zhu Z, Wu S, Li W (2012) CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28:3150–3152
45. Fischer S, Brunk BP, Chen F, Gao X, Harb OS, Iodice JB, Shanmugam D, Roos DS, Stoeckert CJ (2011) Using OrthoMCL to assign proteins to OrthoMCL-DB groups or to cluster proteomes into new ortholog groups. Curr Protoc Bioinformatics Chapter 6:Unit 6.12.1-19
46. Train C-M, Glover NM, Gonnet GH, Altenhoff AM, Dessimoz C (2017) Orthologous Matrix (OMA) algorithm 2.0: more robust to asymmetric evolutionary rates and more scalable hierarchical orthologous group inference. Bioinformatics 33:i75–i82
47. Callister SJ, McCue LA, Turse JE, Monroe ME, Auberry KJ, Smith RD, Adkins JN, Lipton MS (2008) Comparative bacterial proteomics: analysis of the core genome concept. PLoS One 3:e1542. https://doi.org/10.1371/journal.pone.0001542
48. The UniProt Consortium (2017) UniProt: the universal protein knowledgebase. Nucleic Acids Res 45:D158–D169
49. GenBank Sample Record. https://www.ncbi.nlm.nih.gov/Sitemap/samplerecord.html. Accessed 27 Jun 2019
50. Yu NY, Wagner JR, Laird MR et al (2010) PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics 26:1608–1615
51. Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305:567–580
52. Prilusky J, Felder CE, Zeev-Ben-Mordehai T, Rydberg EH, Man O, Beckmann JS, Silman I, Sussman JL (2005) FoldIndex: a simple tool to predict whether a given protein sequence is intrinsically unfolded. Bioinformatics 21:3435–3438
53. Conchillo-Solé O, de Groot NS, Avilés FX, Vendrell J, Daura X, Ventura S (2007) AGGRESCAN: a server for the prediction and evaluation of "hot spots" of aggregation in polypeptides. BMC Bioinformatics 8:65
54. Magnan CN, Baldi P (2014) SSpro/ACCpro 5: almost perfect prediction of protein secondary structure and relative solvent accessibility using profiles, machine learning and structural similarity. Bioinformatics 30:2592–2597
55. Dhanda SK, Mahajan S, Paul S et al (2019) IEDB-AR: immune epitope database-analysis resource in 2019. Nucleic Acids Res 47: W502. https://doi.org/10.1093/nar/gkz452
56. Jurtz V, Paul S, Andreatta M, Marcatili P, Peters B, Nielsen M (2017) NetMHCpan-4.0: improved peptide-MHC class i interaction predictions integrating eluted ligand and peptide binding affinity data. J Immunol 199:3360–3368
57. Jensen KK, Andreatta M, Marcatili P, Buus S, Greenbaum JA, Yan Z, Sette A, Peters B, Nielsen M (2018) Improved methods for predicting peptide binding affinity to MHC class II molecules. Immunology 154:394–406
58. Karosiene E, Lundegaard C, Lund O, Nielsen M (2012) NetMHCcons: a consensus method for the major histocompatibility complex class I predictions. Immunogenetics 64:177–186
59. Peri C, Solé OC, Corrada D, Gori A, Daura X, Colombo G (2015) Prediction of antigenic B and T cell epitopes via energy decomposition analysis: description of the web-based prediction tool BEPPE. Methods Mol Biol 1348:13–22
60. Fiorucci S, Zacharias M (2010) Prediction of protein-protein interaction sites using electrostatic desolvation profiles. Biophys J 98:1921–1930
61. Rice P, Longden I, Bleasby A (2000) EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet 16:276–277

62. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549
63. Garcia-Boronat M, Diez-Rivero CM, Reinherz EL, Reche PA (2008) PVS: a web server for protein sequence variability analysis tuned to facilitate conserved epitope discovery. Nucleic Acids Res 36:W35–W41
64. Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A (2017) DnaSP 6: DNA sequence polymorphism analysis of large data sets. Mol Biol Evol 34:3299–3302
65. Xu B, Yang Z (2013) PAMLX: a graphical user interface for PAML. Mol Biol Evol 30:2723–2724
66. Pond SLK, Frost SDW, Muse SV (2005) HyPhy: hypothesis testing using phylogenies. Bioinformatics 21:676–679
67. Weaver S, Shank SD, Spielman SJ, Li M, Muse SV, Kosakovsky Pond SL (2018) Datamonkey 2.0: a modern web application for characterizing selective and other evolutionary processes. Mol Biol Evol 35:773. https://doi.org/10.1093/molbev/msx335
68. Baum BR (1989) PHYLIP: phylogeny inference package. Version 3.2. Joel Felsenstein. Q Rev Biol 64:539–541
69. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–321
70. Kane TL, Carothers KE, Lee SW (2018) Virulence factor targeting of the bacterial pathogen *Staphylococcus aureus* for vaccine and therapeutics. Curr Drug Targets 19:111–127
71. The Gene Ontology Consortium (2019) The gene ontology resource: 20 years and still GOing strong. Nucleic Acids Res 47:D330-D338
72. El-Gebali S, Mistry J, Bateman A et al (2019) The Pfam protein families database in 2019. Nucleic Acids Res 47:D427–D432
73. Mitchell AL, Attwood TK, Babbitt PC et al (2019) InterPro in 2019: improving coverage, classification and access to protein sequence annotations. Nucleic Acids Res 47: D351–D360
74. Carroll MC (2008) Complement and Humoral Immunity. Vaccine 26:I28–I33
75. Backert L, Kohlbacher O (2015) Immunoinformatics and epitope prediction in the age of genomic medicine. Genome Med 7:119
76. Gonzalez-Galarza FF, McCabe A, Melo Dos Santos EJ, Takeshita L, Ghattaoraya G, Jones AR, Middleton D (2018) Allele frequency net database. Methods Mol Biol 1802:49–62
77. HLA allele frequencies and reference sets with maximal population coverage. IEDB Solutions Center. http://help.iedb.org/hc/en-us/articles/114094151851-HLA-allele-frequencies-and-reference-sets-with-maximal-population-coverage. Accessed 26 Jun 2019
78. Greenbaum J, Sidney J, Chung J, Brander C, Peters B, Sette A (2011) Functional classification of class II human leukocyte antigen (HLA) molecules reveals seven different supertypes and a surprising degree of repertoire sharing across supertypes. Immunogenetics 63:325–335
79. Weiskopf D, Angelo MA, de Azeredo EL et al (2013) Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. Proc Natl Acad Sci U S A 110:E2046–E2053
80. Lassaux P, Peri C, Ferrer-Navarro M et al (2013) A structure-based strategy for epitope discovery in *Burkholderia pseudomallei* OppA antigen. Structure 21:167–175
81. Gourlay LJ, Peri C, Ferrer-Navarro M et al (2013) Exploiting the *Burkholderia pseudomallei* acute phase antigen BPSL2765 for structure-based epitope discovery/design in structural vaccinology. Chem Biol 20:1147–1156
82. Gourlay LJ, Thomas RJ, Peri C et al (2015) From crystal structure to in silico epitope discovery in the *Burkholderia pseudomallei* flagellar hook-associated protein FlgK. FEBS J 282:1319–1333
83. Gaudesi D, Peri C, Quilici G et al (2015) Structure-based design of a B cell antigen from *B. pseudomallei*. ACS Chem Biol 10:803–812
84. van Gunsteren WF, Bakowies D, Baron R et al (2006) Biomolecular modeling: goals, problems, perspectives. Angew Chem Int Ed Eng 45:4064–4092
85. Telford JL (2008) Bacterial genome variability and its impact on vaccine design. Cell Host Microbe 3:408–416
86. Khan AM, Hu Y, Miotto O, Thevasagayam NM, Sukumaran R, Abd Raman HS, Brusic V, Tan TW, Thomas August J (2017) Analysis of viral diversity for vaccine target discovery. BMC Med Genet 10:78
87. Graves CJ, Ros VID, Stevenson B, Sniegowski PD, Brisson D (2013) Natural selection promotes antigenic evolvability. PLoS Pathog 9:

e1003766. https://doi.org/10.1371/journal.ppat.1003766
88. Xu Z, Chen H, Zhou R (2011) Genome-wide evidence for positive selection and recombination in *Actinobacillus pleuropneumoniae*. BMC Evol Biol 11:203
89. Cao P, Guo D, Liu J, Jiang Q, Xu Z, Qu L (2017) Genome-wide analyses reveal genes subject to positive selection in *Pasteurella multocida*. Front Microbiol 8:961. https://doi.org/10.3389/fmicb.2017.00961
90. Petersen L, Bollback JP, Dimmic M, Hubisz M, Nielsen R (2007) Genes under positive selection in *Escherichia coli*. Genome Res 17:1336–1343
91. Nandi T, Ong C, Singh AP et al (2010) A genomic survey of positive selection in *Burkholderia pseudomallei* provides insights into the evolution of accidental virulence. PLoS Pathog 6:e1000845
92. O'Connor DH, McDermott AB, Krebs KC et al (2004) A dominant role for CD8+-T-lymphocyte selection in simian immunodeficiency virus sequence variation. J Virol 78:14012–14022
93. de Oliveira T, Salemi M, Gordon M, Vandamme A-M, van Rensburg EJ, Engelbrecht S, Coovadia HM, Cassol S (2004) Mapping sites of positive selection and amino acid diversification in the HIV genome: an alternative approach to vaccine design? Genetics 167:1047–1058
94. Yang Z (1997) PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl Biosci 13:555–556
95. Kosakovsky Pon S, Poon A, Frost S (2009) Estimating selection pressures on alignments of coding sequences. In: The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing, 2nd edn. Cambridge University Press, New York, NY, pp 419–452
96. Anisimova M, Nielsen R, Yang Z (2003) Effect of recombination on the accuracy of the likelihood method for detecting positive selection at amino acid sites. Genetics 164:1229–1236
97. Kosakovsky Pond SL, Posada D, Gravenor MB, Woelk CH, Frost SDW (2006) GARD: a genetic algorithm for recombination detection. Bioinformatics 22:3096–3098
98. Dobson R, Stockdale C, Lapsley C, Wilkes J, McCulloch R (2011) Interactions among *Trypanosoma brucei* RAD51 paralogues in DNA repair and antigenic variation. Mol Microbiol 81:434–456
99. Holden NJ, Uhlin BE, Gally DL (2001) PapB paralogues and their effect on the phase variation of type 1 fimbriae in *Escherichia coli*. Mol Microbiol 42:319–330
100. Tsuru T, Kobayashi I (2008) Multiple genome comparison within a bacterial species reveals a unit of evolution spanning two adjacent genes in a tandem paralog cluster. Mol Biol Evol 25:2457–2473
101. Rappuoli R (2011) The challenge of developing universal vaccines. F1000 Med Rep 3:16. https://doi.org/10.3410/M3-16
102. Bambini S, Piet J, Muzzi A et al (2013) An analysis of the sequence variability of meningococcal fHbp, NadA and NHBA over a 50-year period in the Netherlands. PLoS One 8:e65043
103. Wachter J, Hill S (2016) Positive selection pressure drives variation on the surface-exposed variable proteins of the pathogenic neisseria. PLoS One 11:e0161348. https://doi.org/10.1371/journal.pone.0161348
104. Counoupas C, Pinto R, Nagalingam G, Hill-Cawthorne GA, Feng CG, Britton WJ, Triccas JA (2016) *Mycobacterium tuberculosis* components expressed during chronic infection of the lung contribute to long-term control of pulmonary tuberculosis in mice. NPJ Vaccines 1:16012

Chapter 6

Alphavirus-Based Antigen Preparation

Kenneth Lundstrom

Abstract

Alphavirus-based vectors present an efficient approach for antigen preparation applied for vaccine development. Semliki Forest virus, Sindbis virus, and Venezuelan equine encephalitis virus have been engineered for high-level expression of antigens targeting infectious diseases and tumors. Alphaviruses possess a large application range as vectors can be delivered as naked RNA replicons, recombinant viral particles, and layered DNA plasmids. Immunization studies in animal models have provided protection against challenges with lethal doses of pathogenic infectious agents and tumor cells. So far, a limited number of clinical trials have been conducted for alphavirus vectors in humans.

Key words Alphavirus vectors, Immunization, Viral vaccines, Cancer vaccines, Protection against lethal challenges

1 Introduction

Alphaviruses belong to the family *Togaviridae* consisting of a positive sense single-stranded RNA (ssRNA) genome encapsulated in a capsid protein covered by a membrane envelope structure [1] (Fig. 1). Among the approximately 30 alphaviruses, Semliki Forest virus (SFV) [2], Sindbis virus (SIN) [3], and Venezuelan equine encephalitis virus (VEE) [4] have been engineered as vectors for recombinant gene expression. Typically, three types of expression vector systems have been developed, represented by replication-deficient viral particles, replication-proficient viral particles, and layered DNA/RNA plasmid vectors [5]. The common feature of all three systems relates to the presence of the alphavirus nonstructural genes, which makes these vectors self-replicating and resulting in high-level RNA replication in infected cells with extreme transgene expression. In the case of recombinant particles, in vitro RNA is transcribed from plasmid DNA, electroporated into mammalian host cells, preferentially baby hamster kidney (BHK) cells, and mature viral particles are harvested. In contrast, layered DNA/RNA vectors where the SP6 RNA polymerase promoter

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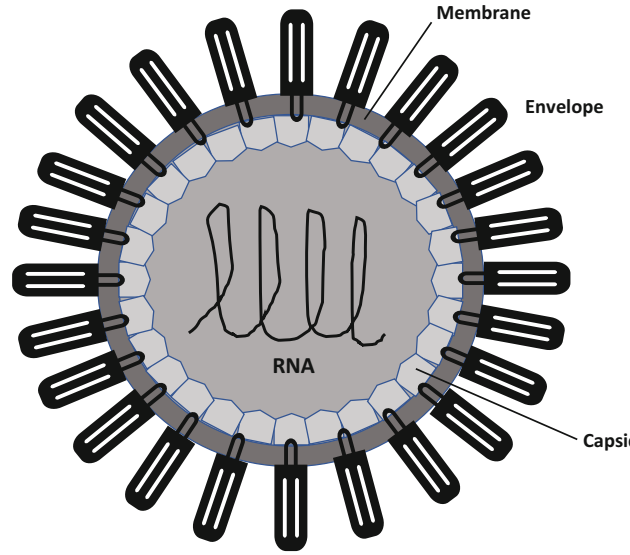

Fig. 1 Structure of alphavirus particle. In alphavirus particles, the genomic single-stranded RNA is surrounded by the capsid protein, the membrane, and envelope spike protein trimers consisting of E1, E2, and E3 (only for SFV) glycoproteins

has been replaced by a CMV promoter allows for direct transfection/immunization with plasmid DNA [5]. Alphavirus vectors have demonstrated a favorable flexibility related to immunization as delivery is possible as naked RNA replicons, recombinant viral particles, and DNA plasmids.

In the context of immunization studies in animal models, VEE particles expressing the hemagglutinin (HA) gene from the Hong Kong influenza A isolate A/HK/156/97 protected vaccinated chicken against challenges with lethal doses of influenza virus [6]. In another approach, immunization of mice with SFV particles expressing the HA and nucleoprotein (NP) genes provided protection against influenza virus challenges [7]. In the context of Lassa virus vaccine development, VEE particles expressing Lassa virus glycoproteins of distantly related clades I and IV from a bicistronic VEE vector with two 26S subgenomic promoters were subjected to immunization studies in mice, resulting in protection against challenges against Lassa virus [8]. Moreover, VEE particles expressing the Ebola virus NP (EBOV-NP) and EBOV glycoprotein (GP) rendered both immunized mice and guinea pigs resistant to challenges with lethal doses of EBOV [9, 10]. In another study, vaccination of primates with VEE particles resulted in protection against intramuscular and aerosol challenges with EBOV [11]. Related to RNA-based delivery, in a comparative study, synthetic mRNA and self-amplifying VEE RNA expressing influenza HA both provided protection against influenza HA challenges, but

64 times less self-amplifying VEE RNA (1.25 μg) was required compared to synthetic mRNA (80 μg) [12]. In the context of plasmid DNA-based delivery, a single intramuscular immunization of a SIN DNA vector expressing the herpes simplex virus type I glycoprotein B (HSV-1-gB) protected mice from lethal HSV-1 challenges [13]. Moreover, SIN DNA-based expression of the p85 antigen generated long-term protection against *Mycobacterium tuberculosis* in mice [14]. One aspect of utilizing alphavirus DNA replicons instead of conventional DNA plasmids for immunization relates to the much lower dose needed for reaching the same level of response [15, 16]. For example, 100- to 1000-fold lower doses of SIN-HSV-1-gB elicited strong immune responses and protection against lethal HSV-1 challenges in mice [13].

Related to cancer vaccines, reduction in tumor volume (87%) and significantly prolonged survival were observed after administration of SFV particles expressing interleukin-12 (IL-12) in a syngeneic RG2 rat glioma model [17]. Moreover, tumor cell replication was targeted by intraperitoneal administration of SFV particles carrying six micro-RNAs (miRNAs), which resulted in glioma targeting, limited spread in the central nervous system, and significantly prolonged survival in BALB/c mice with tumor xenografts [18]. In another study, the naturally oncolytic M1 alphavirus was evaluated in a liver tumor model resulting in selective killing of zinc-finger antiviral protein (ZAP)-deficient cancer cells and potent oncolytic activity [19]. Related to RNA-based delivery, a single injection of SFV-LacZ RNA provided complete protection of mice against colon tumor challenges [20]. Moreover, vaccination 2 days after tumor cell administration prolonged the survival of mice by 10–20 days. In the context of alphavirus-based DNA vectors, intradermal administration of SFV vectors expressing the human papilloma virus (HPV) E6 and E7 antigens generated effective therapeutic antitumor activity with 85% tumor-free mice [21]. In comparison to conventional DNA plasmids, a 200-fold lower equimolar dose of 0.05 μg of SFV replicon DNA was sufficient to achieve therapeutic efficacy. In the context of melanoma, immunization with SIN DNA-based vectors expressing the melanoma cell adhesion molecule (MCAM/MUC18) provided protection against lethal challenges with tumor cells in both primary and metastatic mouse tumor models [22].

So far, alphaviruses have been subjected to a limited number of clinical trials. In this context, VEE particles carrying a CMV gB or a PP65/IE1 fusion protein were subjected to a randomized double-blind, phase I clinical trial in CMV seronegative volunteers [23]. Intramuscular or subcutaneous administration proved safe and elicited neutralizing antibody and multifunctional T cell responses. Moreover, subcutaneous administration of VEE-Gag in healthy HIV-negative volunteers in a randomized, double-blind, placebo-controlled phase I study in the USA and

South Africa showed good tolerance, but only modest immune responses [24]. In another clinical trial, VEE particles expressing the carcinoembryonic antigen (CEA) were administered at intramuscular doses of 4×10^7 IU to 4×10^8 IU in four cycles every third week [25]. Repeated administration induced clinically relevant CEA-specific T cell and antibody responses and longer survival was seen in patients with CEA-specific T cell responses. Another phase I trial was conducted with VEE particles expressing the prostate-specific membrane antigen (PSMA) in patients with castration resistant metastatic prostate cancer (CRPC) [26]. Administration of five doses of 0.9×10^7 IU or 3.6×10^7 IU of VEE-PSMA was well tolerated although only weak PSMA-specific responses were detected, most likely due to suboptimal dosing.

The use of alphavirus vectors describing the steps from plasmid DNA preparation to virus particle production and immunization studies including cell culture techniques, virus purification and concentration and titer determination methods is presented below. All methods describe here are for SFV, although they are, in most cases, similarly applicable for SIN and VEE.

2 Materials

2.1 Reagents and Equipment

1. Restriction endonucleases *Nru*I, *Spe*I.
2. 0.8% agarose gel.
3. Gel electrophoresis apparatus.
4. Phenol–chloroform–isoamyl alcohol 25:24:1 (v/v/v).
5. 3 M Sodium acetate, pH 4.8.
6. 95% and 70% (v/v) ethanol.
7. 10× SP6 Buffer: 400 mM HEPES, pH 7.4, 60 mM magnesium acetate, 20 mM spermidine.
8. 10 mM $m^7G(5')$ ppp (5′) G sodium salt (Roche Molecular Biochemicals).
9. 50 mM Dithiothreitol (DTT).
10. rNTP Mix: 10 mM rATP, 10 mM rCTP, 10 mM rUTP, 5 mM rGTP.
11. 10–50 U/μL RNase inhibitor.
12. 10–20 U/μL SP6 RNA polymerase (Amersham Pharmacia Biotech).
13. RNase-free water (DEPC treated).
14. Phosphate buffered saline (PBS).
15. Trypsin–ethylenediamine tetraacetic acid (EDTA): 0.25% trypsin, 1 mM EDTA × 4 Na.

16. Sterile electroporation cuvettes, 0.2 and 0.4 cm (Bio-Rad or BTX).
17. Electroporator (Bio-Rad Gene Pulser).
18. Tissue culture flasks (T25, T75, and T175).
19. Microwell plates (6-, 12-, and 24-well plates).
20. Falcon tubes (15 and 50 mL).
21. Plastic syringes (1, 10, and 50 mL).
22. Sterile 0.22 μm filters.
23. MicroSpin™ S-200 HR Columns (Amersham).
24. Dulbecco's modified F-12 medium.
25. Iscove's modified Dulbecco's medium.
26. Opti-MEM I reduced-serum medium.
27. X-gal stock solution: 50 mM K ferricyanide, 50 mM K ferrocyanide, 1 M MgCl, 2% X-gal in DMF or DMSO.
28. X-gal staining solution: 1× PBS, 5 mM K ferricyanide, 5 mM K ferrocyanide, 2 mM MgCl, 1 mg/mL X-gal.
29. Moviol 4-88 containing 2.5% DABCO (1,4-diazobicyclo-[2.2.2]-octane).
30. Lysis buffer: 50 mM Tris–HCl pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Nonidet P-40 (NP40).
31. Hybond ECL nitrocellulose filter.
32. TBST: Tris-buffered saline with 0.1% Tween 20.
33. ECL Chemiluminescence kit (Amersham).
34. Starvation medium: methionine-free MEM, 2 mM glutamine, 20 mM HEPES.
35. Chase medium: E-MEM, 2 mM glutamine, 20 mM HEPES, 150 μg/mL unlabeled methionine.

2.2 Cell Lines

The following cell lines are used for alphavirus production and studies on expression of recombinant proteins:

1. BHK-21 cells (Baby hamster kidney) (ATCC-CCL-10).
2. CHO-K1 cells (Chinese ovary cells) (ATCC-CCL-61).
3. COS7 cells (African green monkey cells) (ATCC-CRL-1651).
4. HEK293 cells (Human embryonic kidney) (ATCC CRL-1573).

2.3 Cell Culture Media

1. BHK-21, CHO-K1, and HEK293 cells are cultured in a 1:1 mixture of Dulbecco's modified F-12 medium (Gibco BRL) and Iscove's modified Dulbecco's medium (Gibco BRL) supplemented with 4 mM glutamine and 10% fetal calf serum (FCS).

2. COS7 cells are cultured in DMEM (Dulbecco's Modified Eagles Medium) supplemented with 5% fetal calf serum, 5 mM glutamine and 0.1% penicillin/streptomycin.

2.4 Alphavirus Plasmid Vectors

For the three most commonly used alphaviruses, SFV, SIN and VEE, similar expression and helper vectors have been engineered and most methods are the same. However, the focus here is on SFV only and more detailed information on SIN can be found elsewhere [27]. The following vectors were applied for generation of replicon RNA and recombinant viral particles:

1. pSFV1 (basic vector, minimal cloning sites: *Bam*HI, *Sma*I) [2].
2. pSFV2gen (multilinker cloning sites: *Apa*I, *Bam*HI, *Xho*I, *Spe*I, *Sma*I) [28].
3. pSFV-Helper2 (second generation helper vector) [29].
4. pSFV4 (full-length vector) [30].
5. VA7(74) (full-length vector from avirulent strain) [31].
6. pSCA1 (layered DNA-RNA vector with CMV promoter) [32].
7. pSCA-Helper (layered DNA-RNA helper vector with CMV promoter) [33].
8. pCMV-SFV4 (full length layered DNA-RNA vector) [34].

For the generation of replication-deficient recombinant SFV particles, either pSFV1 [4] or pSFV2gen (also called pSFV4.2) [28] vectors are used together with the pSFV-Helper2 [29] vector (Fig. 2). In contrast, pSFV4 [30] or VA7(74) [31] vectors are applied for the production of full-length replication-proficient particles. Layered DNA-RNA vectors [33] can be used directly for transfection of host cells for recombinant proteins or alternatively together with the layered DNA-RNA helper vector or as a full-length layered DNA-RNA vector [34] for production of replication-deficient and proficient particles, respectively.

3 Methods

3.1 Subcloning into SFV Vectors

Genes of interest can be introduced into the cloning sites of the chosen SFV expression vector and restriction endonuclease digestions and nucleotide sequencing can be used for verification of inserts. Preparation of high-purity DNA (Midiprep or Maxiprep DNA) is highly recommended to provide the best possible quality and yields of in vitro–transcribed RNA for recombinant viral particle production and/or immunization (*see* **Note 1**).

3.2 DNA Linearization

Efficient in vitro transcription requires complete linearization of plasmid DNA. The pSFV1 and pSFV-Helper2 vectors are linearized by *Spe*I and pSFV2gen by *Nru*I.

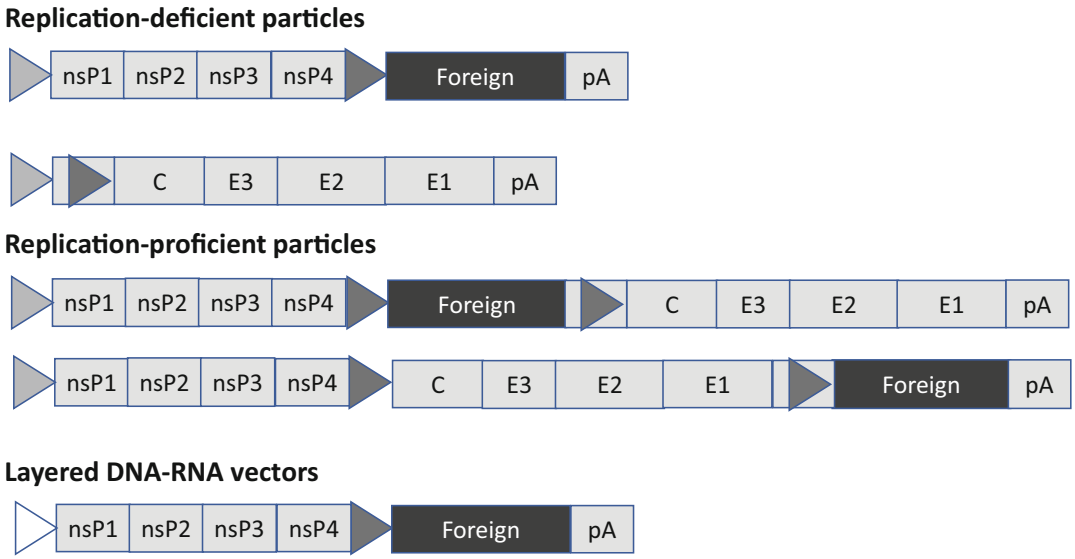

Fig. 2 Schematic illustration of alphavirus vectors. The application of different alphavirus vectors for expression studies: **Replication-deficient particles** are generated by cotransfection of expression vector and helper vector RNA into BHK-21 cells. **Replication-proficient particles** are produced by transfection of a full-length RNA genome including the foreign gene of interest introduced downstream of the nonstructural replicase genes (nsP1–4) or the structural genes (C-E3-E2-E1). **DNA-RNA layered vectors** are used for direct plasmid DNA transfections. SP6 RNA polymerase promoter illustrated as light gray triangle, subgenomic 26S promoter as dark gray triangle, and CMV promoter as white triangle

1. Recombinant SFV plasmids are linearized by *Spe*I or *Nru*I under standard restriction digestion conditions.
2. Complete digestions are confirmed by agarose gel electrophoresis and linearized DNA constructs are purified by phenol/chloroform extraction followed by precipitation with 0.3 M sodium acetate (final) and 2.5× volume of 95% ethanol (overnight at −20 °C or 15 min at −80 °C).
3. The ethanol precipitates are centrifugated for 15 min at 18,000 × *g* at +4 °C and washed with 70% ethanol. DNA pellets are air-dried or lyophilized after repeated centrifugation for 5 min and resuspended in RNase-free H_2O at a final concentration of 0.5 μg/μL (measured in a spectrophotometer).

Alternatively, MicroSpin™ S-200 HR Column purification can be used for DNA purification.

3.3 In Vitro Transcription

It is highly recommended that each batch of in vitro–transcribed RNA is prepared fresh for immunizations or electroporations, although RNA transcripts can be stored for shorter periods (e.g., weeks) at −80 °C. It is most important to set up the in vitro transcription reactions at room temperature as the spermidine in the SP6 buffer might precipitate at lower temperatures. It is

recommended that the optimized SP6 RNA polymerase buffer (*see* Subheading 2.1) is used instead of available commercial in vitro transcription buffers (*see* **Note 2**). Add enzyme components last because they are the least stable reactants in the reaction.

In Vitro Transcription Reaction
5 μL (2.5 μg) linearized plasmid DNA.
5 μL 10× SP6 buffer.
5 μL 10 mM $m^7G(5')ppp(5')G$.
5 μL 50 mM DTT.
5 μL rNTP mix.
x μL RNase-free H_2O to reach a final volume of 50 μL.
1.5 μL (50 U/μL) RNase inhibitor.
3.5 μL (20 U/μL) SP6 RNA polymerase.

1. All reaction components are mixed and incubated for 1 h at 37 °C. However, in case of large inserts, yields can be improved by prolonging the incubation time (*see* **Note 3**).
2. The quality of in vitro–transcribed RNA is assessed by loading 1–4 μL aliquots on 0.8% agarose gels. High-quality RNA is visible as thick bands without smearing along the axis of migration with an approximate mobility of 8 kb (compared to DNA markers) from the expression vector and a slightly faster mobility of helper RNA. Each in vitro transcription reaction is anticipated to generate 20–50 μg of RNA. The RNA is quantified by comparison of the intensity of RNA bands to DNA markers or by spectrophotometric measurements.
3. In vitro–transcribed RNA is directly applied to electroporation or lipid-mediated transfection. In case RNA transcripts are stored at −80 °C, the quality of RNA should be reevaluated by agarose gel electrophoresis before use.

3.4 Electroporation of RNA

Generally, the use of BHK-21 cells is preferred for alphavirus production as they are known to produce high-titer virus stocks [2]. Alternative host cells can be considered, but the production process needs to be optimized for each cell line employed. The following conditions have been found optimal for obtaining high-titer SFV stocks (e.g., 1×10^9 infectious particles/mL) in BHK-21 cells.

1. Cells are cultured under 85–95% humidity and 5% CO_2 with a low passage number in T175 flasks to no more than 80% confluency, washed once with PBS, and treated with 6 mL trypsin–EDTA per T175 flask for 5 min at 37 °C to detach the cells.

2. Cells are resuspended in 25 mL cell culture medium, centrifugated for 5 min at 800 × *g* and cell pellets are resuspended in a small volume (<5 mL) of PBS.
3. The volume is increased to 25 mL with PBS followed by recentrifugation for 5 min at 800 × *g*.
4. Cells are resuspended in approximately 2.5 mL PBS per T175 flask, equivalent to 1–2 × 10^7 cells/mL. Cells should be subjected to electroporation immediately although short-term storage (<1 h) on ice is acceptable.
5. BHK-21 cell suspension (0.4 mL) is transferred to 0.2 cm cuvettes (0.8 mL to 0.4 cm cuvettes). In vitro–transcribed recombinant RNA (20–45 μL) and helper RNA (20 μL) are added to the cell suspension and two consecutive pulses are applied with the following settings for the Bio-Rad Gene Pulser:

	0.2 cm cuvette	0.4 cm cuvette
Capacitance extender	960 μF	960 μF
Voltage	1500 V	850 V
Capacitor	25 μF	25 μF
Resistance (pulse controller)	∝ Ω	disconnected
Expected time constant (TC)	0.8 s	0.4 s

The Bio-Rad Gene Pulser II requires the following modifications:

1. The pulse controller should be set to "high range" and "∝".
2. The capacitance rotary should be switched to "high capacitance."
3. The following settings should be applied: 360 V and 75 μF.
4. The resistance for 0.2 cm cuvettes should be 10 Ω and the time constant 0.7–0.8 s.
5. Cells are immediately diluted 25-fold in cell culture medium and transferred to T25 flasks or 100 mm culture plates for overnight incubation at 37 °C in an incubator with 5% CO_2.

3.5 Lipid-Mediated Transfection of RNA

As an alternative to electroporation, DMRIE-C and other transfection reagents can be used to transfect BHK-21, COS7, or CHO-K1 cells.

1. BHK-21 cells (1.5–3 × 10^5) are cultured in 35 mm petri dishes or on 6-well plates to approximately 80% confluency.
2. Cells are washed with Opti-MEM I reduced-serum medium.
3. Cationic lipid-RNA complexes are prepared as follows:

(a) For each transfection sample, 0, 3, 6, 9, 12, and 15 μL of DMRIE-C is added to a 1.5 mL microcentrifuge tube containing 1 mL Opti-MEM I reduced-serum medium at room temperature.

(b) In vitro–transcribed recombinant RNA (10 μL, ~5 μg) and helper RNA (5 μL, ~2.5 μg) are mixed.

(c) The RNA (15 μL total) is added to each tube containing the DMRIE and vortexed briefly.

4. The lipid-RNA complex is immediately added to the cells and incubated at 37 °C for 4 h.
5. The transfection medium is replaced with prewarmed (37 °C) complete BHK medium and the BHK-21 cells are incubated at 37 °C overnight in an incubator with 5% CO_2.

3.6 Harvest of Recombinant SFV Particles

Production of recombinant SFV particles occurs within the first 24 h, resulting in high titers of approximately 10^9 infectious particles/mL. The titers can be increased to some extent by extending the incubation time by another 24 h.

1. Virus particles are harvested by carefully removing the medium from the BHK-21 cells.
2. Virus stocks are sterilized and purified by forcing the harvested medium through a 0.22 μm filter to remove cell debris and possible contaminants.
3. Virus stocks are aliquoted before storage at −20 °C (for weeks) or at −80 °C (for years) as repeated cycles of freezing and thawing can reduce the titers significantly.

3.7 Activation of Recombinant SFV Particles

Although the conventional pSFV-helper vector has been initially used, it is recommended to utilize the second-generation vector pSFV-Helper2, as it contains three point mutations at the cleavage site between the E2 and E3 proteins in the p62 precursor [29]. Generation of infectious particles requires activation with α-chymotrypsin as described below. In contrast to SFV particles generated with the pSFV-helper vector, particles produced with the pSFV-Helper2 vector are conditionally infectious and thereby prevent accumulation of replication-proficient particles through recombination, thus providing an additional level of biosafety.

1. Particles are activated by addition of α-chymotrypsin at a final concentration of 500 μg/mL for 20 min at room temperature.
2. The reaction is terminated by addition of aprotinin (trypsin inhibitor) at a final concentration of 250 μg/mL and the activated particles are ready to use.

3.8 Verification of Virus Titers

The titers of replication-proficient virus can be verified by standard plaque assay methods [35]. In contrast, as replication-deficient particles do not generate plaques, methods for titer determination are limited although indirect titer estimations are possible by calculating the number of infected cells visualized by reporter gene expression.

1. BHK-21 (or other) cells are cultured to a defined confluency on 6- or 12-well plates or on sterile coverslips before infection with serial dilutions (e.g., fivefold dilutions in the range expected to give 20–50 positive cells per microscope field) of virus stocks expressing green fluorescent protein (GFP) or β-galactosidase.
2. Cells expressing the reporter gene (fluorescent or stained cells, see below) are counted after 24 h incubation at 37 °C. Certain SFV mutant vectors may provide a weak signal at 24 h post-infection and should be incubated for 48 h to reach optimal expression levels.

3.8.1 GFP Detection

1. The number of GFP-positive cells are counted applying fluorescence microscopy.
2. The approximate titers are estimated as infectious particles/mL based on the number of GFP-positive cells per well, by taking into account the virus dilution.

3.8.2 X-gal Staining

1. SFV-infected cells are washed with PBS, fixed in cold methanol (99.8%) at −20 °C for 5 min and washed again three times with PBS.
2. Cells are stained for at least 2 h in X-gal staining solution at 37 °C or at room temperature.
3. The number of β-galactosidase (blue) positive cells are counted applying light microscopy.
4. Approximate titers are estimated as described for GFP detection above.

For comparison of GFP and X-gal methods, please *see* **Note 4**.

3.8.3 Immunofluorescence

If antibodies are available against the recombinant protein itself, or against tags engineered in the vector construct, immunofluorescence methods can be applied for titer determination.

1. SFV-infected cells are cultured on sterile coverslips, rinsed twice with PBS and fixed for 6 min at −20 °C in methanol.
2. Coverslips are washed three times in PBS and incubated for 30 min at room temperature in PBS containing 0.5% gelatin and 0.25% BSA to prevent nonspecific binding.

3. The blocking buffer is replaced with a primary antibody in the same buffer for 30 min at room temperature and washed three times with PBS and incubated with a secondary antibody for 30 min at room temperature.
4. Washed coverslips are rewashed three times with PBS, once more with H_2O, and air-dried.
5. Coverslips are mounted on glass slides using 10 μL Mowiol 4-88 containing 2.5% DABCO (1,4-diazobicyclo-[2.2.2]-octane), the number of positive cells is counted, and titers estimated as described for GFP detection above.

As alphavirus infection causes changes to the cell morphology (i.e., they round up) microscopic examination can also be applied to provide an approximate estimate of titers. Similar to fluorescence and staining (described above), the titers can be estimated by counting the rounded cells.

3.8.4 RT-PCR-Based Titer Determination

Another alternative for titer determination is to apply RT-PCR [36].

1. RNA from SFV stocks is extracted using the RNeasy Kit (Qiagen).
2. qRT-PCR is applied for sequence amplification within the nsP1 gene of SFV.
3. A serial dilution is made of pSFV plasmid DNA to obtain a standard curve for SFV-RNA.
4. Mean Cts are applied to a standard curve equation to determine the cDNA copy number in each sample.
5. The total SFV RNA copy number present in the original SFV sample is calculated by multiplying the cDNA copy number by the conversion factor specific to each sample considering all dilutions made and the yield of RNA extraction.

3.9 Evaluation of Gene Expression

Initial confirmation of recombinant protein expression is recommended before proceeding to immunization studies as it enables verification of expression levels and the size of gene products. Expression evaluation can be performed by Western blotting if antibodies are available against the target protein or engineered tag fusions. Alternatively, expression can be evaluated by metabolic labeling of SFV-infected cells with ^{35}S-methionine.

3.9.1 Western Blotting

1. Host cells (BHK-21, CHO-K1, HEK293) cultured on 6-, 12-, or 24-well plates are infected with serial dilutions of virus stocks and incubated for 1–2 days at 37 °C.
2. Cells are lysed with 250 μL, 125 μL, and 62.5 μL of lysis buffer per 6-, 12-, and 24-well plate, respectively, incubated for 10 min on ice and samples are loaded onto 10–12% SDS-PAGE (polyacrylamide gel electrophoresis) gels.

3. Electrophoresed protein material is transferred to Hybond ECL nitrocellulose filters for 30 min.
4. Filters are treated with 5% nonfat dry milk at +4 °C for 30 min followed by primary and secondary antibody treatment, each for 30 min at room temperature.
5. Specific bands are visualized with the ECL Chemiluminescence kit.

3.9.2 Metabolic Labeling

1. Host cells (BHK-21, CHO-K1, or HEK293) cultured on 6-, 12-, or 24-well plates are infected with serial dilutions of virus stocks and incubate for 1–2 days at 37 °C.
2. The medium is removed, cells washed once with PBS, Starvation medium added, and cells are incubated for 30 min at 37 °C.
3. The medium is replaced with Starvation medium containing 50–100 μCi/mL of ^{35}S-methionine and cells are incubated for 20 min at 37 °C.
4. The medium is removed, cells washed twice with PBS, and Chase medium added for appropriate time (e.g., 15 min to 3 h).
5. The Chase medium is removed, cells washed once with PBS, 250 μL lysis buffer added per 6-well plate, and cells are incubated 10 min on ice.
6. Samples are loaded on 10–12% SDS-PAGE under standard conditions, fixed in 10% acetic acid, 30% methanol for 30 min at room temperature and replaced with Amplify® for 30 min at room temperature.
7. The gel is dried and exposed on Hyperfilm-MP for 2–24 h (depending on signal) at room temperature or at −80 °C applying radioactivity-intensifying screens for visualization (*see* **Note 5**).

3.10 Virus Stock Purification

Although it is possible to use alphavirus particles directly for expression studies in cell lines after a single filter-sterilization procedure, in vivo applications for immunization in animal models gain from additional purification steps. Moreover, rigid purification procedures are mandatory for application of alphavirus particles for clinical trials. For this purpose, various methods based on ultracentrifugation and affinity chromatography can be employed as described below.

3.10.1 Ultracentrifugation of Virus Stocks

1. A step gradient is prepared in ultracentrifuge tubes by addition of 1 mL of 50% sucrose solution (bottom) and 3 mL of 20% sucrose solution (top).

2. Virus stock solution is added (9 mL for SW 40 Ti or 8 mL for SW 41 Ti) onto the sucrose gradient and centrifugated at 160,000 × *g* (30,000 rpm in SW 40 Ti or SW41 Ti rotor) for 90 min at +4 °C.
3. Virus settled near the interface between the 20% and 50% sucrose layers is collected by discarding the medium fraction and the bottom 0.8 mL consisting of 50% sucrose.

3.10.2 Centriprep Concentration

1. Virus stocks are loaded onto the sample container of the Centriprep concentrator as described by the manufacturer.
2. The assembled concentrator is centrifugated at an appropriate *g*-force (according to the manufacturer's recommendations), until the fluid levels inside and outside the filtrate collector equilibrate.
3. The device is removed, the airtight seal cap is snapped off, the filtrate is decanted, the cap is replaced, and the concentrator is centrifugated a second time.
4. The filtrate is decanted, the twist-lock cap is loosened, and the filtrate collector is removed.
5. The concentrated virus sample is collected with a 1 mL disposable plastic pipette. If further concentration of virus is desired, it can be centrifugated again after decanting the filtrate.

3.10.3 Affinity Chromatography Concentration

Efficient removal of endotoxins and other contaminants can be achieved by application of Matrex® Cellufine™ Sulfate columns, which also provides a convenient procedure for virus stock concentration. The manufacturer's recommendations should be followed as described below.

1. The affinity matrix column is equilibrated with adsorption buffer (0.01 M phosphate, 0.1 M NaCl, pH 7.5) and samples are loaded at pH 7.5.
2. The column is washed with several bed volumes of adsorption buffer to remove nonbinding contaminants and the concentrated virus is eluted with elution buffer (1–2 M NaCl or KCl).

3.11 Immunizations

Although the topic of this chapter relates to antigen preparation it is essential to include some information of the immunization process for RNA, viral particles, and DNA. In the context of alphavirus-based vaccine development, a large number of immunization studies have indeed been conducted [5].

3.11.1 Immunization of Mice with RNA

In vitro–transcribed alphavirus RNA can be directly used for administration of RNA replicons for immunization experiments in mice, as follows [20].

1. In vitro–transcribed SFV-LacZ RNA (100 μg) is administered intramuscularly into BALB/c mice.
2. The immune response is evaluated by monitoring the presence of IgG antibodies against recombinant β-galactosidase protein by ELISA 21 days post-injection.
3. Splenocytes are isolated 21 days after immunization and are restimulated in vitro for 6 days in the presence of Ld-restricted peptide β-gal 876–884 (1 μg/mL) for monitoring of β-galactosidase-specific $CD8^+$ T cell recognition.

Evaluation of tumor protection of mice immunized with in vitro–transcribed RNA can be performed as follows.

1. Mice are administered intravenously with 5×10^5 CT26.CL25 tumor cells (from mouse colon) and tumor protection is evaluated 21 days postimmunization.
2. The number of pulmonary metastases is counted after 12 days.
3. In case of preestablished tumors, BALB/c mice are injected intravenously with 1×10^5 CT26.CL25 cells and tumors are grown for 2 days before immunization with 100 μg SFV-LacZ RNA.
4. Animal survival is assessed.

3.11.2 Immunization of Mice with Recombinant VEE Particles

1. Alphavirus particles (10^6) are diluted in PBS and injected subcutaneously into the plantar surface of each footpad of C57BL/6 mice three times at 2 weeks intervals [37].
2. Vaccinated mice are challenged with 7.5×10^4 B16F10 tumor cells (from mouse melanoma) intradermally 2 weeks after immunization for tumor protection evaluation.
3. Therapeutic efficacy is addressed by an initial inoculation of 7.5×10^4 B16F10 tumor cells (either intradermally or intravenously) followed by three weekly vaccinations with alphavirus particles.

3.11.3 Immunization of Macaques with Recombinant VEE Particles

1. Immunization with 10^{10} VEE-EBOV GP focus forming units (FFUs) is conducted intramuscularly in the quadriceps muscle of naïve cynomolgus macaques for vaccine development against Ebola virus [11].
2. Vaccinated animals are challenged intramuscularly and intranasally with approximately 1000 PFU of Ebola virus and are monitored closely for at least 28 days.

3.11.4 DNA Immunization of C57BL/6 Mice with DNA

1. C57BL/6 mice are immunized with 3 μg layered DNA-RNA plasmid vectors by five weekly intramuscular injections, which can be enhanced by plasmid-coated gold particles applying gene gun technology [38].

2. Mice are inoculated with 1×10^5 B16F10 tumor cells 1 week after the last immunization and monitored for tumor growth for at least 3 weeks.

3.11.5 DNA Immunization of BALB/c Mice with Recombinant VEE Particles

1. SFV plasmid DNA vectors expressing membrane proteins PrM and E of Murray Valley encephalitis virus (MVE) are diluted in saline to a concentration of 1 mg/mL and doses of 100–125 mg DNA and are injected intramuscularly into BALB/c mice [39].
2. Immunized mice are challenged intraperitoneally with 1.3×10^8 PFU of MVE and signs of encephalitis are observed for 21 days. Alternatively, SPF mice are immunized intramuscularly with 100 mg DNA into multiple sites in the hind leg muscles and are boosted after 21 days.
3. Mice are intracranially challenged with 1000 $TCID_{50}$ of MVE 2 weeks after the final immunization and are monitored for signs of encephalitis for 21 days.

3.12 Future Directions and Potential for Other Applications

Antigen production applying alphaviruses has been straight forward and successful. The clear advantages of alphavirus-based approaches relate to easy and rapid vector preparation, the self-amplifying RNA replication leading to enhanced transgene expression, and for example in the case of DNA-based delivery, the requirement of 100- to 1000-fold lower dose concentrations for immunization [13]. Likewise, it has been demonstrated that 64 times less self-amplifying RNA (1.25 μg) compared to synthetic mRNA (80 μg) was required to provide protection in immunized mice [12]. In addition to alphavirus vector development including less cytotoxic mutants [40, 41] (*see* **Note 6**) and translation enhancement signals [42], delivery issues have been addressed particularly for RNA by lipid nanoparticle encapsulation [43]. In this context, self-amplifying VEE RNA encapsulated in lipid nanoparticles enhanced immunogenicity compared to delivery of unformulated RNA providing a novel approach for improved vaccine development.

4 Notes

1. To ensure the production of both good quality and high quantity of in vitro–transcribed RNA, it is recommended to use plasmid DNA preparations of high purity.
2. Optimization of in vitro transcription yields can be achieved by titration of the CAP analogue $m^7G(5')ppp(5')G$ concentration and the use of an appropriate transcription buffer. Although commercially available buffers might generate high RNA

yields, the quality is not always compatible with efficient production of high titer virus.

3. The length of gene inserts introduced into the expression vector might affect the RNA yields, typically when inserts exceed 4 kb. The RNA yields can be improved to some extent by extension of the incubation time for in vitro transcription reactions.
4. In comparison of the GFP and β-gal methods for the verification of virus titers, the fluorescence-based GFP approach is easier to carry out as no fixing or staining of cells is required. It also allows one to follow the duration of expression. The drawback is the requirement of a fluorescence microscope.
5. Visualization of radioactively labeled proteins via SDS-PAGE can efficiently be enhanced by application of film cassettes with double-sided X-ray intensifying screens [44].
6. Due to the cytopathic effects caused by alphaviruses on host cells, the time of host cell survival and transgene expression levels might be reduced. To address these problems, several mutant SFV [40] and SIN [41] vectors have been engineered. Increased expression has also been achieved by applying alphavirus vectors with' translation enhancement signals [42].

References

1. Strauss J, Strauss E (1994) The alphaviruses; gene expression, replication and evolution. Microbiol Rev 58:491–562
2. Liljestrom P, Garoff H (1991) A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Bio/Technology 9:1356–1361
3. Xiong C, Levis R, Shen P, Schlesinger S, Rice CM, Huang HV (1989) Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. Science 243:1188–1191
4. Davies NL, Brown KW, Johnston RE (1989) In vitro synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant. Virologie 171:189–204
5. Lundstrom K (2014) Alphavirus-based vaccines. Viruses 6:2392–2415
6. Schultz-Cherry S, Dybing JK, Davis NL, Williamson C, Suarez DL, Johnston R et al (2000) Influenza virus (A/HK/156/97) hemagglutinin expressed by an alphavirus replicon system protects against lethal infection with Hong Kong-origin H5N1 viruses. Virology 278:55–59
7. Fleeton MN, Chen M, Berglund P, Rhodes G, Parker SE, Murphy M et al (2001) Self-replicative RNA vaccines elicit protection against influenza A virus, respiratory syncytial virus, and a tickborne encephalitis virus. J Infect Dis 183:1395–1398
8. Wang M, Jokinen J, Tretyakova I, Pushko P, Luikashevich IS (2018) Alphavirus vector-based replicon particles expressing multivalent cross-protective Lassa virus glycoproteins. Vaccine 36:683–690
9. Wilson JA, Hart MK (2001) Protection from Ebola virus mediated by cytotoxic T-lymphocytes specific for the viral nucleoprotein. J Virol 75:2660–2664
10. Pushko P, Bray M, Ludwig GV, Parker M, Schmaljohn A, Sanchez A et al (2000) Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. Vaccine 19:142–153
11. Herbert AS, Kuehne AI, Barth JF, Ortiz RA, Nichols DK, Zak SE et al (2013) Venezuelan equine encephalitis virus replicon particle vaccine protects nonhuman primates from intramuscular and aerosol challenge with Ebolavirus. J Virol 87:4952–4964
12. Vogel AB, Lambert L, Kinnear E, Busse D, Erbar S, Reuter KC et al (2018) Self-amplifying

RNA vaccines give equivalent protection against influenza to mRNA vaccines but at much lower doses. Mol Ther 26:446–455

13. Hariharan MJ, Driver DA, Townsend K, Brumm D, Polo JM, Belli BA et al (1998) DNA immunization against herpes simplex virus: enhanced efficacy using a Sindbis virus-based vector. J Virol 72:950–958
14. Kirman JR, Turon T, Su H, Li A, Kraus C, Polo JM et al (2003) Enhanced immunogenicity to Mycobacterium tuberculosis by vaccination with an alphavirus plasmid replicon expressing antigen 85A. Infect Immun 71:575–579
15. Reddy JR, Kwang J, Varthakavi V, Lechtenberg KF, Minocha HC (1999) Semliki Forest virus vector carrying the bovine viral diarrhea virus NS3 (p80) cDNA induced immune responses in mice and expressed BVDV protein in mammalian cells. Comp Immunol Microbiol Infect Dis 22:231–246
16. Knudsen ML, Ljungberg K, Tatoud R, Weber J, Esteban M, Liljestrom P (2015) Alphavirus replicon DNA expressing HIV antigens is an excellent prime for boosting with recombinant Ankara (MVA) or with HIV gp140 protein antigen. PLoS One 10: e0117042
17. Roche FP, Sheahan BJ, O'Mara SM, Atkins GJ (2010) Semliki Forest virus-mediated gene therapy of the RG2 rat glioma. Neuropathol Appl Neurobiol 36:648–660
18. Ylosmaki E, Martikainen M, Hinkkanen A, Saksela K (2013) Attenuation of Semliki Forest virus neurovirulence by microRNA-mediated detargeting. J Virol 87:335–344
19. Lin Y, Zhang H, Liang J, Li K, Zhu W, Fu L, Wang F, Zheng X, Shi H, Wu S et al (2014) Identification and characterization of alphavirus M1 as a selective oncolytic virus targeting ZAP-defective human cancers. Proc Natl Acad Sci U S A 111:E4504–E4512
20. Ying H, Zaks TZ, Wang R-F, Irvine KR, Kammula US, Marincola FM et al (1999) Cancer therapy using a self-replicating RNA vaccine. Nat Med 5:823–827
21. Van de Wall S, Ljungberg K, Ip PP, Boerma A, Knudsen ML, Nijman HW et al (2018) Potent therapeutic efficacy of an alphavirus replicon DNA vaccine expressing human papilloma virus E6 and E7 antigens. Oncoimmunology 7:e1487913
22. Leslie MC, Zhao YJ, Lachman LB, Hwu P, Wu GJ, Bar-Eli M (2007) Immunization against MUC18/MCAM, a novel antigen that drives melanoma invasion and metastasis. Gene Ther 14:316–323
23. Bernstein DI, Reap EA, Katen K, Watson A, Smith K, Norberg P et al (2010) Randomized, double-blind, Phase I trial on an alphavirus replicon vaccine for cytomegalovirus in CMV negative volunteers. Vaccine 28:484–493
24. Wecker M, Gilbert P, Russell N, Hural J, Allen M, Pensiero M et al (2012) Phase I safety and immunogenicity evaluations of an alphavirus replicon HIV-1 subtype C gag vaccine in healthy HIV-1-uninfected adults. Clin. Vaccine Immunol 19:1651–1660
25. Morse MA, Hobelka AC, Osada T, Berglund P, Hubby B, Negri S et al (2010) An alphavirus vector overcomes the presence of neutralizing antibodies and elevated numbers of Tregs to induce immune responses in humans with advanced cancer. J Clin Investig 120:3234–3241
26. Slovin SF, Kehoe M, Durso R, Fernandez C, Olson W, Gao JP et al (2013) A phase I dose escalation trial of vaccine replicon particles (VRP) expressing prostate-specific membrane antigen (PSMA) in subjects with prostate cancer. Vaccine 31:943–949
27. Ehrengruber MU, Lundstrom K (2016) Recombinant alphavirus-mediated expression of ion channels and receptors in the brain. In: Luján R, Ciruela F (eds) Receptor and ion channel detection in the brain: methods and protocols, Neuromethods, vol 110. Springer Science + Business Media, New York, NY. https://doi.org/10.1007/978-1-4939-3064-7_7
28. Schweitzer C, Kratzeisen C, Adam G, Lundstrom K, Malherbe P, Ohresser S et al (2000) Characterization of [^{3}H] LY-354740 binding of rat $mGlu_2$ and $mGlu_3$ receptors expressed in CHO cells using Semliki Forest virus vectors. Neuropharmacology 39:1700–1706
29. Berglund P, Sjöberg M, Garoff H, Atkins GJ, Sheahan BJ, Liljestrom P (1993) Semliki Forest virus expression system: production of conditionally infectious recombinant particles. Bio/-Technology 11:916–920
30. Liljestrom P, Lusa S, Huylebroeck D, Garoff H (1991) In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular weight membrane protein modulates virus release. J Virol 65:4107–4113
31. Vähä-Koskela MJ, Tuittila MT, Nygardas PT, Nyman JK, Ehrengruber MU, Renggli M (2003) A novel neurotropic expression vector based on the avirulent A7(74) strain of Semliki Forest virus. J Neurovirol 9:1–15
32. DiCiommo DP, Bremner R (1998) Rapid, high-level protein production using

DNA-based Semliki Forest virus vectors. J Biol Chem 273:18060–18066

33. DiCiommo DP, Duckett A, Burcescu I, Bremner R, Gallie BR (2004) Retinoblastoma protein purification and transduction of retina and retinoblastoma cells using improved alphavirus vectors. Invest. Ophthalmol Vis Sci 45:3320–3329
34. Ulper L, Sarand I, Rausalu K, Merits A (2008) Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with a n inserted intron. J Virol Methods 148:265–270
35. Puglia AL, Rezende AG, Jorge SA, Wagner R, Pereira CA, Astray RM (2013) Quantitative RT-PCR for titration of replication-defective recombinant Semliki Forest virus. J Virol Methods 193:647–652
36. Juare D, Long KC, Aguilar P, Kochel TJ, Halsey JS (2013) Assessment of plaque assay methods for alphaviruses. J Virol Methods 187:185–189
37. Avogadri F, Merghoub T, Maughan MF, Hirschhorn-Cymerman D, Morris J, Ritter E et al (2010) Alphavirus replicon particles expressing TRP-2 provide potent therapeutic effect on melanoma through activation of humoral and cellular immunity. PLoS One 5: e12670
38. Leitner WW, Hwang NL, de Veer MJ, Zhou A et al (2003) Alphavirus-based DNA vaccine breaks immunological tolerance by activating innate antiviral pathways. Nat Med 5:33–39
39. Colombage G, Hall R, Pavy M, Lobigs M (1998) DNA-based and alphavirus-vectored immunization with PrM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. Virology 250:151–163
40. Lundstrom K, Abenavoli A, Malgaroli A, Ehrengruber MU (2003) Novel Semliki Forest virus vectors with reduced toxicity and temperature-sensitivity for long-term enhancement of transgene expression. Mol Ther 7:202–209
41. Agapov EV, Frolov I, Lindenbach BD, Pragal PM, Schlesinger S, Rice CM (1998) Noncythopathic Sindbis virus RNA vectors for heterologous gene expression. Proc Natl Acad Sci U S A 95:12989–12994
42. Sjöberg EM, Suomalainen M, Garoff H (1994) A significantly improved Semliki Forest virus expression system based on translation enhancer segments from the viral capsid gene. Bio/Technology 12:1127–1131
43. Geall AJ, Verma A, Otten GR, Shaw CA, Hekele A, Banerjee K et al (2012) Nonviral delivery of self-amplifying RNA vaccines. Proc Natl Acad Sci U S A 109:14604–14609
44. Voytas D, Ke N (2001) Detection and quantitation of radiolabeled proteins and DNA in gels and blot. Curr Protoc Mol Biol Appendix 3:3A

Chapter 7

Production of Chimeric Hepatitis B Virus Surface Antigens in Mammalian Cells

Mihaela-Olivia Dobrica, Catalin Lazar, and Norica Branza-Nichita

Abstract

The small (S) envelope protein of the Hepatitis B Virus (HBV), HBV-S, has the unique ability to self-assemble into highly immunogenic subviral particles (SVPs), in the absence of other viral factors, in eukaryotic cells, including those of nonhepatic origin. This feature is currently exploited for generation of SVPs exposing heterologous epitopes on their surface that can be used as vaccine candidates to target various diseases. Here, we describe a simple and robust method for production of such chimeric HBV-S protein-based SVPs in transiently transfected HEK293T cells and purification from cell supernatants by ultracentrifugation on sucrose cushion and sucrose step gradients. The SVPs obtained by this methodology have been successfully used in immunogenicity studies in animal models.

Key words HBV, Antigens, Protein production, Purification, Subviral particles, Vaccine

1 Introduction

A variety of expression systems are available for production of protein antigens and vaccine development. The ideal vaccine candidate should be highly immunogenic and suitable for high yield production at low costs. While *Escherichia coli* is the most cost-efficient production platform for many soluble proteins, mammalian transmembrane and secretory proteins undergoing complex folding and posttranslational processing are usually not compatible with expression in prokaryotic cells [1]. These transformations, requiring intra- and intermolecular disulfide bonds and specific glycosylation patterns, are often crucial for the biological properties, including protein antigenicity. By providing the appropriate eukaryotic milieu for protein processing as well as scalability, yeast and insect cell cultures have become popular alternative hosts to prokaryotes for high yield expression of many biopharmaceutical

Mihaela-Olivia Dobrica and Catalin Lazar contributed equally to this work.

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proteins. However, the limited capacity of their N-glycosylation pathway to produce only high-mannose oligosaccharides remains an important drawback when expressing proteins that are structurally or functionally dependent on N-linked glycan processing to complex type structures [2, 3]. In this case, the mammalian cell is the host of choice as it provides all posttranslational modifications of the native protein.

The Hepatitis B Virus (HBV) small (S) surface antigen (HBV-S) is a multispanning transmembrane protein that forms disulfide bridge-stabilized dimers within the endoplasmic reticulum (ER) of the host cells [4]. The dimers spontaneously associate into 20 nm-diameter subviral particles (SVPs) that do not incorporate the viral capsid and genetic material and are secreted from cells, independent of virions (Fig.1) [5]. These virus-like particles (VLPs) are highly immunogenic, non-infectious and can be produced in large amounts in heterologous expression systems in the absence of any other viral components, which has led to their development into efficient and safe vaccines against HBV [6]. These remarkable properties of the HBV-S protein have been exploited to generate chimeric SVPs carrying foreign and HBV-derived epitopes either fused or co-expressed with HBV-S [7–11]. Several insertion sites have been tested for their ability to accommodate epitopes of different lengths within the luminal domain of the S proteins, containing the major B-cell epitopes (the "a" determinant), and two have been shown to be compatible with SVP production and secretion. Chimeric HBV-S proteins bearing foreign peptide sequences allowed for VLP formation and secretion and triggered specific humoral and cellular immune responses against the native protein [7, 8].

Here we describe a method for mammalian cell production and purification of chimeric HBV particles that combines relevant virus neutralization epitopes of the large (L) and S envelope proteins for further use in immunological investigations. Our previous studies have indicated that the HBV S/preS1$^{21–47}$ chimera obtained by insertion of the 21–47 amino acids sequence of the preS1 domain of the L protein between residues 126 and 127 of the "a" determinant of S (genotype D) preserves the SVPs properties and is secretion competent. Moreover, the chimeric protein is a more efficient immunogen than the HBV-S protein, the major component of the current vaccine [10, 11]. Our approach employs transient transfection of adherent HEK293T cells with plasmids encoding for HBV S/preS1$^{21–47}$ and HBV-S, followed by purification of corresponding SVPs from cell supernatant by ultracentrifugation on sucrose cushion and step gradients. This method is simple and scalable and may be applied to similar chimeric HBV particles displaying relevant immunogenic peptides derived from other pathogens of medical interest.

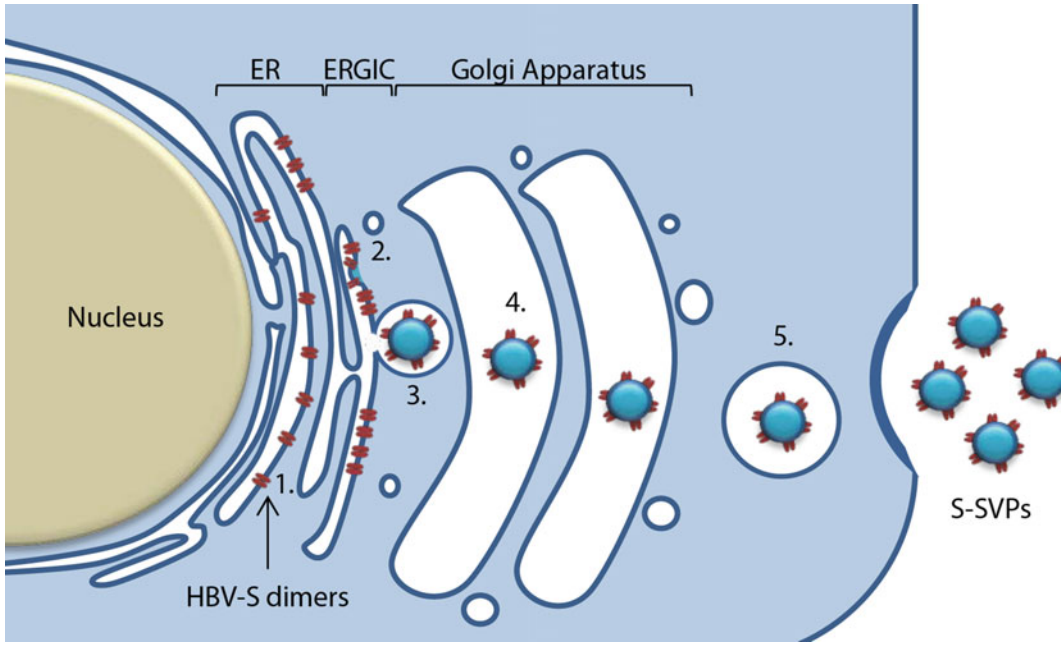

Fig. 1 Assembly of HBV-S subviral particles (S-SVPs). The HBV-S protein is cotranslationally inserted into the ER membrane where it forms disulfide-linked dimers (1) that are further transported to the ER-Golgi intermediate compartment (ERGIC). The dimers associate into higher molecular weight disulfide-linked oligomers that self-assemble with lipids and bud into the ERGIC compartment (2). The resulting 20 nm-particles are sorted into transport vesicles (3), trafficked through the Golgi (4) and exported from cells via the constitutive secretory pathway (5)

2 Materials

Prepare all buffers and solutions using deionized water and analytical grade reagents. All cell culture steps are performed in a Biosafety Level 2 laboratory. Manipulation of cells and cell culture reagents is performed in a laminar flow hood using sterile consumables, unless specified otherwise.

2.1 Cell Culture

1. HEK293T cells (European Collection of Animal Cell Culture, Porton Down, UK).
2. Dulbecco's Modified Eagle Medium with GlutaMAX™-I (DMEM, 1×). Add 50 mL fetal bovine serum (*see* **Note 1**) and 5 mL nonessential amino acids 100× to a 500 mL DMEM bottle to obtain complete medium. Store at 4 °C.
3. Cell detaching reagent: 0.05% trypsin, 0.05% EDTA in PBS.
4. Cell culture petri dishes (55 cm^2) or flasks (75 cm^2).
5. Falcon tubes.
6. Disposable serological pipettes.
7. CO_2 incubator, set at 37 °C and 5% CO_2.

8. Laminar flow hood.
9. For large-scale production: 1700 cm^2 ribbed-surface roller bottles and Roll-In CO_2 control incubator.

2.2 Cell Transfection

1. Dissolve plasmids pCi-S (encoding for the wild-type HBV-S protein) and pCi-S/preS1$^{21–47}$ (encoding for the chimeric HBV S/L protein) in 10 mM Tris–HCl, pH 8.5 at final concentrations higher than 1 μg DNA/μL (*see* **Note 2**).
2. Transfection reagent (TR): 1 mg/mL polyethylenimine (PEI), pH 7.
3. Transfection buffer (TB): Opti-MEM I-GlutaMAX-I.

2.3 Detection of Denatured HBV Antigens

1. Cell lysis buffer: 0.01 M Tris–HCl, pH 7.5, 0.002 M EDTA, 0.150 M NaCl, 0.5% Triton X-100 and protease inhibitor cocktail (1×) (*see* **Note 3**).
2. Reducing SDS sample loading buffer (5×): 0.25 M Tris–HCl (pH 6.8), 10% SDS, 25% β-mercaptoethanol, 0.1% bromophenol blue, 50% glycerol. Store aliquots at −20 °C.
3. Nonreducing SDS sample loading buffer (5×): 0.25 M Tris–HCl (pH 6.8), 10% SDS, 0.1% bromophenol blue, 50% glycerol. Store aliquots at −20 °C.
4. SDS 10% polyacrylamide (PAA) gels.
5. SDS-PAA gel electrophoresis (SDS-PAGE) running buffer: 0.025 M Tris–HCl, pH 8.2, 0.190 M glycine, 0.1% SDS.
6. Peptide: N-glycosidase F (PNG-ase F) and Endoglycosidase H (Endo H) (*see* **Note 4**).
7. Nitrocellulose membrane.
8. Western-blot semidry transfer buffer: 0.025 M Tris–HCl, 0.192 M glycine, 20% methanol.
9. Phosphate buffer saline (PBS; 10×): 1.4 M NaCl, 0.027 M KCl, 0.1 M Na_2HPO_4, 0.018 M KH_2PO_4, pH 7.4.
10. Western-blot blocking solution: 10% nonfat milk in PBS (1×).
11. Western-blot washing solution: 0.1% Tween in PBS (1×).
12. Primary mouse anti-preS1 antibody, secondary anti-mouse-HRP antibody.
13. Antibody dilution solution: 1% nonfat milk, 0.1% Tween in PBS (1×).
14. Enhanced Chemiluminescence detection kit.
15. Purified HBV-L protein for the standard curve.
16. BCA protein assay kit.
17. Prestained molecular weight standards.
18. Autoradiography films.

19. Refrigerated centrifuge.
20. Mini PROTEAN Tetra system.
21. Semidry transblot system.
22. Heater.

2.4 Detection of Native HBV Antigens

1. ELISA—Monolisa HBsAg ULTRA kit (Bio-Rad) (*see* **Note 5**).
2. Multichannel pipette.
3. 96-well plate absorbance reader.

2.5 Antigen Purification

1. Sucrose solutions: 15%, 20%, 25%, 35%, 45%, and 60% sucrose in water. All solutions are heat-sterilized and stored at 4 °C (*see* **Note 6**).
2. Ultracentrifuge (SW32Ti and SW41Ti rotors).
3. Ultracentrifuge tubes.
4. Vortex.
5. Dialysis membranes, 10 MWCO.
6. Liquid nitrogen.
7. Lyophilizer.

3 Methods

Perform all steps at room temperature, unless otherwise indicated.

3.1 Small-Scale HBV Antigen Expression

1. Seed 5×10^5 HEK293T cells in 6-well plates; add 2 mL of complete DMEM and grow in a CO_2 incubator for 24 h.
2. Replace cell media with 2 mL of fresh DMEM and transfect cells with either pCi-S or pCi-S/preS1$^{21–47}$ plasmids, using a mixture of 200 μL TB, 6 μL TR, and 2 μg plasmid DNA per well (*see* **Note 7**). Keep nontransfected HEK293T cells as a control (*see* **Note 8**).

3.2 Biochemical Characterization of HBV Antigens

1. Harvest the HEK293T cells and supernatants at 48 h posttransfection.
2. Briefly collect cells by centrifugation at 1200 × *g* for 2 min then incubate resulting pellets with lysis buffer, for 30 min, on ice.
3. Clarify lysates by centrifugation at 10,000 × *g*, for 10 min, at 4 °C. Determine the total protein concentration using the BCA kit, as indicated by the supplier.
4. To monitor the N-glycosylation status of HBV proteins, treat samples either with PNG-ase F or EndoH enzymes, following the protocol provided by the supplier (*see* **Note 9**).
5. Heat-denature sample volumes (equivalent of 30 μg of total protein/lane) and molecular standards (equivalent of 2 μg protein/lane) in the presence of either nonreducing or reducing SDS sample loading buffer, at 95 °C, for 5 min (*see* **Note 10**).

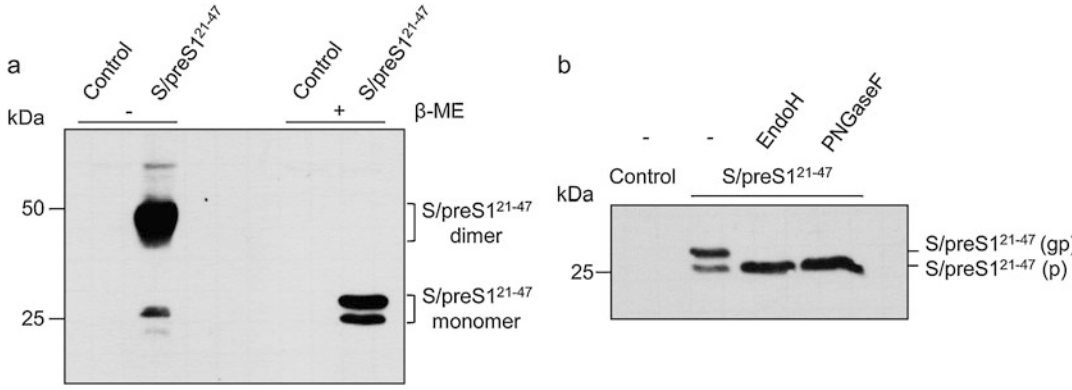

Fig. 2 Expression, oligomerization, and N-glycosylation of the HBV-S/preS1$^{21–47}$ chimeric antigen in HEK293T cells. Lysates of pCi-HBV-S/preS1$^{21–47}$- or mock-transfected (control) HEK293T cells were subjected to SDS-PAGE in the presence (+) or absence (−) of β-mercaptoethanol (β-ME) (**a**). The gels show the migration pattern of antigen monomers and dimers. Cell lysates were also treated with either PNGase F or Endo H prior to SDS-PAGE (**b**). Proteins were detected by western blot using anti-preS1 antibodies. The de-glycosylated (p) and glycosylated (gp) forms of the HBV antigen are shown

6. Load samples on SDS-10% PAA gels and electrophorese at 30 mA until the blue dye front reaches the bottom of the gel.
7. Wet nitrocellulose membranes in western-blot transfer buffer and transfer proteins from gels using a semidry blotter, for 75 min at 60 mA.
8. Block the membranes with 10% nonfat milk in PBS for 1 h.
9. Incubate membranes with mouse anti-preS1 antibody (1/1000 in antibody dilution buffer), for 1 h (*see* **Note 11**).
10. Wash the membranes 3× with western-blot washing solution, 10 min each time.
11. Incubate membrane with anti-mouse-HRP secondary antibody (1/10,000, in antibody dilution buffer) for 1 h.
12. Incubate membranes with ECL substrate as recommended by the manufacturer and visualize proteins by autoradiography (Fig. 2).
13. Determine secretion of HBV antigens in serially diluted extracellular medium, by using the Monolisa HBsAg ULTRA and the protocol provided by the manufacturer.
14. Quantify the level of secreted HBV antigens considering the cut-off of the assay (0.06 ng/mL) and the sample dilution factor (*see* **Note 5**).

3.3 Large-Scale Cell Culture and Transfection

1. Seed 1 × 10^6 or 1.5 × 10^6 HEK293T cells in 55 cm^2 Petri dishes or 75 cm^2 flasks, respectively. Grow the cells in complete DMEM for 2 days to become 80–90% confluent.
2. Wash cells with PBS, add trypsin solution to cover the cell monolayer and return to the incubator for 2 min (*see* **Note 12**).

3. Add 10 mL DMEM and gently tap the sides of the culture dishes to detach the cells. Pipette cells up and down using a 10 mL serological pipette, until all clumps are dispersed and a homogenous suspension is obtained.
4. Transfer the cell suspension to Falcon tubes and centrifuge at 1200 × *g* for 2 min. Remove the supernatant and resuspend the pellet in 10 mL complete DMEM.
5. Transfer cell suspensions from 5 × 55 cm^2 petri dishes or 4 × 75 cm^2 flasks to 1700 cm^2 ribbed-surface roller bottles and add 240 mL complete DMEM. Grow the cells in a Roll-In CO_2 Control Incubator for 72 h.
6. Replace cell media with 200 mL fresh, complete DMEM. Transfect the HEK293T cells either with pCi-S or pCi-S/preS1$^{21–47}$ plasmids, using a mixture of 50 mL TB, 750 μL TR, and 500 μg plasmid DNA per bottle (*see* **Note 7**). Keep a bottle of nontransfected HEK293T cells as control. Return cells to the Roll-In CO_2 Control Incubator for 72 h.
7. Collect cell media and clarify by centrifugation at 10,000 × *g*, for 10 min, at 4 °C. Store at 4 °C until further use.
8. Add 200 mL fresh, complete DMEM to the cells and return the bottles to the incubator for another 72 h period (*see* **Note 13**).
9. Repeat **step 3**.
10. Harvest cells by flushing up and down 50 mL PBS using a 10 mL serological pipette. Centrifuge cell suspensions at 1200 × *g* for 10 min. Weigh resulting pellets and store at −20 °C until further use.
11. Analyze expression and secretion of HBV antigens by ELISA and western blot, as above.

3.4 Antigen Purification

1. Pour 4 mL of 20% sucrose in 38.5 mL ultracentrifuge tubes. Gently add 34 mL media on top of the sucrose cushion. Centrifuge samples at 32,000 rpm (125,755 × *g*, SW32Ti rotor) for 5 h, at 4 °C.
2. Carefully remove supernatants by using a serological pipette, leaving about 0.8 mL of samples at the bottom of the tube. Add 0.2 mL PBS and leave the tubes overnight on ice (*see* **Note 14**).
3. Gently vortex the tube for 10 s and pool resuspended pellets corresponding to a specific antigen to a final volume of 1.7 mL.
4. Analyze secretion of HBV antigens by ELISA and western blot, as in Subheading 3.2. Include transfected HEK293T cell lysates to compare migration patterns of intra- and extracellular antigens (Fig. 3) (*see* **Note 15**).
5. Pipet 2 mL of each of 60%, 45%, 35%, 25%, and 15% sucrose solutions in a 12 mL ultracentrifuge tube, starting with the higher concentration at the bottom. Gently layer the 1.7 mL of

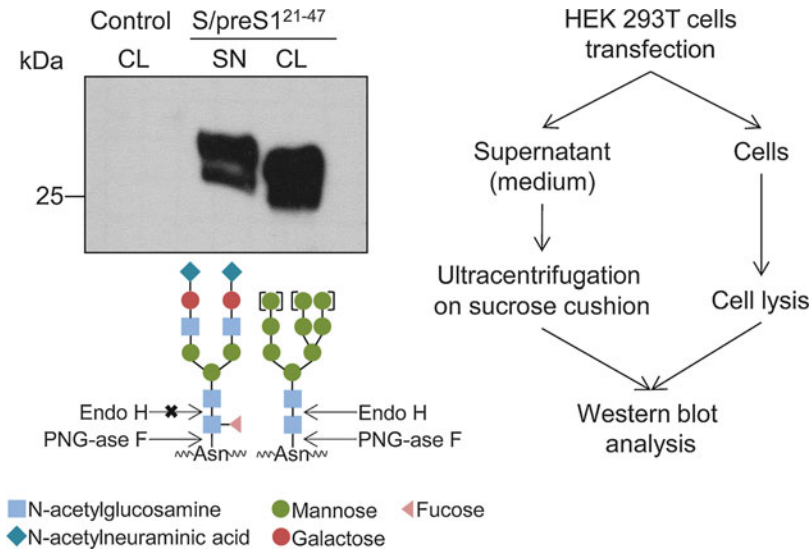

Fig. 3 Secretion of the HBV-S/preS1$^{21–47}$ chimeric antigen from HEK293T cells. Supernatant of pCi-HBV-S/preS1$^{21–47}$-transfected cells were concentrated by ultracentrifugation on a 20% sucrose cushion and loaded on SDS-PAGE along with corresponding cell lysates (CL), as indicated in the experimental flow chart. Proteins were detected by western blot using anti-preS1 antibodies. Mock-transfected cells were included as control (CL). The diagram below the gel shows a typical composition of complex (left)—and high-mannose N-linked oligosaccharides (right) and the corresponding endoglycosidases cleavage sites. The residues in brackets are usually trimmed in the ER by specific mannosidases

pooled sample on the top of the sucrose gradient using a 5 mL serological pipette. Centrifuge samples at 30,000 rpm (111,132 × *g*, SW41Ti rotor) for 16 h, at 4 °C (*see* **Note 16**).

6. Collect 750 μL fractions from the top of the gradient and perform ELISA using the Monolisa HBsAg ULTRA kit.
7. Pool HBV antigen-positive fractions and dialyze 3× against 3 L PBS 0.1× using SnakeSkin Dialysis Tubing with 10 kDa pores (*see* **Note 17**).
8. Freeze-dialyzed samples in liquid N_2 and concentrate by lyophilization (*see* **Note 18**).
9. Add sterile water to the protein powder to a final volume of 300 μL/antigen/1700 cm^2 roller bottle.
10. Quantify HBV antigens levels in the concentrated samples by using Monolisa HBsAg ULTRA kit. Estimate the yields per gram fresh cell weight.
11. Quantify HBV chimeric antigens by western blot using a standard curve made of known amounts of commercial L protein (the preS1 antigen, Beacle). A typical example of purified chimeric HBV protein is shown in Fig. 4 (*see* **Note 19**).
12. Determine the protein concentration in the concentrated samples by using the BCA protocol and calculate the purity of HBV antigens (*see* **Note 20**).

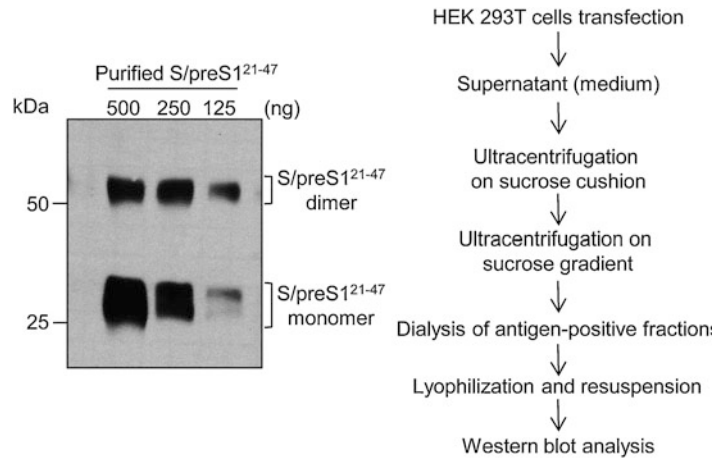

Fig. 4 Quantification of purified HBV-S/preS1$^{21–47}$ by western blot. Serial dilutions of the HBV-S/preS1$^{21–47}$ antigen purified from HEK293T supernatant were loaded on SDS-PAGE followed by western blot and detection with anti-preS1 antibodies. The bands shown correspond to protein monomers and dimers. The numbers above the panel indicate the amount of antigen/lane, as quantified using a preS1 standard curve and the same detection methodology. The protein purification approach is schematically depicted in the experimental flow chart

4 Notes

1. The fetal bovine serum is heat-inactivated at 56 °C for 30 min, aliquoted in 50 mL Falcon tubes, and stored at −20 °C.
2. Higher plasmid DNA concentration is desirable as it results in increased stability of the solution while minimizing the volume used in transfection reactions and hence the amount of salts from the solvent. The DNA quality is also essential for optimal transfection and we note that OD_{260}/OD_{280} ratios of 1.8 or higher are suitable. To ensure results reproducibility, prepare large stocks of plasmid DNA and store them at 4 °C for short term use (weeks) or aliquot and store at −20 °C. Repeated freeze–thaw cycles should be avoided as it damages the DNA solution.
3. The protease cocktail is dissolved and stored according to instructions provided by the manufacturer. When organic solvents are used, prepare stock solutions at least 100× concentrated to minimize cell toxicity. Always add the protease cocktail to the cell lysis buffer prior to use.
4. PNG-ase F de-glycosylates *N*-linked glycoproteins, regardless of their oligosaccharides structure. Endo H removes only high mannose and some hybrid types of the N-linked carbohydrates. Therefore, acquirement of complex glycans by proteins,

especially in the Golgi, results in resistance to Endo H digestion. Both PNG-ase F and Endo H treatments reduce the apparent molecular weight of the glycoprotein, which will change the migration pattern of the protein on SDS gels (as in Fig. 2b).

5. Detection of the HBV surface antigens and SVPs by the Monolisa HBsAg ULTRA kit (Bio-Rad) is based on the reactivity of a mixture of monoclonal antibodies that are highly dependent on the conformation of the "a" determinant of the S domain. Therefore, chimeric HBV proteins containing insertions of foreign epitopes within this region may be less well recognized and their quantification underestimated. In our laboratory we use western blotting under denaturing conditions and antibodies against linear epitopes (preS1) to complete this analysis.
6. Autoclave sucrose solutions at 100 °C for 20 min and store at 4 °C. Higher temperature and increased heat exposure may result in sucrose breakdown and sugar caramelization, which will turn the solution yellow-brown. Filter-sterilization is not an option for concentrated sucrose solutions as filters tend to clog.
7. Split TB in equal volumes and add TR and the plasmid DNA solution to each half, then mix them before cell transfection. This will prevent DNA being precipitated when in contact with concentrated TR.
8. We have also run experiments using HEK293T cells transfected with empty pCi vector as a control and found no difference in reactivity of cellular background proteins against anti-HBV antibodies when compared to nontransfected cells.
9. Samples subjected to PNG-ase F and Endo H digestions are denatured to increase de-glycosylation efficiency, which sometimes results in precipitation of proteins and signal loss in western blots. This can be prevented by reducing the amount of total proteins in the reaction volume.
10. Comparing samples migrated on SDS-PAGE under nonreducing and reducing conditions will provide a first indication on the ability of the HBV chimeric proteins to form disulfide-linked oligomers, a prerequisite for SVPs assembly (as in Fig. 2a).
11. Current commercial antibodies against HBV-S are conformation dependent; therefore, to detect HBV chimeric antigens by western blot, antibodies against linear epitopes are preferred. Membrane incubation with primary antibodies for 1 h is usually sufficient for good signals in western blot, when using monoclonal anti-preS1 antibodies. However, overnight incubation is recommended when the antigen is more diluted in analysed samples. No additional membrane washing is necessary.
12. HEK cells are easily detachable; therefore, any washing steps must be performed with care. Pipet solutions on the flask wall

rather than the cell monolayer. Check cellular morphology under a microscope and incubate a little longer if cells are still attached or not round-shaped yet.

13. This second incubation step is based on our observation that HEK293T cells still contain an important amount of HBV antigens at 72 h post-transfection and ensures complete release of assembled SVPs into the cell medium.
14. We have observed in our laboratory that some of the SVP sample always remains in the proximity of the pellet as a fine layer that is lost if the entire sucrose solution is removed. By leaving the tubes to rest overnight with a small volume of sample diluted 1:1 in PBS, the pellet gently self-detaches from the bottom of the tube resulting in a concentrated SVP solution that contains at most 10% sucrose.
15. Comparing the migration pattern of intra- and extracellular antigens on SDS-PAGE under reducing conditions provides valuable information about protein processing along the secretory pathway. Secreted glycosylated HBV antigens will always migrate slower than their intracellular counterparts due to trimming of the N-linked glycan to complex structures (as in Fig. 3). This process can be further confirmed by digestions with PNGase F and Endo H [10].
16. At this stage the SVP sample contains about 10% sucrose and can be loaded on top of the first, 15% sucrose-layer of the sucrose step gradient without additional dilution (*see* also **Note 13**).
17. Samples are dialyzed against diluted PBS to avoid concentration of salts in the antigen sample after lyophilization.
18. Lyophilization of the HBV antigens results in a protein powder that dissolves easily in water. However, we have observed a slight loss of antigen recognition by the highly conformation-dependent monoclonal antibodies of the Monolisa HBsAg ULTRA kit, suggesting that some epitopes may be denatured during this procedure.
19. Serially dilute a protein containing preS1 epitope of known concentration to generate a standard curve (500–20 ng). Proceed as in Subheading 3.2 above, **steps 6–11** [10].
20. Typically, this protocol leads to production of 28–30 μg/g and 3.75–4 μg/g cell fresh weight of HBV-S and HBV-S/preS1$^{21-47}$ SVPs, respectively, of about 10–15% purity, which is compatible with further analysis of the antigenic properties in animal models [10, 12]. Due to its simplicity and rapidity, this purification approach is particularly suitable for primary screening of a large number of antigens. It can also be used as a precursory step in more sophisticated purification protocols when production of highly pure SVPs might be required.

Acknowledgments

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References

1. Dalton AC, Barton WA (2014) Over-expression of secreted proteins from mammalian cell lines. Protein Sci 23(5):517–525
2. Herscovics A, Orlean P (1993) Glycoprotein biosynthesis in yeast. FASEB J 7(6):540–550
3. Altmann F, Staudacher E, Wilson IB, Marz L (1999) Insect cells as hosts for the expression of recombinant glycoproteins. Glycoconj J 16 (2):109–123
4. Siegler VD, Bruss V (2013) Role of transmembrane domains of hepatitis B virus small surface proteins in subviral-particle biogenesis. J Virol 87(3):1491–1496
5. Gilbert RJ, Beales L, Blond D, Simon MN, Lin BY, Chisari FV, Stuart DI, Rowlands DJ (2005) Hepatitis B small surface antigen particles are octahedral. Proc Natl Acad Sci U S A 102(41):14783–14788
6. Komatsu H (2014) Hepatitis B virus: where do we stand and what is the next step for eradication? World J Gastroenterol 20 (27):8998–9016
7. Lee IH, Kim CH, Ryu WS (1996) Presentation of the hydrophilic domains of hepatitis C viral E2 envelope glycoprotein on hepatitis B surface antigen particles. J Med Virol 50 (2):145–151
8. Netter HJ, Macnaughton TB, Woo WP, Tindle R, Gowans EJ (2001) Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C virus epitopes. J Virol 75(5):2130–2141
9. Patient R, Hourioux C, Vaudin P, Pages JC, Roingeard P (2009) Chimeric hepatitis B and C viruses envelope proteins can form subviral particles: implications for the design of new vaccine strategies. New Biotechnol 25 (4):226–234
10. Dobrica MO, Lazar C, Paruch L, Skomedal H, Steen H, Haugslien S, Tucureanu C, Caras I, Onu A, Ciulean S, Branzan A, Clarke JL, Stavaru C, Branza-Nichita N (2017) A novel chimeric Hepatitis B virus S/preS1 antigen produced in mammalian and plant cells elicits stronger humoral and cellular immune response than the standard vaccine-constituent, S protein. Antivir Res 144:256–265
11. Dobrica MO, Lazar C, Paruch L, van Eerde A, Clarke JL, Tucureanu C, Caras I, Ciulean S, Onu A, Tofan V, Branzan A, Urban S, Stavaru C, Branza-Nichita N (2018) Oral administration of a chimeric Hepatitis B Virus S/preS1 antigen produced in lettuce triggers infection neutralizing antibodies in mice. Vaccine 36(38):5789–5795
12. Beaumont E, Roingeard P (2015) Chimeric hepatitis B virus (HBV)/hepatitis C virus (HCV) subviral envelope particles induce efficient anti-HCV antibody production in animals pre-immunized with HBV vaccine. Vaccine 33 (8):973–976

Chapter 8

Bioreactor-Based Antigen Production Process Using the Baculovirus Expression Vector System

Julie Harnischfeger, Lukas Käßer, Jan Zitzmann, Denise Salzig, and Peter Czermak

Abstract

Several vaccines are already produced using the baculovirus expression vector system (BEVS). This chapter describes methods for generating recombinant baculoviral DNA (also called bacmid) for cultivating *Spodoptera frugiperda Sf*-9 cells and producing a baculovirus stock from the recombinant bacmid and for producing a protein-based vaccine with the BEVS in a stirred tank reactor.

Key words Process analytical technology, Online process monitoring, Dielectric spectroscopy, *Spodoptera frugiperda*, *Sf*-9 cells, Antigen production, Cell cultivation

1 Introduction

Various competing production platforms, based on different host organisms, are involved in the production of vaccines. Within this field, the baculovirus expression vector system (BEVS) is, although established more than 30 years ago, just becoming a state-of-the-art technology. Several BEVS-based vaccines for veterinary and human use, which are based on protein subunits (e.g., Flublock®, Protein Sciences Corporation, human influenza vaccine) or virus like particles (e.g., Cervarix®, GSK, human papillomavirus vaccine), have been approved and marketed in the past decades [1]. Consequently, BEVS can be considered to be an established tool, not only for research purposes but also for industrial production. The BEVS technology itself is based on insect cell-lines as such as *Sf*-9 or *Sf*-21 (*Spodoptera frugiperda*) and a corresponding recombinant *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV), carrying the genetic information for the target vaccine to be expressed. Because the production is based on virus infection, rather than on a stable chromosomal integration of the foreign genetic information, BEVS offers manufacturing speed, high flexibility, and yields

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high product titers at the same time. Posttranslational modification and protein folding are well supported, although the glycosylation pattern is not human. Genetically and metabolically engineered insect cell lines can be used for the production of recombinant products with humanized glycosylation [2]. As an additional benefit, insect cell-specific viruses, such as the *Ac*NPV, are not capable of gene expression in mammalian organisms, thus, meeting required safety regulations [3]. Due to the lytic nature of BEVS, a tight process control is necessary for large-scale bioreactor processes to ensure the effective timing of the key events—infection and harvesting. Therefore, advanced biomass monitoring strategies, for example based on dielectric spectroscopy (DS), have to be employed to generate data beyond the standard parameters: pH, dissolved oxygen, and stirrer speed [4, 5].

The following chapter describes all steps for the setup of a BEVS-based production process in a bioreactor, including advanced process monitoring (Fig. 1). The protocol can be used for the production of vaccine-related proteins, such as the exemplarily shown ORF2 protein (*see* **Note 1**), but is also applicable to other proteins of interest.

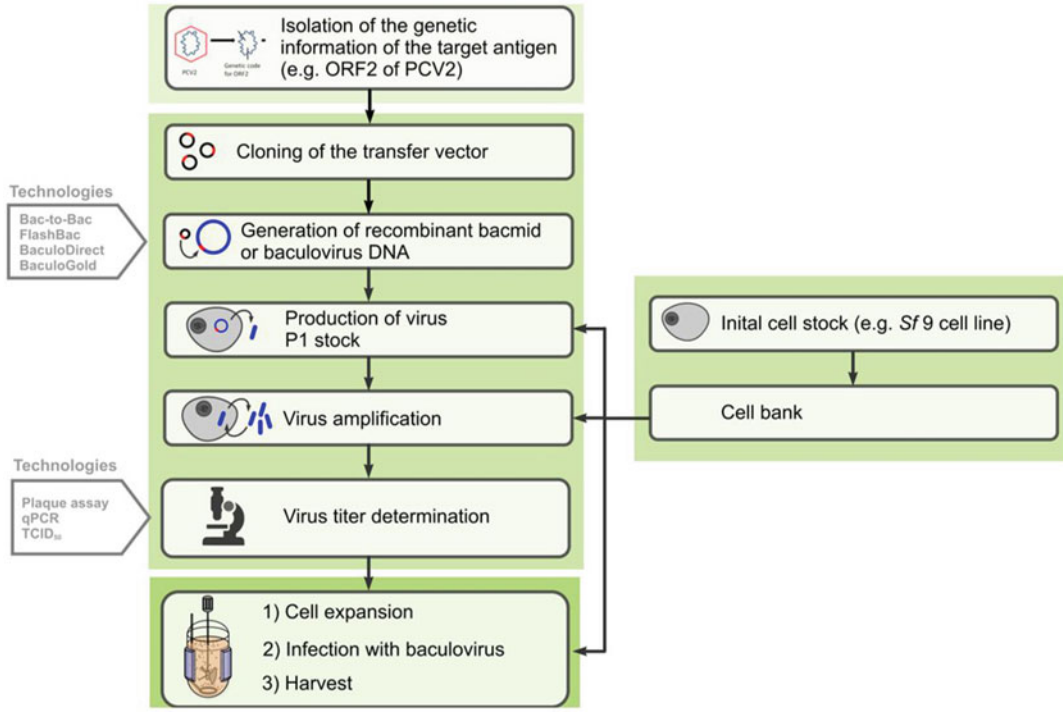

Fig. 1 Process flowchart for the production of recombinant proteins using BEVS

2 Materials

2.1 Transfer Vector Cloning

In this part of the chapter, the required materials for the cloning of the transfer vector are described. In addition to the genetic sequence of interest, which codes for the target protein, it is assumed that a lacZα cassette for blue–white screening is integrated.

1. Transfer cloning vector (e.g., pFastBac-1, Thermo Fisher Scientific, Waltham, MA, USA).
2. Genetic sequence of interest (e.g., ORF2 of PCV2).
3. Other sequences of genetic elements (a reporter gene, a secretion signal, etc.).

2.2 Generation of Recombinant Bacmid (Baculoviral DNA)

This part of the chapter describes the materials for the generation and analysis of recombinant bacmid DNA, based on the Bac-to-Bac™ Baculovirus Expression Vector System using MAX Efficiency® DH10-Bac™ (*see* **Note 2**).

2.2.1 Materials for Transformation of Competent MAX Efficiency® DH10-Bac™

1. Baculovirus expression vector system kit (e.g., Bac-to-Bac™ Baculovirus Expression System, Thermo Fisher Scientific, Waltham, MA, USA).
2. Cloned transfer vector (*see* Subheading 2.1).
3. Crushed ice for thawing the MAX Efficiency® DH10-Bac™.
4. S.O.C. medium (e.g., New England Biolabs, Ipswich, Massachusetts, USA).
5. LB medium for casting agar plates (e.g., granulated LB medium, Carl Roth, Karlsruhe, Germany, prepare according to the manufacturer's instructions).
6. Liquid LB medium for the dilution of the bacterial cell suspension (e.g., granulated LB medium, Carl Roth, Karlsruhe, Germany).
7. Agar plates containing 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) (*see* **Note 3**) and also kanamycin (working concentration 25 μg/mL), tetracycline (working concentration 15 μg/mL). Depending on the transfer vector, further or other antibiotics may be required (*see* **Note 4**).
8. Sterile single-use pipette tips (e.g., pipette tips, Sarstedt, Nümbrecht, Germany).
9. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
10. 1.5 mL centrifuge tubes (e.g., Sarstedt, Nümbrecht, Germany).

11. Thermomixer (e.g., Thermomixer comfort, Eppendorf, Hamburg, Germany).
12. Incubator (e.g., INCU-Line® IL 23, VWR, Radnor, Pennsylvania, USA).

2.2.2 Materials for Analysis of the Recombinant Bacmid Using LongAmp Taq Polymerase

1. MAX Efficiency® DH10-Bac™ colonies (e.g., Bac-to-Bac™ Baculovirus Expression System, Thermo Fisher Scientific, Waltham, MA, USA).
2. 1.5 mL centrifuge tubes (e.g., Sarstedt, Nümbrecht, Germany).
3. 5× LongAmp *Taq* Polymerase (e.g., New England Biolabs, Ipswich, Massachusetts, USA).
4. LongAmp *Taq* Reaction buffer (e.g., New England Biolabs, Ipswich, Massachusetts, USA).
5. Deoxyribonucleoside triphosphates (e.g., Deoxyribonucleoside (dNTP) Solution Mix, New England Biolabs, Ipswich, Massachusetts, USA).
6. M13 forward (-40) primer and M13 reverse primer (Source: e.g., Biomers.de, Ulm, Germany; Synthesis: e.g., Sigma-Aldrich, St. Louis, Missouri, USA).
7. Distilled water, cell-culture grade (e.g., Milli-Q Academic, Millipore, Burlington, Massachusetts, USA).
8. Sterile single-use pipette tips (e.g., pipette tips, Sarstedt, Nümbrecht, Germany).
9. Stained agarose gel (1% (w/v) agarose with for example SYBR Safe DNA Gel Stain, Invitrogen, Carlsbad, California, USA).
10. DNA loading buffer (e.g., Gel Loading Dye, Purple (6×), New England Biolabs, Ipswich, Massachusetts, USA).
11. DNA ladder (e.g., 2 Log DNA Ladder, New England Biolabs, Ipswich, Massachusetts, USA).
12. PCR tubes (e.g., Strip tubes for PCR, Thermo Fisher Scientific, Waltham, MA, USA).
13. Piston pipettes (e.g., Research Plus, Eppendorf, Hamburg, Germany).
14. Single-use pipette tips (e.g., pipette tips, Sarstedt, Nümbrecht, Germany).
15. Thermocycler (e.g., peqSTAR, VWR, Radnor, Pennsylvania, USA).
16. Electrophoresis chamber (e.g., Biozym Scientific, Hessisch Oldendorf, Germany).
17. Gel imager (e.g., ChemiDoc™XRS+ Imager, Bio-Rad Laboratories, Hercules, California, USA).

2.3 Isolation of Bacmid DNA and Buffer Preparation

This part of the chapter describes the materials for the isolation of the bacmid DNA and the materials for the required buffers.

2.3.1 Materials for Buffer Preparation

1. Tris hydrochloride buffer (Tris–HCl, e.g., Thermo Fisher Scientific, Waltham, MA, USA).
2. Ethylenediaminetetraacetic acid (EDTA, e.g., VWR, Radnor, Pennsylvania, USA).
3. RNase A (e.g., Omega Bio-tek, Norcross, GA, USA).
4. Sodium hydroxide (NaOH, e.g., Merck, Darmstadt, Germany).
5. Sodium dodecyl sulfate (SDS, pure Ph. Eur., AppliChem, Darmstadt, Germany).
6. Potassium acetate (e.g., Carl Roth, Karlsruhe, Germany).
7. Distilled water, cell-culture grade (e.g., Milli-Q Academic, Millipore, Burlington, Massachusetts, USA).
8. Sterile single-use pipette tips (e.g., serological pipettes, Sarstedt, Nümbrecht, Germany).
9. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
10. 100 mL glass bottle (e.g., Glasgerätebau Ochs, Bovenden, Germany).
11. Measuring cylinder (e.g., Glasgerätebau Ochs, Bovenden, Germany).
12. pH meter (e.g., FiveEasy™ FE20, Mettler Toledo, Columbus, Ohio, USA).

2.3.2 Materials for Isolation of Bacmid DNA

1. Positive clones (clones with insert) (*see* **Note 5**).
2. Liquid LB medium for the dilution of the cell suspension (e.g., granulated LB medium, Carl Roth, Karlsruhe, Germany, prepare according to the manufacturer's instructions).
3. Kanamycin and tetracycline in corresponding working concentrations (*see* **Note 4**).
4. Cultivation tubes (e.g., Sarstedt, Nümbrecht, Germany).
5. Resuspension buffer (containing Tris-HCl and EDTA).
6. Lysis buffer (containing NaOH and SDS).
7. Precipitate buffer (containing potassium acetate).
8. Absolute isopropanol (e.g., Merck, Darmstadt, Germany).
9. 70% (v/v) ethanol (e.g., Merck, Darmstadt, Germany).
10. Distilled water, cell-culture grade (e.g., Milli-Q Academic, Millipore, Burlington, Massachusetts, USA).

11. Crushed ice for incubation.
12. 1.5 mL centrifuge tubes (e.g., Sarstedt, Nümbrecht, Germany).
13. Sterile single-use pipette tips (e.g., serological pipettes, Sarstedt, Nümbrecht, Germany).
14. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
15. Incubator (e.g., INCU-Line® IL 23, VWR, Radnor, Pennsylvania, USA) with orbital shaker (e.g., Multitron Standard, Infors HT, Basel, Switzerland).
16. Centrifuge (e.g., Sigma 1-16K, Sigma-Aldrich, Hamburg, Germany).
17. Vortexer (e.g., Lab dancer, VWR, Radnor, Pennsylvania, USA).

2.4 Cell Bank

This part of the chapter describes the materials used for the operational steps described in Subheading 3.4.

2.4.1 Materials for Thawing Insect Cells

1. *Spodoptera frugiperda* cell suspension (1 mL) with a cell density of 2×10^7 cells/mL (e.g., TriEx *Sf*-9, Merck, Darmstadt, Germany).
2. 100 mL baffled shake flask (e.g., Glasgerätebau Ochs, Bovenden, Germany).
3. Insect cell culture medium (e.g., Sf900 II, Thermo Fisher Scientific, Waltham, MA, USA).
4. Sterile single-use pipette tips (e.g., serological pipettes, Sarstedt, Nümbrecht, Germany).
5. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
6. Centrifuge (e.g., Sigma 1-16K, Sigma-Aldrich, Hamburg, Germany).
7. Aspiration system (e.g., Vacusafe, Integra, Biebertal, Germany).
8. 15-mL centrifuge tubes (e.g., Sarstedt, Nümbrecht, Germany).
9. Incubator (e.g., B-line, Binder, Tuttlingen, Germany) with orbital shaker (e.g., Celltron, Infors HT, Basel, Switzerland).

2.4.2 Materials for Cell Counting by Trypan Blue Staining

1. 0.4 % trypan blue (e.g., Sigma-Aldrich, Hamburg, Germany).
2. Phosphate-buffered saline (PBS, e.g., Biochrom, Berlin, Germany).
3. Incident light microscope (e.g., DM1i, Leica, Wetzlar, Germany).

4. Neubauer improved counting chamber (e.g., Marienfeld, Königshofen, Germany).
5. Piston pipettes (e.g., Research Plus, Eppendorf, Hamburg, Germany).
6. Pipette tips (e.g., Sarstedt, Nümbrecht, Germany).

2.4.3 Materials for Cell Passaging

1. 100 mL baffled shake flask (e.g., Glasgerätebau Ochs, Bovenden, Germany).
2. Insect cell culture medium (e.g., Sf900 II, Thermo Fisher Scientific, Waltham, MA, USA).
3. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
4. Sterile single-use pipette tips (e.g., serological pipettes, Sarstedt, Nümbrecht, Germany).
5. Incubator (e.g., B-line, Binder, Tuttlingen, Germany) with orbital shaker (e.g., Celltron, Infors HT, Basel, Switzerland).

2.4.4 Materials for Freezing the Cells

1. *Spodoptera frugiperda* cell suspension in exponential growth phase with a cell density of 2–6 $\times$ 10^6 cells/mL and a viability >95%.
2. Sterile 1.5 mL Cryo-Vials (e.g., Sarstedt, Nümbrecht, Germany).
3. Insect cell culture medium (e.g., Sf900 II, Thermo Fisher Scientific, Waltham, MA, USA).
4. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
5. Sterile serological pipettes (e.g., Sarstedt, Nümbrecht, Germany).
6. 15-mL centrifuge tubes (e.g., Sarstedt, Nümbrecht, Germany).
7. Aspiration system (e.g., Vacusafe, Integra, Biebertal, Germany).
8. Freezing container (e.g., Mr. Frosty, Thermo Fisher Scientific, Waltham, MA, USA).
9. −80 °C freezer.
10. −140 °C liquid nitrogen freezing tank.
11. Dimethyl sulfoxide (DMSO).

2.4.5 Materials for Cultivation of Insect Cells in a Stirred-Tank Bioreactor

1. Bioreactor with 1 L working volume (e.g., 2L-Labfors, Infors HT, Basel, Switzerland).
2. Bioreactor control unit (e.g., Labfors 5 cell, Infors HT, Basel, Switzerland).

3. Preculture of *Sf*-9 cells.
4. Insect cell culture medium (e.g., Sf900 II, Thermo Fisher Scientific, Waltham, MA, USA).
5. Temperature probe (e.g., Labfors 5 cell, Infors HT, Basel, Switzerland).
6. pH and DO probe (e.g., EasyFerm/VisiFerm, Hamilton, Bonaduz, Switzerland).
7. Glass bottles with connector caps and bottom drain for acid, base, medium and inoculation.
8. Male and female Luer lock adapters.
9. Luer lock lids.
10. Sterile, hydrophobic air filter with Luer lock adapter (e.g., Minisart, Sartorius, Göttingen, Germany).
11. Sterile, single-packed, 10-mL syringe with Luer lock adapter (e.g., B. Braun, Melsungen, Germany).
12. 1 M phosphoric acid.
13. 1 M sodium hydroxide.
14. 70% (v/v) ethanol.

2.4.6 Materials for Online Dielectric Spectroscopy

1. Dielectric spectroscopy probe (e.g., InCyte, Hamilton, Bonaduz, Switzerland).
2. Signal transformation box (e.g., Cell Density ComBox, Hamilton, Bonaduz, Switzerland).
3. Documentation software (e.g., Cell Density software, Hamilton, Bonaduz, Switzerland).
4. Cleaning solution (15 g/L Na_2SO_3).

2.5 Production of a P1 Baculovirus stock and Amplification

The required materials for the production of a P1 virus stock as well as for the amplification of the virus stock are described in this part of the chapter.

2.5.1 Materials for the Transfection of Sf-9 Cells for the Generation of a P1 Baculovirus Stock

1. Material for cell counting (*see* Subheading 2.4.2).
2. *Spodoptera frugiperda* cell suspension (2.5 mL) with a cell density of 0.3×10^6 cells/mL (e.g., TriEx *Sf*-9, Merck, Darmstadt, Germany).
3. Insect cell culture medium (e.g., Sf900 II, Thermo Fisher Scientific, Waltham, MA, USA).
4. Isolated Bacmid DNA (e.g., Bacmid DNA of *Ac*MNPV-Orf2-PCV2; *see* Subheading 3.3).
5. Transfection reagent (e.g., TransIT®-Insect Transfection Reagent, Mirus Bio LL, Madison, Wisconsin, USA).

6. Grace's Insect Medium (e.g., Sigma-Aldrich, St. Louis, Missouri, USA).
7. 6-well plates for suspension culture (e.g., Sarstedt, Nümbrecht, Germany).
8. Sterile single-use pipette tips (e.g., serological pipettes, Sarstedt, Nümbrecht, Germany).
9. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
10. Microscope (e.g., Inverted Microscope Dmi1, Leica Camara, Wetzlar, Germany).
11. Aspiration system (e.g., Vacusafe, Integra, Biebertal, Germany).
12. Multifuge (e.g., Multifuge X1R, Thermo Fisher Scientific, Waltham, MA, USA).
13. 15-mL centrifuge tubes, lightsafe (e.g., Sarstedt, Nümbrecht, Germany).
14. Incubator (e.g., KB 115 E 3.1, Binder, Tuttlingen, Germany).
15. Centrifuge (e.g., 6-16 KS, Sigma-Aldrich, St. Louis, Missouri, USA).

2.5.2 Materials for Amplification of the P1 Baculovirus Stock to P2

1. Material for cell counting (*see* Subheading 2.4.2).
2. *Spodoptera frugiperda* cell suspension with a cell density of 1.0×10^6 cells/mL (e.g., TriEx *Sf*-9, Merck, Darmstadt, Germany).
3. Insect cell culture medium (e.g., Sf900 II, Thermo Fisher Scientific, Waltham, MA, USA).
4. P1 baculovirus stock (2 mL) (e.g., *Ac*MNPV-Orf2-PCV2; *see* **Note 6**).
5. 250 mL baffled shake flask (e.g., Glasgerätebau Ochs, Bovenden, Germany).
6. Sterile single-use pipette tips (e.g., serological pipettes, Sarstedt, Nümbrecht, Germany).
7. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
8. 50-mL centrifuge tubes, lightsafe (e.g., Sarstedt, Nümbrecht, Germany).
9. Incubator (e.g., KB 115 E 3.1, Binder, Tuttlingen, Germany).
10. Centrifuge (e.g., 6-16 KS, Sigma-Aldrich, St. Louis, Missouri, USA).

2.6 Determination of the Baculovirus Titer

In this part of the chapter, three alternatives for the determination of the baculovirus titer are presented: the quantification using qPCR with SYBR Green, the performance of a viral plaque assay, and the determination the $TCID_{50}$.

2.6.1 Materials for the Quantification of Baculoviruses Using qPCR with SYBR Green

1. Kit for the isolation of baculoviral DNA (e.g., PureLink® viral RNA/DNA Mini Kit, Thermo Fisher Scientific, Waltham, MA, USA).
2. qPCR Kit (e.g., QuantiNova™ SYBR® Green, QIAGEN, Venlo, Netherlands).
3. Suitable Primer for the detection of a specific gene sequence of baculoviral DNA.
4. Crushed ice.
5. Sterile single-use filter tips (e.g., Biosphere® filter tips, Sarstedt, Nümbrecht, Germany).
6. Piston pipettes (e.g., Research Plus, Eppendorf, Hamburg, Germany).
7. 1.5-mL DNA Low Bind centrifuge tubes (e.g., Eppendorf, Hamburg, Germany).
8. PCR tubes (e.g., Multiply®-Pro 0.2 mL (PP), Sarstedt, Nümbrecht, Germany).
9. PCR cooler (e.g., Eppendorf, Hamburg, Germany).
10. 96-well plate, semi skirted (e.g., twin.tec. real-time PCR Plate 96, Eppendorf, Hamburg, Germany).
11. PCR film (e.g., Masterclear real-time PCR film, Eppendorf, Hamburg, Germany).
12. qPCR System (e.g., Mastercycler® ep gradient S *realplex*, Eppendorf, Hamburg, Germany).
13. Multifuge (e.g., Multifuge X1R, Thermo Fisher Scientific, Waltham, MA, USA).
14. Centrifuge (e.g., Sigma 1-16K, Sigma-Aldrich, Hamburg, Germany).
15. Thermomixer (e.g., HLC Cooling-Thermomixer, DITABIS, Pforzheim, Germany).
16. Vortexer (e.g., Lab dancer, VWR, Radnor, Pennsylvania, USA).

2.6.2 Materials for Quantification of Baculoviruses Using a Viral Plaque Assay

1. *Spodoptera frugiperda* cell suspension with a cell density of 1.0×10^6 cells/mL (e.g., *Sf*-9 Insect Cells, Merck, Darmstadt, Germany).
2. Insect cell culture medium (e.g., Sf900 II, Thermo Fisher Scientific, Waltham, MA, USA).
3. Generated P2 virus stock.
4. 4% agarose Gel (e.g., Plaque agarose, Biozym Scientific, Hessisch Oldendorf, Germany).
5. Neutral red, high purity (e.g., Carl Roth, Karlsruhe, Germany).

6. Sterile single-use pipette tips (e.g., serological pipettes, Sarstedt, Nümbrecht, Germany).
7. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
8. 6-well tissue-culture plates (e.g., Sarstedt, Nümbrecht, Germany).
9. Distilled water, cell-culture grade, sterile (e.g., Milli-Q Academic, Millipore, Burlington, Massachusetts, USA).
10. 100 mL glass bottle, sterile (e.g., Glasgerätebau Ochs, Bovenden, Germany).
11. Water bath (e.g., Memmert, Schwabach, Germany).
12. Microwave.
13. Incubator (e.g., KB 115 E 3.1, Binder, Tuttlingen, Germany).

2.6.3 Materials for the Determination of the 50% Tissue Culture Infective Dose ($TCID_{50}$)

1. *Spodoptera frugiperda* cell suspension with a cell density of 1.0×10^5 cells/mL (e.g., *Sf*-9 Insect Cells, Merck, Darmstadt, Germany).
2. Baculovirus stock P2 (e.g., *Ac*MNPV-Orf2-PCV2).
3. Insect cell culture medium (e.g., Sf900 II, Thermo Fisher Scientific, Waltham, MA, USA).
4. 96-well plates (e.g., TC Plate 96, Sarstedt, Nümbrecht, Germany).
5. Reagent reservoir (e.g., VWR, Radnor, Pennsylvania, USA).
6. Multichannel pipette (e.g., Research Plus, Eppendorf, Hamburg, Germany).
7. Single-use pipette reservoirs (e.g., 25-mL pipette reservoir, Argos, Vernon Hills, IL, USA).
8. Single-use pipette tips (e.g., Sarstedt, Nümbrecht, Germany).
9. Incubator (e.g., KB 115 E 3.1, Binder, Tuttlingen, Germany).

2.7 Materials for Infection of Sf-cells with Baculovirus and Protein Production in a Stirred-Tank Bioreactor

In this part of the chapter, the materials for the infection of insect cells, using BEVS for protein production in a stirred-tank bioreactor, and the harvest of those produced recombinant proteins, are described.

2.7.1 Materials for the Infection of Sf-cells with Baculovirus

1. *Spodoptera frugiperda* cell suspension (1 L) with a cell density of 1×10^6 cells/mL (e.g., TriEx *Sf*-9, Merck, Darmstadt, Germany).
2. Insect cell culture medium (e.g., Sf900 II, Thermo Fisher Scientific, Waltham, MA, USA).

3. Baculovirus stock P2 (e.g., *Ac*MNPV-Orf2-PCV2).
4. 5 mL sterile syringe.
5. Luer lock lids.
6. Materials for the cultivation of insect cells in a stirred-tank bioreactor (*see* Subheading 2.4.5).
7. Materials for online dielectric spectroscopy (*see* Subheading 2.4.6).
8. Materials for cell counting (*see* Subheading 2.4.2).

2.7.2 Materials for the Harvesting of the Produced Recombinant Proteins

1. Glass bottles with connector cap for harvesting (e.g., the connected inoculation bottle, *see* Subheadings 2.4.5 and 3.7.2).
2. Protease inhibitor (e.g., Protease Inhibitor Cocktail, VWR, Radnor, Pennsylvania, USA).
3. Sterile single-use pipette tips (e.g., serological pipettes, Sarstedt, Nümbrecht, Germany).
4. Pipette holder/rubber bulb (e.g., pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
5. Centrifuge tubes (e.g., Sarstedt, Nümbrecht, Germany).
6. Centrifuge (e.g., 6-16 KS, Sigma-Aldrich, St. Louis, Missouri, USA).
7. −80 °C freezer.

3 Methods

3.1 Transfer Cloning Vector

The DNA sequence of the desired gene which encodes for the protein of interest must be known, and can either be synthesized de novo, or amplified by PCR. The desired target sequence must then be integrated into an acceptor vector. Depending on which Baculovirus Expression Vector System is used, different acceptor vectors are available. To insert the desired gene sequence into the acceptor vector, Golden Gate cloning, or classical restriction ligation cloning, can be used. Other genetic modifications, such as a lacZα cassette (which is assumed in this case), a reporter gene, or others, should be considered too (*see* **Note 7**).

3.2 Generation of Recombinant Baculoviral DNA

This chapter describes the methods for generating baculoviral DNA (also called bacmid). The protocol corresponds, apart from a few deviations, to the Bac-to-Bac Manual of Life Technologies [6].

3.2.1 Transformation of Competent MAX Efficiency® DH10-Bac™ E. coli

1. Prepare agar plates containing kanamycin, tetracycline, X-Gal, and IPTG for the selection of positive *E. coli* transformants.
2. Thaw one vial competent MAX Efficiency® DH10-Bac™ on ice.

Table 1
Master mix for the analysis of recombinant bacmid DNA

Master mix for one reaction	Volume [μL]
Long Amp *Taq* Polymerase	1 μL
5× Long Amp *Taq* reaction buffer	5 μL
10 mM dNTPs	0.75 μL
10 μM M13 Forward (-40) Primer	1 μL
10 μM M13 Reverse Primer	1 μL
ddH_2O	16.5 μL
Σ	25 μL

3. Gently add 100 ng cloned transfer vector plasmid DNA to the MAX Efficiency® DH10-Bac™.
4. Place the vial with the bacteria suspension on ice for 30 min.
5. Induce a heat-shock at 42 °C for 45 s without shaking to enforce a plasmid uptake.
6. Incubate the bacteria on ice for an additional 2 min.
7. Add 750 μL S.O.C. Medium.
8. Incubate the bacteria at 37 °C and 450 rpm for 4 h.
9. Add LB medium to dilute the suspension 1:10.
10. Seed 100 μL of the diluted bacteria suspension on the agar plates containing kanamycin, tetracycline, X-Gal, and IPTG.
11. Incubate the plates overnight at 37 °C.
12. Select white *E. coli* colonies and restreak them on fresh agar plates containing kanamycin, tetracycline, X-Gal, and IPTG.
13. Incubate the agar plate overnight at 37 °C.

3.2.2 Analysis of the Recombinant Bacmid Using LongAmp Taq Polymerase

1. Pick one white colony from restreaked agar plates and suspend it into tubes containing 5 mL LB medium and kanamycin, tetracycline each—follow the procedure 6–10-times (meaning that you have 6–10 colonies in culture at the end).
2. Incubate the liquid cultures at 37 °C and 250 rpm overnight.
3. Prepare the master mix for the analysis of the recombinant bacmid DNA (*see* Table 1).

3.3 Isolation of Recombinant Baculoviral DNA and Buffer Preparation

Three different buffers are required for the isolation of baculoviral DNA: (1) resuspension buffer, containing Tris–HCl and EDTA, (2) lysis buffer containing NaOH and SDS, and (3) precipitation buffer containing potassium acetate. The buffer preparation and isolation of recombinant bacmid is carried out in according to the protocol of Sung et al. [7].

3.4 Suspension Culture of Sf-9 Cells

This chapter comprises a brief description of the strain maintenance, seeding train, preparation, and initiation of an insect-cell bioprocess in a bioreactor. The authors point out, that a closely related and even more detailed description of the setup handling, process initiation and sampling has previously been published in Animal Cell Biotechnology—Methods and Protocols in 2019 by Käßer et al., with a focus on the cultivation of stable insect cell lines [8].

3.4.1 Thawing the Cells

1. Thaw a cryovial containing 2×10^7 *Sf*-9 cells/mL in your hand.
2. Gently add 9 mL of insect cell medium to the cells and mix them properly by pipetting them gently up and down (*see* **Note 8**).
3. Centrifuge the cell suspension at $200 \times g$ for 5 min.
4. Aspirate the supernatant and therefore remove the DMSO (*see* **Note 9**).
5. Resuspend the cells in 10 mL fresh medium.
6. Transfer the 10 mL of cell suspension into a 100 mL baffled Erlenmeyer flask.
7. Cultivate the cells in the incubator on the orbital shaker at 28 °C and a suitable shaking rate.
8. After 3–4 days, passage cells to a concentration of 0.5×10^6 cells/mL (*see* **Note 10**).

3.4.2 Cell Counting by Trypan Blue Staining

1. Assemble the counting chamber as recommended by the manufacturer.
2. Take a sample (about 100 µL).
3. Dilute sample by mixing with PBS to obtain a concentration suitable for counting (typical ratios are 1:1 to 1:20).
4. Mix 75 µL of diluted cell suspension with 75 µL trypan blue (*see* **Note 11**).
5. Pipet the stained cell suspension immediately into the counting chamber.
6. Count the cells and calculate the cell density according to the manufacturer's instructions (*see* **Note 12**).

3.4.3 Passaging the Cells

1. Count cells according to Subheading 3.4.2 using the Neubauer chamber (*see* **Note 13**).
2. Calculate the volume of cell suspension and medium needed to passage the cells to 0.5×10^6 cells/mL (*see* **Note 10**).
3. Pipet the calculated volume of fresh medium into a new shake flask and add the calculated volume of the cell suspension (*see* **Note 8**).

4. As a precaution, keep the previous passage.
5. Cultivate the cells in the incubator on the orbital shaker at 28 °C and a suitable shaking rate.

3.4.4 Freezing the Cells

1. Calculate the volume of the *Spodoptera frugiperda* cell suspension needed to obtain a total of 3.6×10^8 cells.
2. Split up the volume into 50-mL centrifuge tubes.
3. Centrifuge for 5 min at 200 × *g*.
4. Fill 8.1 mL of supernatant into a 50-mL centrifuge tube and add 1.8 mL DMSO to it.
5. Aspirate the remaining supernatant and resuspend the cells in 8.1 mL fresh medium.
6. Add the cell suspension to the prepared medium with DMSO (*see* **Note 14**).
7. Fill 1 mL of the prepared cell suspension in each cryo-vial.
8. Transfer the 18 cryo-vials into the freezing container and freeze at a rate of −1 °C/min at −80 °C for at least 4 h.
9. For long-term storage, transfer vials to the liquid nitrogen freezing tank (−140 °C).
10. To verify a successful freezing process, rethaw one vial (described in Subheading 3.4.1). Observe cell growth and viability. If a viability of >90% and a doubling time between 20 and 35 h can be observed 3–4 passages after rethawing, successful freezing and rethawing is indicated.

3.5 Production of the P1 Virus Stock and Amplification

This part of the chapter describes the generation of a P1 virus stock by a transfection of *Sf*-9 insect cells and the multiplication of the P1 virus stock to obtain the P2 virus stock.

3.5.1 Transfection of Sf-9 Cells for the Generation of a P1 Baculovirus Stock

1. Add 2 mL of *Sf*-9 cell suspension with a cell density of 0.3×10^6 cells/mL to each well of a 6-well plate and incubate at 28 °C overnight.
2. Analyze the cell morphology with the microscope before infection. The cells should look round and not granulated. Further no other particles (e.g., bacterial or fungal contamination) should be visible.
3. Prewarm transfection reagent and vortex it.
4. Defrost the bacmid DNA (*see* **Note 15**).
5. Transfer 250 µL Grace's Insect Medium into a 1.5 mL centrifuge tube.
6. Add 2.5 µg bacmid DNA to the Grace's Insect Medium.
7. Mix the bacmid DNA by gently inverting the centrifuge tube 10 times.

8. Incubate the transfection solution at room temperature for 15 min.
9. Dropwise add the transfection solution to the cell suspension of each well.
10. Incubate the 6-well plates at 28 °C for 72 h, without shaking.
11. Again, analyze the morphology of the insect cells under the microscope and check the cells for cytopathic effects (*see* **Note 16**).
12. Gently transfer the cell suspension to sterile 15 mL centrifuge tubes.
13. Centrifuge the tubes for 5 min at 1000 × *g*.
14. Transfer the P1 virus stock-containing supernatant into new 15 mL centrifuge tubes that are impervious for light because of the virus's light sensitivity.
15. Store the centrifuge tubes at 4 °C, protected from light.

3.5.2 Amplification of the P1 Baculovirus Stock to P2

1. Add 50 mL of *Sf*-9 cell suspension with a cell density of 1×10^6 cells/mL to a baffled shake flask with a working volume of 250 mL (*see* **Note 17**).
2. Add 2 mL of P1 virus stock to the cell suspension.
3. Wrap the flask in aluminum foil and incubate the cell suspension containing viruses at 80 rpm and 28 °C for 72–96 h.
4. Determine the cell concentration and the viability directly after infection, and continue sampling every 24 h to monitor the progress of infection.
5. As soon as the viability of the *Sf*-9 cells drops below of 75–80%, harvest the virus containing cell suspension (*see* **Note 18**).
6. Transfer the harvested cell suspension to sterile 50 mL centrifuge tubes and centrifuge them at 250 × *g* for 10 min.
7. Transfer the supernatant into new 50 mL centrifuge tubes and centrifuge the tubes at 3000 × *g* for 10 min.
8. Again, transfer the P2 virus-containing supernatant to a fresh 50 mL centrifuge tubes (*see* **Note 19**).
9. Store the centrifuge tubes at 4 °C protected from light.

3.6 Determination of the Baculoviral Titer

This part of the chapter describes three different methods for virus titration: the quantification using qPCR with SYBR Green (*see* **Note 20**), the performance of a viral plaque assay (*see* **Note 21**), and the determination of a $TCID_{50}$ (*see* **Note 22**).

3.6.1 Quantification of Baculoviruses Using qPCR with SYBR Green

The quantification of the baculoviral titer using qPCR with SYBR Green is carried out according to the manufacturer's specifications by Thermo Fisher for the corresponding qPCR Kit [9].

3.6.2 Plaque Assay

The performance of a viral plaque assay using *Sf*-9 insect cells is carried out according to the protocol of Bac-to-Bac™ Manual of Life Technologies [6].

3.6.3 Determination of the $TCID_{50}$

1. Determine the cell density of *Sf*-9 cells from a shake flask as described in Subheading 3.4.2.
2. For each sample of baculovirus, dilute exponentially growing *Sf*-9 cells with a fresh medium to a density of 1×10^5 cells/mL in a total volume of 10 mL.
3. Transfer 10 mL of the cell suspension into a reagent reservoir.
4. Fill 100 µL cell suspension in each well of a 96-well plate, using a multichannel pipette (one 96-well plate is required as a seeding plate for each virus sample).
5. Incubate at 28 °C for 4 h.
6. In the meantime, carry out the following:
   (a) Take a second 96-well plate for the dilution of the virus sample (per virus sample eight wells are required).
   (b) Add 135 µL of fresh medium into seven of the eight required wells (an undiluted virus sample will be placed in the first well).
   (c) Add 150 µL of the virus sample to the first well and resuspend the mixture with the pipette.
   (d) Change the pipette tip for a new one.
   (e) Transfer 15 µL from the first well to the second well and resuspend the mixture again (*see* **Note 23**).
   (f) Change the pipette tip for a new one.
7. Retry the **steps** (**c**)–(**f**) until the eighth well is reached.
8. Create 12 replicates for each virus dilution by adding 10 µL of the diluted virus sample from the virus dilution plate to the cell seed plate using a multichannel pipette.
9. Incubate the cells at 28 °C for 120 h.
10. Identify infected cells in each well by detecting the reporter protein GFP.
11. Label infected cells, for example with +, and count the infected wells per column.
12. For the calculation of the $TCID_{50}$ use the following Eq. 1:

$$\log(\mathrm{TCID}_{50}) = |x| + D(\mathrm{Sp} - 0.5) \quad (1)$$

with $|x|$ = log of 100% infected wells in the last column, D = log of the dilution factor (in a total volume of 300 µL suspension, 30 µL virus suspension results in a dilution factor of 10, consequently $\log(10) = 1$), and Sp = sum of the fraction of the first column with 100% infected wells to the column with 0% infected wells.

3.7 Preparation of a Bioreactor for the Cultivation of Sf-9 Insect Cells and Viral Infection

3.7.1 Setup preparation for the Cultivation

1. Attach filters and tubing to the connector caps of medium, inoculum, base and acid bottles (*see* **Note 24**).
2. Fill phosphoric acid and sodium hydroxide into the acid and base bottles and close the caps (*see* **Note 24**).
3. Attach tubing clamps to all tubes.
4. Connect tubes for sampling and pH regulation to the bioreactor.
5. Mount sterile air filters to the gas in-let and outlet.
6. Calibrate the pH probe according to the manufacturer's instructions.
7. Install the pH and DO probe in the bioreactor head plate at a suitable depth.
8. Use aluminum foil to cover all probe connectors and filters.
9. Open all tubing clamps, except those for acid and base, and autoclave the bottles and the bioreactor.
10. After autoclaving, close all tubing clamps and let the equipment cool down to room temperature.

3.7.2 Initiation of the Cultivation

1. Count the cells in the shake flask culture (*see* Subheading 3.4.2).
2. Calculate the inoculum volume needed for an initial cell concentration of 1×10^6 cells/mL.
3. Transfer the cultivation medium into a sterile medium bottle while working under the clean hood.
4. Disinfect the connectors and connect the medium bottle to the bioreactor.
5. Open the tubing clamps of the medium bottle tube and use gravity to fill the reactor with the medium.
6. Set and start the controllers for stirrer and temperature (e.g., 28 °C, 70 rpm).
7. Start aeration with 100% air and 0.1 vvm to equilibrate the medium with ~21% oxygen.
8. One-point calibrate the dissolved oxygen to 100% after the DO value is stabilized, the system is mixed, and a temperature of 28 °C is reached.
9. Activate acid and base pumps until the tubing is filled up to the reactor inlet. Set pH controller to pH 6.3 and activate the pH controller.
10. Transfer the calculated inoculum volume from the baffled shake flask into the inoculum flask while working under the clean hood.

11. Disinfect the connectors and connect the inoculation bottle to the bioreactor.
12. Open the tubing clamps of the inoculation bottle and use gravity to inoculate the reactor.
13. Start data acquisition.

3.7.3 Viral Infection of the Culture

1. Calculate the inoculum volume of the baculovirus stock using the following Eq. 2:

$$\text{Inoculum of the baculovirus stock} = \frac{\text{MOI} \times \text{Total cell count}}{\text{Virus titer}} \quad (2)$$

2. The MOI is the multiplicity of infection (*see* **Note 25**), and the total cell count is composed of the inoculation density of the insect cells multiplied by the culture volume.
3. Draw up the calculated inoculation volume of the baculovirus with a sterile syringe under sterile conditions, and close with a Luer lock lid (*see* **Note 26**).
4. Disinfect the plug of the connect tube and the Luer lock lid of the syringe with 70% (v/v) ethanol.
5. Remove closures of the syringe and the connect tube, and connect them quickly.
6. Open the tubing clamp of the connect tube.
7. Hold the syringe with the baculovirus suspension vertical, and transfer the virus into the culture.
8. Close the tubing clamp.
9. Take a sample of the infected culture directly after the addition of the virus (*see* **Note 27**).
10. For the monitoring of the cell infection, determine the cell concentration and the viability every 24 h.

3.7.4 Harvesting Procedure of the Produced Recombinant Proteins

1. Start the harvest as soon as the insect cells have reached a viability of 70–80% (*see* **Note 28**).
2. Connect the tubing to the harvest pipe of the bioreactor and clamp it into the peristaltic pump.

   Transfer the cell suspension into a 100 mL glass bottle with the help of the peristaltic pump (at this point, it is advisable to use the empty medium bottle, as it is still sterilely connected to the reactor).
3. Aliquot the 1 L suspension into centrifuge tubes and centrifuge 10 min at 250 × *g*. If the proteins are produced intracellularly, the supernatant is discarded at this point and the cell pellet is dissolved using lysis buffer. If the proteins are secreted into the supernatant of the culture, continue with **step 4**.

4. Transfer the supernatant into fresh centrifuge tubes and centrifuge 10 min at 3000 × *g*.
5. Again, transfer the supernatant into fresh centrifuge tubes.
6. Add a protease inhibitor to the protein-containing supernatant.
7. Store the supernatant at −80 °C until further downstream processing.

4 Notes

1. The Porcine Circovirus 2 (PCV2) infection is related to the Post-Weaning Multisystemic Wasting Syndrome (PMWS) and therefore a target for vaccination. The genome of this virus comprises three open reading frames (ORF). ORF2 encodes an approximately 30 kDa-sized capsid protein, which is the main immunostimulating agent in infected animals.
2. The transfer vector carries the desired genetic sequences that needs to be integrated into the baculoviral genome. For this purpose, several commercial kits are available: (1) flashBAC (Oxford Expression Technologies), (2) BaculoGold™ (BD Biosciences), (3) BaculoDirect™ Baculovirus Expression System (Thermo Fisher Scientific), and (4) Bac-to-Bac® Baculovirus Expression System (Thermo Fisher Scientific), which was used for this chapter.
3. If a lacZα cassette is integrated in the transfer vector, X-Gal and IPTG should be added to the agar plates to allow for an identification of positive clones by blue–white screening. The lacZα cassette expresses β-galactosidase, which converts the yellow dye X-Gal into a bluish dye with the help of the inductor IPTG, due to which the negative clones appear blue on the agar plate. If the transduction is successful, the lacZα cassette is interrupted and the colonies appear white.
4. The *E. coli* strain MAX Efficiency® DH10-Bac™ contains the bacmid DNA pMON14727, which carries a kanamycin resistance, and the helper plasmid, which codes for a tetracycline resistance. Depending on which antibiotic resistances are carried by the transfer vector, it is necessary to add these antibiotics to the medium in the corresponding working concentration. For example, if using the pFastBac-1 transfer vector, gentamicin (working concentration 15 μg/mL) and ampicillin (working concentration 50 μg/mL) need to be added [6].
5. Vectors carrying the lacZα cassette allow blue–white screening. Depending on the application, white (e.g., lacZα cassette is replaced by gene of interest) or blue colonies (e.g., lacZα

cassette marks transformed clones) should be picked. If this is not possible, restriction digestion can be performed with randomly picked clones and corresponding restriction enzymes.

6. Alternatively, the titer of the generated baculovirus stock P1 can be determined after transfection. Amplification is then performed with a specific multiplicity of infection (MOI).
7. Specific genetic elements, that can be integrated into the transfer vector, can be of great benefit. For example, a lacZ cassette enables a blue–white screening to identify bacterial clones after the transformation with the transfer vector. A reporter gene is useful for carrying out a $TCID_{50}$, as it codes for a fluorescent protein, allowing infected cells to be identified (*see* Subheading 3.6.3). Another example is a secretion signal that allows for the target protein to be secreted from the cell and that facilitates protein harvesting.
8. Preheating the insect cell culture medium to 37 °C before use may damage cell culture medium components. The insect cell culture medium is designed for cultivation temperatures at 27–28 °C, and preheating refrigerated medium is not necessary.
9. Add fresh medium immediately after aspiration in order to avoid cell damage.
10. Do not seed *Sf*-9 cells below 0.5×10^6 cells/mL in order to avoid reduced growth or even the death of the culture.
11. Living cells appear as bright spheres with a smooth round shape and are not stained by trypan blue. Dead cells appear as blue spheres, as the trypan blue is able to permeate the membrane of damaged cells. For cultivating or freezing *Sf*-9 cells, a viability of >90% is appropriate.
12. Calculate the cell concentration in accordance to the manufacturer's instructions. Consider the dilution of the cell suspension with PBS and trypan blue in the dilution factor. Usually, the cell concentration can be calculated as follows:

$$\text{Cell concentration in cells/mL} = \frac{\text{Counted Cells} \times \text{Chamber Factor} \times \text{Dilution Factor}}{\text{Number of Counted Greater Squares}}$$

13. With an initial cell concentration of $0.5–1 \times 10^6$ cells/mL, cell densities around $0.6–1 \times 10^7$ cells/mL can be expected after 3–4 days of incubation.
14. After mixing the cells with preconditioned medium, add fresh medium and the cryoprotectant DMSO. Proceed in a timely manner, to prevent cell damage.
15. For transfection, use baculoviral DNA in a concentration of 500 ng/μL. For this, the bacmid DNA concentration must be measured at $\lambda = 260$ nm after isolation by using a plate reader

(e.g., Cytation3, BioTek, Winooski, Vermont, USA). For an indication of the purity, the ratio of E_{260} to E_{280} can be determined.

16. The infection of insect cells with baculoviral DNA usually leads to structural changes and cytopathic effects [10, 11]. Therefore, it is useful to morphologically analyze the host cells before and after a viral transfection.
17. The inoculum concentration is calculated for a total culture volume of 50 mL—however, only 48 mL culture volume is required. The viral infection is carried out by adding the 2 mL of P1 virus stock.
18. Virus harvesting should be performed before the insect cells enter the stationary phase. Therefore, the viability of the insect cells should be monitored. If the viability decreases too much, viral surface proteins can be degraded by cell lysis-related host cell proteins and, as a result, the infectivity of the virus decreases.
19. Further measurements can be carried out with the supernatant, e.g., concentration of amino acids, or glucose, or lactate.
20. To quantify the baculovirus using qPCR with SYBR Green, a specific gene sequence of the bacmid DNA must be selected, and corresponding primers need to be designed. In addition, the copy number needs to be calculated using the following equation:

$$\text{Copy Number} = \frac{\text{Amount (ng)} \times 6.022 \times 10^{23}}{\text{Length (bp)} \times 1 \times 10^{9} \times 660}$$

The Amount (ng) corresponds the amount of DNA. 6.022×10^{23} is the Avogadro's constant, which indicates the number of particles in 1 mol. The Length (bp) is the length of the DNA in bp. 1×10^{9} represents the conversion factor for ng, and 660 is the average mass of 1 bp double stranded DNA.

21. For performing a viral plaque assay, *Sf*-9 Insect Cells (e.g., *Sf*-9 Insect Cells Novagen, Merck, Darmstadt, Germany) are required. For this purpose, the insect cells are cultivated semi-adherently in T-bottles, as specified by the manufacturer.
22. The $TCID_{50}$ is determined, for example, by the cytopathic effect of insect cells, or by a reporter gene that codes for a fluorescent protein, such as the green fluorescent protein (GFP).
23. Use a multichannel pipette for the virus dilutions 10^{-0} to 10^{-7} in order to process all samples simultaneously.
24. It is recommended, to attach one type of Luer lock adapter to the tubes connected to the headplate (e.g., male Luer lock), and the opposite adapter (e.g., female Luer lock) to all tubes connected to bottles. This prevents confusion.

25. The MOI indicates the ratio of plaque-forming units (pfu) at the time of the infection of the cells. For the viral infection of insect cells, a MOI between 0.01 and 1 should be chosen, as a low MOI reduces the number of defective viral particles [12–14].
26. The remaining volume of the syringe should be filled with air (under sterile conditions), so that the virus suspension does not remain in the tube when the reactor is infected.
27. Bioreactor sampling has been described previously in Animal Cell Biotechnology - Methods and Protocols in 2019 by Käßer et al. [8].
28. The time of harvest is one of the most important factors in protein production when using a lytic production strategy, and usually depends on the viability of infected cells. Harvesting can be indicated by a drop in viability (i.e., viability below 90%) due to the baculovirus-related late expression promoter. Harvesting before an observed viability loss can result in a poor protein yield. Harvesting too late (i.e., viability below 70%) can result in poor protein yield too, due to cell lysis-related protein degradation.

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References

1. Felberbaum RS (2015) The baculovirus expression vector system: a commercial manufacturing platform for viral vaccines and gene therapy vectors. Biotechnol J 10 (5):702–714. https://doi.org/10.1002/biot.201400438
2. Tomiya N, Narang S, Lee YC et al (2004) Comparing N-glycan processing in mammalian cell lines to native and engineered lepidopteran insect cell lines. Glycoconj J 21(6):343–360. https://doi.org/10.1023/B:GLYC.0000046275.28315.87
3. Kost TA, Condreay JP (2002) Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol 20(4):173–180 https://doi.org/10.1016/S0167-7799(01)01911-4
4. Grein TA, Loewe D, Dieken H et al (2018) High titer oncolytic measles virus production process by integration of dielectric spectroscopy as online monitoring system. Biotechnol Bioeng 115(5):1186–1194. https://doi.org/10.1002/bit.26538
5. Zitzmann J, Weidner T, Eichner G et al (2018) Dielectric spectroscopy and optical density measurement for the online monitoring and control of recombinant protein production in stably transformed drosophila melanogaster S2

cells. Sensors (Basel) 18(3). https://doi.org/10.3390/s18030900

6. Life Technologies (2015) Bac-to-Bac baculovirus expression system: an efficient site-specific transposition system to generate baculovirus for high-level expression of recombinant proteins. Life Technologies, Carlsbad, CA
7. Sung L-Y, Chen C-L, Lin S-Y et al (2014) Efficient gene delivery into cell lines and stem cells using baculovirus. Nat Protoc 9 (8):1882–1899. https://doi.org/10.1038/nprot.2014.130
8. Käßer L, Zitzmann J, Grein TA et al (2019) Turbidimetry and dielectric spectroscopy as process analytical technologies for mammalian and insect cell cultures. Methods Mol Biol 2095:335–364. https://doi.org/10.1007/978-1-0716-0191-4_20
9. Thermo Fisher Scientific PureLink Viral RNA DNA Mini Kit User Guide (Pub. No. MAN0000562 Rev. A.0)
10. Üstün-Aytekin Ö, Gürhan İD, Ohura K et al (2013) Monitoring of the effects of transfection with baculovirus on Sf9 cell line and expression of human dipeptidyl peptidase IV. Cytotechnology 66(1):159–168. https://doi.org/10.1007/s10616-013-9549-3
11. Folgueras-Flatschart AV, Flatschart RB, Resende M et al (2000) Early detection of productive baculovirus DNA transfection. Biotechniques 29(3):430–432., 434. https://doi.org/10.2144/00293bm05
12. Wickham TJ, Davis T, Granados RR et al (1991) Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. Biotechnol Lett 13 (7):483–488. https://doi.org/10.1007/BF01049204
13. Licari P, Bailey JE (1991) Factors influencing recombinant protein yields in an insect cell-bacuiovirus expression system: multiplicity of infection and intracellular protein degradation. Biotechnol Bioeng 37(3):238–246. https://doi.org/10.1002/bit.260370306
14. Maranga L, Brazão TF, Carrondo MJT (2003) Virus-like particle production at low multiplicities of infection with the baculovirus insect cell system. Biotechnol Bioeng 84 (2):245–253. https://doi.org/10.1002/bit.10773

Chapter 9

High-Throughput Process Development for the Chromatographic Purification of Viral Antigens

Shaleem I. Jacob, Spyridon Konstantinidis, and Daniel G. Bracewell

Abstract

Chromatography is a widely used method in the biotechnology industry and functions to separate the desired product from process and product related impurities. There is a multitude of resins available based on different modalities (such as charge, hydrophobicity, and affinity) to provide a spectrum of approaches to meet the separation challenges of the diverse products. The challenge of developing viral antigen purification processes is addressed in this method. A unique feature of this product class is that in order to protect against more than one strain of an antigen, vaccines are often multivalent. This entails multiple production processes for each antigen, all of which will require separate development and validation. Ideally, a universal purification method is sought, but differences in the protein subunits (frequently used as the antigens) make this challenging and often-bespoke purification steps are required. This means process development for the chromatographic stages of these products can be particularly challenging and labour intensive. With the numerous choices available, making critical process decisions that are usually unique to each product, process, and strain, can be costly and time-consuming. To address this, scale down purification at <1.0 mL column volume and automation approaches are increasingly applied to increase throughput. In this work, a method is described wherein a Tecan Freedom EVO® automated liquid handler is deployed for the evaluation of different resin chemistries and buffer conditions to find a suitable purification strategy. This method allows for the rapid evaluation of the separation viral antigens where limited information on chromatography behavior is known at the early stages of process development. Here, we demonstrate the methodology firstly by explaining the automated purification script and secondly by applying the script for an efficient purification development for different serotypes of rotavirus antigens.

Key words Process development, High-throughput process development (HTPD), Rotavirus antigens, Purification, Chromatography

1 Introduction

High-throughput (HT) development activities are currently performed in both upstream and downstream unit operations and benefit by the advancements of automated liquid handlers [1–3]. Such screening activities, even from the early stages of process development, can lead to optimal or near optimal conditions in a systematic and efficient fashion [4–7]. In downstream applications,

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and in particular in the development of chromatographic separations, the techniques employed are separated into batch and packed bed column chromatography [8, 9]. Recently, applications have been developed that employ both diffusive and convective media, but the latter are still missing a commercially available flow mode based HT technology. While batch HT methods allow the evaluation of multiple conditions in parallel, including binding capacity measurements from small product masses due to the low volumes of stationary phases employed. Moreover, the miniature packed bed column technique (i.e., RoboColumns) can be easily scaled up or down [10]. It can also return an efficient way of evaluating different stationary phases due to the advantage of packing any beaded based resin at a cost. This is an application that is traditionally regarded to be better accomplished with batch methods, requiring, however, sophisticated processes for preparing resin slurry multiwell plates [11]. Jacob and coworkers [12] have demonstrated that it is possible to design and synthesize affinity resins for high recovery of novel influenza antigens and various recombinant proteins (erythropoietin, immunoglobulins, etc.) [13, 14]. Therefore, such HT techniques can be employed to evaluate their performance.

In this instance, the scale-down performance of the miniature columns was leveraged to guide development efforts in a multivariate input space, including stationary phase type and operating conditions, for recombinant vaccine antigen purification. The approach relies heavily on the performance of the miniature columns and as such it employs custom solutions and in-house developed tools that deliver full walk-away automation across the different parts of the HT study, that is, buffer preparation, method definition, experiment completion, and results reporting. The followed workflow employs a Tecan Freedom EVO® 200 automated liquid handling station (Tecan Group Ltd., Männedorf, Switzerland) and MATLAB® (The MathWorks, MA, USA) codes, compiled into executables, which convert user-defined inputs into robotic commands. These are then executed within generic Tecan Freedom EVOware® (Tecan Group Ltd.) based scripts that implement all necessary actions to complete a study with no end-user intervention [10]. Here, both 0.2 and 0.6 mL columns, packed with various types of resin, can be used in flowthrough or bind and elute mode, with the latter employing isocratic, multistep, and multislope gradient elution. The workflow is demonstrated by implementing an early purification strategy in scouting for multimodal resins and conditions for two different strains of rotavirus antigen. A small number of well-planned experiments were sufficient to elucidate a lead resin candidate and separation conditions which were then found to be scalable to a larger pilot scale. Hence, the combination of HT techniques, automation, and a systematic screening approach can lead to attractive and feasible purification process conditions in a rapid fashion.

2 Materials

2.1 Viral Antigen

Rotavirus antigens are expressed in *Pichia pastoris*.

2.2 Miniature Columns

Miniature columns (0.2 and 0.6 mL) are purchased from Repligen® (Waltham, Massachusetts, USA). These are packed with the commercially available resins (Table 1). For custom designed resins, bulk resin can be sent to Repligen, which can then be packed at a desired bed volume in RoboColumns.

2.3 Automated Workstation

The employed automated liquid handling station comprises of a Tecan Freedom Evo 200 robot. The station is controlled by Freedom EVOware v2.6 (Tecan Group Ltd.) on an Intel i5 4670 CPU machine with 4 GB of RAM running Windows 7 (Microsoft Corporation, WA, USA). The robot's layout is depicted schematically in Fig. 1a (*see* **Notes 1–3**). It includes an eight-channel liquid handling (LiHa) arm, using short uncoated stainless-steel tips, and an eccentric robot manipulator (RoMa) arm. Integrated devices include the Te-Shuttle™ and Te-Chrom™ (fraction collection system) modules and an Infinite® M200 Pro plate reader (Tecan Group Ltd.), capable of UV/Vis and fluorescence measurements, and operated by i-control™ software (Tecan Group Ltd.). An in-depth description of the layout is found in [10]. Briefly, all carriers are supplied by Tecan and are responsible for holding plates and troughs filled with buffers. Nine site hotel carriers are also present and these are responsible for storing plates containing collected fractions and newly prepared buffers. These are used to blank correct the plate reader measurements of the collected fractions (*see* **Note 4**). All carrier and labware definitions are edited in EVOware in order to implement robotic operations, including plate transfers and liquid handling, robustly and reliably (*see* **Note 5**). Finally, the deployment of the robotic station made use of standard liquid classes which also included those supplied with

Table 1
List of commercially available multimodal chromatography resins tested at 0.6 mL scale

Type	Resin	Manufacturer
Multimodal	Capto™ MMC	GE Healthcare
	PPA Hypercel™	Pall
	Nuvia™ cPrime™	Bio-Rad
	CMM HyperCel™	Pall
	Toyopearl® MX-Trp-650M	Tosoh Bioscience
	Eshmuno® HCX	Merck KgaA

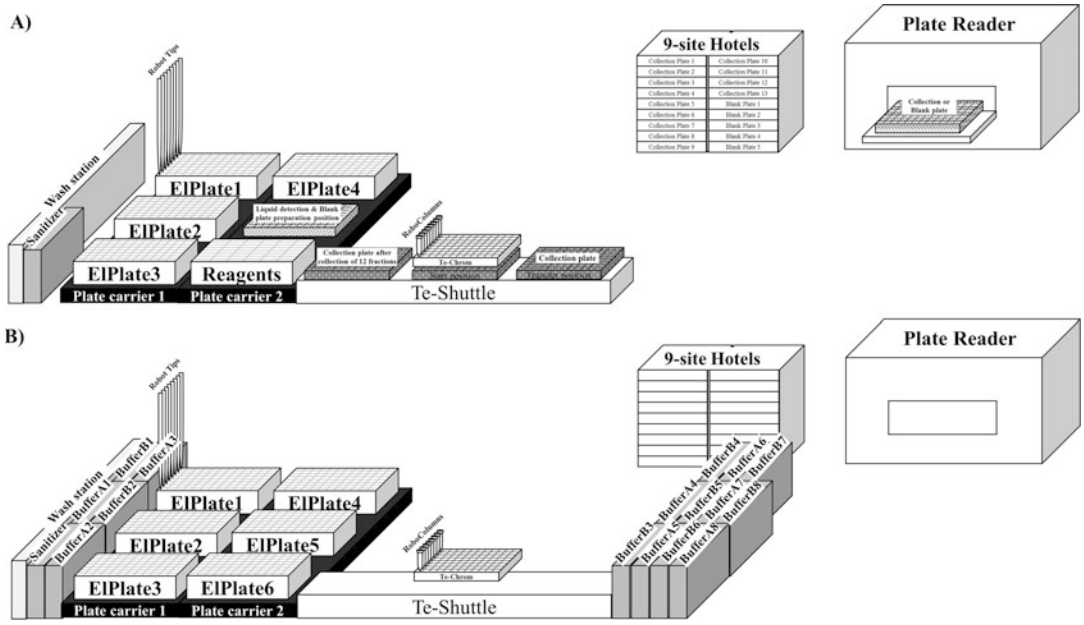


Fig. 1 (**a**) Robotic layout for performing RoboColumn experiments. The Sanitizer is a 100 mL trough containing sanitization solution (typically 0.5 M NaOH) to decontaminate the tips. plates ElPlate1–ElPlate4 are 96-well deep square well plates and contain elution buffers for each RoboColumn in each of their rows respectively (i.e., up to 48 buffers per column). The Reagents plate is a 48-well deep square well plate containing solutions to dispense into the RoboColumns during the Equilibration, Load, Wash, Strip, CIP, and Storage phases (each row of the plate corresponds to the respective RoboColumn). Fill the reagents plate appropriately with the selected buffers and solutions. Place the ElPlate1–4 and Reagents plates onto two plate carriers. The second plate carrier also includes a vacant spot. This is used either as a position to transfer an empty microplate from the hotel, to fill it up with buffers from the Reagents and ElPlates1–4 plates during a Blank Plate preparation or to transfer and then liquid detect a filled Collection plate to determine the volume of the collected fractions. Store the Collection and Blank plates in two 9-site hotels. The Collection plates from these hotels can be transferred to the Transfer position of the Te-Shuttle module which will move them to the start position and eventually to a final position once 12 fractions have been collected. At the start position, the first column of a Collection plate is aligned with the RoboColumns which are held in position on the Te-Chrom module. Finally, Collection and Blank plates are measured in a Plate reader to determine absorbances and also the volumes of their well contents if volume determination occurs with near infrared measurements instead of using the robot's liquid detect function. (**b**) Robotic layout for preparing buffers for up to eight RoboColumn experiments. Same as layout A with the addition of troughs containing eight pairs of Buffers A and B (i.e., BufferA1, BufferB1, BufferA2, BufferB2, . . ., BufferA8, BufferB8) and two more plates containing elution buffers (i.e., ElPlate5, 6). Here, each row in each of these plates also corresponds to the respective RoboColumn (i.e., row A cotains buffers for RoboColumn 1, row B for RoboColumn B, . . ., row H for RoboColumn 8). Hence, a gradient with $6 \times 12 = 72$ steps can be prepared for each RoboColumn across six ElPlates

the Te-Chrom and Te-Shuttle modules for the dispensing into the RoboColumns. Custom liquid classes are employed only for the sanitization of the stainless-steel tips and during the implementation of the robot's liquid detect function (*see* **Notes 6–8**).

3 Methods

3.1 Miniature Column Chromatography

The implementation of the miniature column technique on the aforementioned robotic station follows closely the operation of bench/large scale chromatography throughout eight main steps (i.e., (1) removal of storage solution, (2) equilibration, (3) loading, (4) wash, (5) elution, (6) strip, (7) regeneration, and (8) storage). Hardware differences between HT RoboColumn and conventional chromatography means that analogies need to be made. In HT chromatography, solutions are transferred to the RoboColumns discretely, as opposed to continuously, and they are aspirated across different locations within and between labware. In the case of gradient based separations, a gradient is first broken into a series of small steps with each step being a buffer with a given composition (*see* **Note 9**). Hence, all buffers in HT column experiments need to be prepared in advance at the correct volume and composition and be placed in specific labware and locations within the robotic station. Since a robot is usually equipped with eight channels, and it is not possible to mix liquids continuously, each of these channels play the role of a simple inlet/outlet pump delivering liquids to, up to eight columns in parallel. Moreover, in HT column chromatography, the role of a fractionator is fulfilled by the Te-Shuttle module which collects effluent, or fractions, from each RoboColumn to different wells in 96 well collection plates. Finally, the plate reader integrated with the robot, plays the role of the detector since it reads the plates containing the collected fractions at particular wavelengths and modes compatible with the capabilities of the reader (*see* **Notes 10** and **11**). Figure 2 details how the different components of the robotic station are used during a typical experiment with eight RoboColumns, whereas Fig. 3 depicts an illustrative example of collecting the first 12 elution fractions in a collection plate.

3.2 Robotic Buffer Preparation

Buffer preparation is implemented on the aforementioned robotic station and it includes both stock preparation and elution buffer preparation in the case of HT column experiments employing gradient elution. This employs custom written MATLAB (The MathWorks) codes, compiled into executables, which are launched through in-house developed VBA tools providing an interface for end-user input definition. For buffer stock preparation, the tools require the specification of their conjugate acid–base pairs and salt, along with their desired composition (e.g., buffer concentration, pH and salt concentration), and generate robotic instructions that are loaded and executed in generic Tecan Freedom EVOware scripts and result in the preparation of stocks in troughs (i.e., 100 mL). The same tools are implemented to prepare buffers corresponding to the steps in elution gradients in multiple

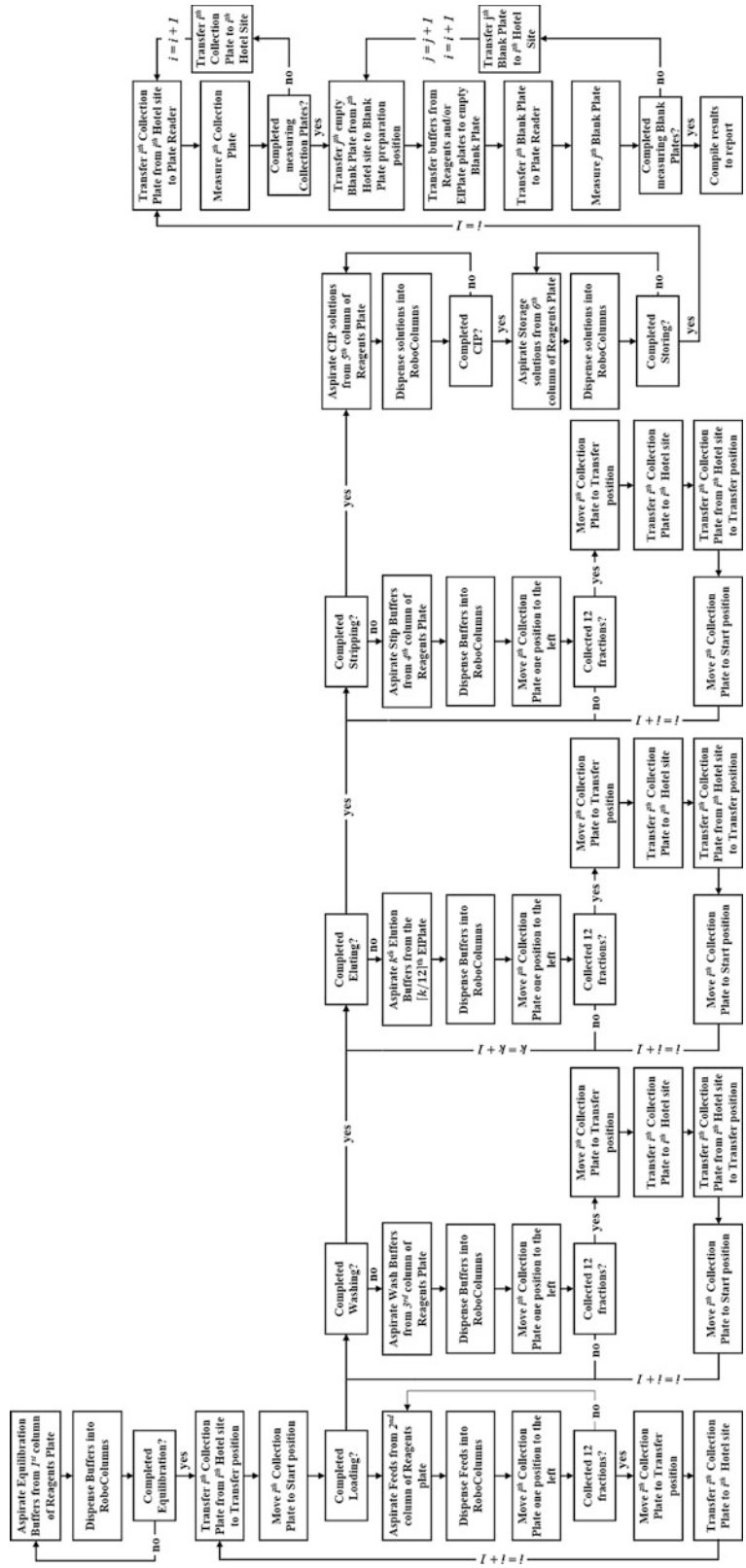


Fig. 2 Flowchart of actions taken during the deployment of RoboColumns on an automated liquid handler

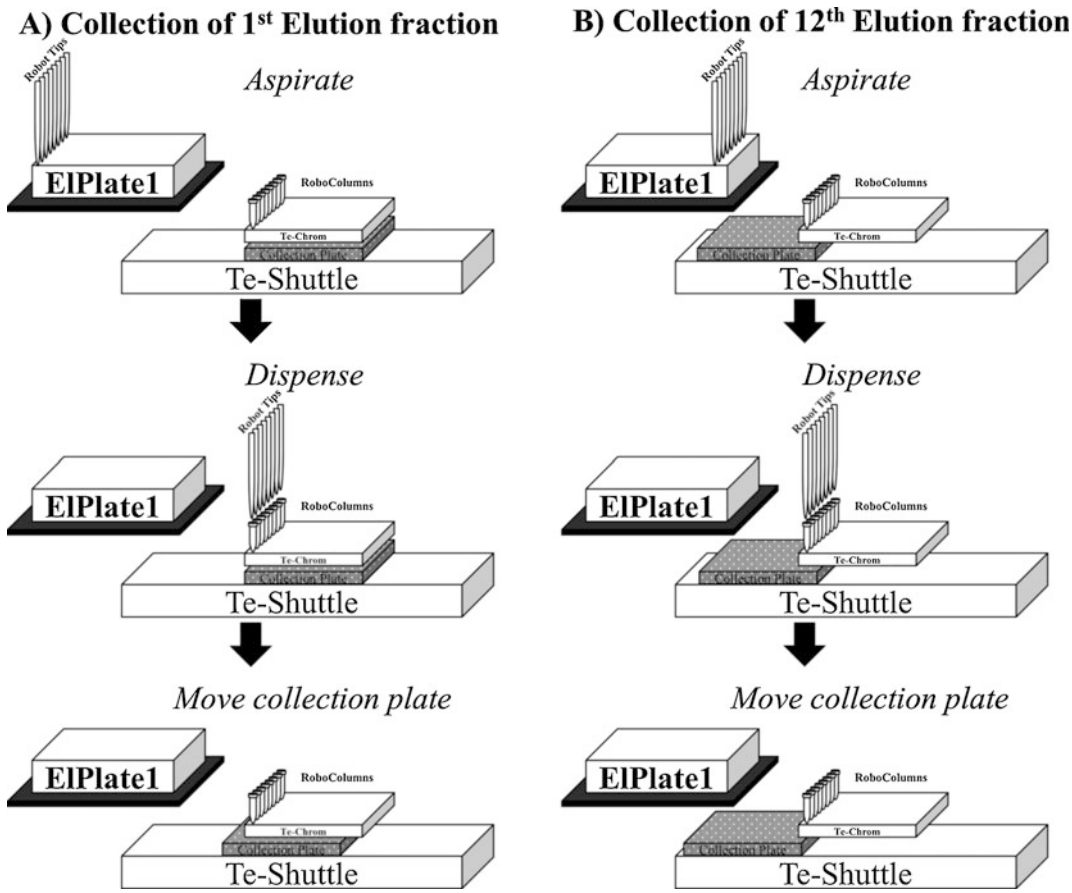


Fig. 3 (**a**, **b**) Depiction of first 12 fraction collections using an automated liquid handler and the Te-Shuttle module which ensures that fraction collection occurs in a different column of a Collection plate for each new fraction

96-well plates. This involves the end-user specification of up to eight Buffer A/B pairs (Fig. 1b), the duration of the gradients in column volumes, the %B at the beginning and end of the gradients, and the desired volume for preparing each buffer/step in the gradients. Here, gradients for up to eight miniature columns are prepared at a time by mixing together pairs from up to 16 buffer stocks at different ratios in order to obtain the desired step compositions per gradient and column. In each of these pairs one stock plays the role of Buffer A whereas the other the role of Buffer B. Upon input definition, the tools result in the generation of robotic instructions to prepare miniature column gradients in an automated fashion through generic Tecan Freedom EVOware scripts (*see* **Notes 12** and **13**).

3.3 Implementation of HT Column Chromatography

The step-by-step methodology below describes the implementation of HT column chromatography for testing various buffered conditions against rotavirus antigens using different multimodal chromatography (MMC) resins.

1. Fill the Reagents plate (Fig. 1a) with 4 mL of the listed buffers (Table 2) (*see* **Note 14**).
2. The elution plates (ElPlates1–ElPlates4 in Fig. 1a) are 96-well deep square well plates. Fill the plates with 2 mL of buffers. These buffers are prepared to return mobile phase conditions (e.g., pH, buffer concentration and species, salt concentration and type, additives) that are believed to affect the separation and from which an optimal condition will be identified. Listed in Table 3 are the selected buffered conditions used for the MMC columns.
3. Once all of the plates are in their positions as shown in Fig. 1a. Place the 96-well microplates (Collection plates) in the hotels. Fill the tip sanitization trough (Fig. 1a) with 0.5 M sodium hydroxide.
4. Launch the EVOware software and select the purification script for 0.6 mL miniature column purification (Appendix 1). The choice of column size, residence time (*see* **Note 15**), and column volume (CV) for each buffered step is decided (Table 4). This can be selected depending on your requirements.

Table 2
Contents of 48 well deep-well square Reagents plate

	Column					
Row	1 (Equilibration)	2 (Wash)	3 (Wash)	4 (Strip)	5 (CIP)	6 (Storage)
A(RC1)	Equilibration	Load	Wash	Strip	1 M NaCl, 0.5 M NaOH	20% EtOH
B(RC2)	Equilibration	Load	Wash	Strip	1 M NaCl, 0.5 M NaOH	20% EtOH
C(RC3)	Equilibration	Load	Wash	Strip	1 M NaCl, 0.5 M NaOH	20% EtOH
D(RC4)	Equilibration	Load	Wash	Strip	1 M NaCl, 0.5 M NaOH	20% EtOH
E(RC5)	Equilibration	Load	Wash	Strip	1 M NaCl, 0.5 M NaOH	20% EtOH
F(RC6)	Equilibration	Load	Wash	Strip	1 M NaCl, 0.5 M NaOH	20% EtOH
G(RC7)	Equilibration	Load	Wash	Strip	1 M NaCl, 0.5 M NaOH	20% EtOH
H(RC8)	Equilibration	Load	Wash	Strip	1 M NaCl, 0.5 M NaOH	20% EtOH

Each row of the plate (A–H) corresponds to RoboColumn (RC1–RC8)

Table 3
List of different buffered conditions trialed for each of the resins

Column	Buffered conditions	Start–Ending elution buffers
1	CIEX	20 mM sodium citrate pH 5—20 mM Sodium Phosphate pH 7
2	CIEX	20 mM Sodium Citrate pH 5—20 mM Sodium Phosphate pH 7
3	MMC	20 mM Sodium Citrate pH 5, 1 M NaCl—20 mM Sodium Phosphate pH 7
4	MMC	20 mM Sodium Citrate pH 5, 1 M NaCl—20 mM Sodium Phosphate pH 7
5	CIEX	50 mM Sodium Citrate pH 4—50 mM Tris-HCl 0.8 M NaCl pH 8
6	CIEX	50 mM Sodium Citrate pH 4—50 mM Tris-HCl 0.8 M NaCl pH 8
7	MMC	50 mM Citrate pH 4, 0.8 M $(NH_4)_2SO_4$—50 mM Tris-HCl pH 8
8	MMC	50 mM Citrate pH 4, 0.8 M $(NH_4)_2SO_4$—50 mM Tris-HCl pH 8

Table 4
List of chromatography steps entered in the automated script

Step	Length of step (CV)
Removal of storage solution	3
Equilibration	5
Fermentation Load	5
Wash	3
Elution	10
Strip	4
CIP	1
Storage	3

In this instance, the column size was 0.6 mL and the residence time 2 min (i.e., flow rate of 5 μL/s)

3.4 Application of HT Column Chromatography for Multimodal Resins (MMC)

Each resin in Table 1 was tested for a different set of buffered conditions and they included changes in pH, sodium chloride concentration, and buffer species (Table 3).

3.4.1 Elution Buffer Plate Preparation for Step Gradients

1. Use the script dedicated to elution buffer preparation for step gradients (Appendix 2) to find the optimal desorption conditions. Its layout is shown in Fig. 1b. Dual elution step gradients are defined to optimize multimodal effectiveness by decreasing

Table 5
Details of tested elution conditions

Elution condition	Start salt concentration (M)	Ending salt concentration (M)	%B buffer change	pH change	No. Column volumes in elution	Fraction volume (mL)
CIEX	0	0.8	0–100	4–8	15	0.2
MMC	0.8	0	0–100	4–8	15	0.2

Table 6
The table below shows the inputs used to create the elution buffer steps per RoboColumn (RC)

RC	Gradient CV	Fraction volume (mL)	Buffer A trough	Buffer B trough	Start % B	End % B	Gradient slope (% B/CV)
1	15	0.2	BufferA1	BufferB1	0	100	6.67
2	15	0.2	BufferA2	BufferB2	0	100	6.67
3	15	0.2	BufferA3	BufferB3	0	100	6.67
4	15	0.2	BufferA4	BufferB4	0	100	6.67
5	15	0.2	BufferA5	BufferB5	0	100	6.67
6	15	0.2	BufferA6	BufferB6	0	100	6.67
7	15	0.2	BufferA7	BufferB7	0	100	6.67
8	15	0.2	BufferA8	BufferB8	0	100	6.67

the concentration of sodium chloride (NaCl) and increasing the pH (i.e., Table 3, Column 7 and 8 for MMC conditions).

2. In the buffer preparation script, the gradients are prepared in a step-wise fashion and the salt concentration changes in a step-wise fashion. However, the pH change will not show such a simple trend; instead it will follow a nonlinear trend common for titration curves. Once prepared, determine the pH of all prepared solution buffers experimentally using an off-line pH probe (*see* **Note 16**).
3. To prepare the elution gradients described in Table 3, prepare the buffer stocks of the starting and ending buffers and input the steps into the script (Table 5).
4. Table 6 demonstrates the inputs used to create step gradient elution buffers in 96 well deep square well plates using the automated protocol. In addition, flow rates can be calculated relating the larger scale to the smaller scale or vice versa.

3.5 A Case Study: Nuvia™ cPrime™ and CMM Hypercel™

Resins Nuvia cPrime and CMM Hypercel were superior compared to the other commercial multimodal resins in terms of achieved product yield and purity. The chemistry of ligands in both resins includes hydrophobic (benzene) and cation exchange (carboxylic acid) groups (Fig. 4). Multiple conditions were evaluated rapidly (pH, salt, and buffer) and these two resins were selected for scale-up using the ÄKTA™ pure (GE Healthcare, Uppsala Sweden).

Initial experiments on the Tecan at 0.6 mL scale resulted in Nuvia cPrime outperforming CMM Hypercel (Table 7) under multimodal buffered conditions to purify rotavirus antigen A. Nevertheless, both antigens (A and B) were purified at smaller (0.6 mL) and larger scale (>1 mL) in order to assess the reproducibility of the HT method. The deployment of CMM Hypercel at HT scale is shown in Fig. 5 as an example of method application (Columns 7 and 8 MMC conditions in Table 3). Further work was undertaken using rotavirus antigen B and the evaluation of both resins in order to develop a purification process.

The chromatogram in Fig. 5 shows the purification of rotavirus antigen A using CMM Hypercel. The employed conditions exploit the multimodal capability of the resin as the equilibration buffer has 0.8 M ammonium sulfate for hydrophobic binding and a pH of 4 to induce attraction between negatively charged ligand and positively charged product. Elution is achieved by reducing the ammonium sulfate concentration and increasing the pH. This sets a dual gradient (Fig. 5) and resulted in 97% purity and a yield of 49% based on densitometry (Table 7). The separation between the impurities

Fig. 4 The structure of multimodal resins Nuvia cPrime and CMM Hypercel

Table 7
Purification of rotavirus antigen A using 0.6 mL Nuvia™ cPrime™ and CMM Hypercel™ under MMC buffered conditions performed on the Tecan EVO® 200

Resin	Scale (mL)	Conditions used to purify	Purity (%)	Yield (%)	$OD_{260/280}$
CMM Hypercel™	0.6	MM	97	49	0.59
Nuvia™ cPrime™	0.6	MM	97	56	0.59

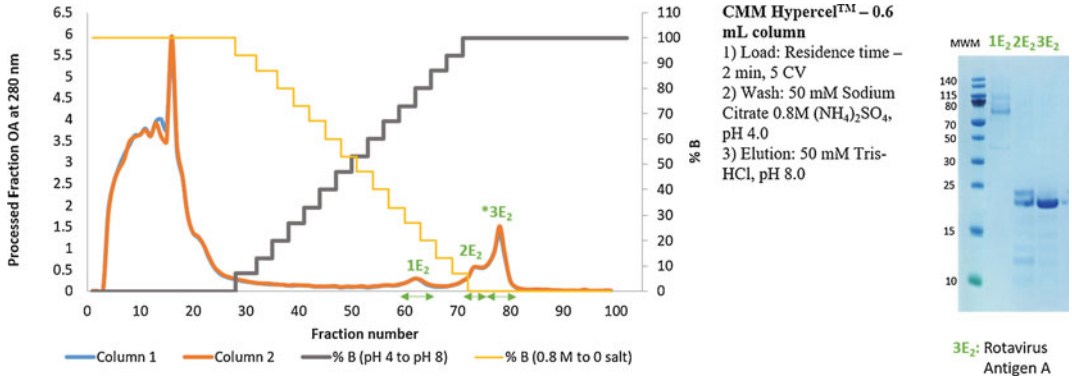


Fig. 5 Purification of rotavirus antigen A using 0.6 mL CMM Hypercel RoboColumn under multimodal buffered conditions performed on the Tecan EVO 200. Left *y*-axis depicts blank corrected and pathlength normalized fraction absorbances at 280 nm (fraction at 15 CVs has increased normalized absorbance due to spuriously low pathlength). The chromatogram shows the purification of two columns under the same conditions to demonstrate reproducibility at a small scale. SDS-PAGE of the ternary elution of the impurities and rotavirus antigen A is also shown on the right

($1E_2$ and $2E_2$) and the rotavirus antigen A ($3E_2$) can also be observed in Fig. 5. The high molecular weight (HMW) impurities are eluting from the column in elution fraction $1E_2$. These are well separated from the smaller molecular weight (LMW) impurities which elute at the end of the gradient and are followed by the elution of the antigen in the column strip phase. While this method is highly capable of clearing one of the main impurity species, the mixing between the LMW impurities and the antigen would require further optimization. For example, the starting ammonium sulfate concentration and pH can be reduced and increased respectively to bind antigen and flow through HMW impurities. This can then be followed by a shallower gradient with the aim of resolving the LMW impurities from the antigen resulting to an even higher purity and, more importantly, a higher yield by increasing the volume of the product pool. To assess the scalability of these initial results, the elution gradients were applied at a 5 mL scale and the ternary elution peaks were observed (data not shown). Therefore, there is reproducibility from 0.6 to 5 mL given the scale up parameters (Table 6) remain consistent and any further improvements with the HT scale columns would also be scalable.

These initial screens (Table 3, Fig 5) are beneficial in determining where the product, product-related impurities, host cell DNA and host cell proteins elute and the difficulty of the separation. In this instance, HMW impurities eluted first and followed by a close elution of LMW impurities and the product at the end of the gradient. This information can then be used to optimize the separation further with steps at a systematically chosen pH and salt

values. Taking into account that a RoboColumn run can be typically completed within a day (the method in Fig. 5 had a duration of <8) with no end-user intervention, other than setting up the robot and the method, demonstrates the power of the RoboColumn technique as a tool for generating valuable process information in an efficient and effective fashion. The benefit of adopting this high-throughput method is further compounded by the fact that viral antigen products are characterized by even larger screening spaces due to the existence of multiple strains and/or serotypes of viruses (i.e., influenza, lentivirus, HPV). Here, the offered parallelization and walk-away automation, can lead to a rapid development of purification processes, which would be impossible to achieve with conventional workflows, and more importantly to assess the potential of establishing a platform process for all different strains of a given virus. This would allow for significant process development simplification and return significant time and cost savings.

4 Notes

1. We use two MP3 pos carriers from Tecan making it possible to store up to six plates on the robot's deck. It is possible to use two MP4 pos carriers allowing the storage of up to eight plates instead.
2. It is possible to place the Te-Shuttle carrier to the front of three MP3 pos carriers resulting in space saving on the robot's deck in the case of EVO® 150 or EVO® 100 instruments. To implement this, the worktable will have to be edited in EVOware by changing the two carrier definitions:
   (a) Locate the MP3 pos carrier in EVOware's CARRIERS tab.
   (b) Right click and select Edit.
   (c) Make note of carrier definitions (take and save a screen capture).
   (d) Change the X and Y Dimensions to 1 and 1 mm.
   (e) Change to Y Reference Offset to 100 mm.
   (f) Add three carriers, for example, grid locations 2, 8, and 14 (leave 6 grids spacing between each carrier).
   (g) Locate the Te-Shuttle carrier in EVOware's CARRIERS tab.
   (h) Right click and select Edit.
   (i) Make note of carrier definitions (take and save a screen capture).

(j) Change the *X* and *Y* Dimensions to 1 and 1 mm.

(k) Add carriers to grid location 3.

(l) Change carrier definitions to their original values.

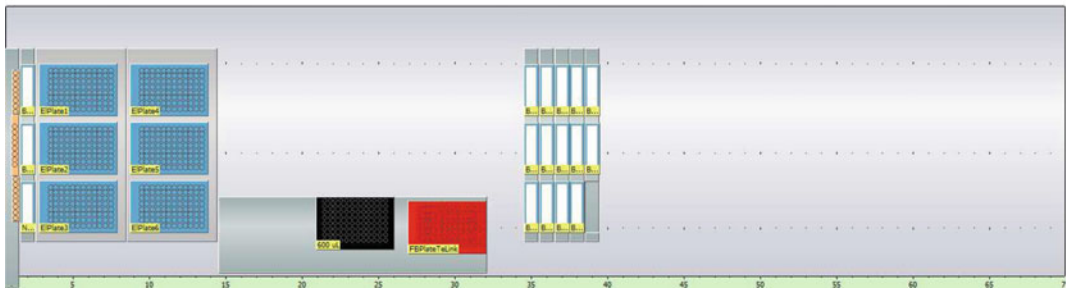
3. If using MP4 pos carriers or MP3 pos carriers placed behind the Te-Shuttle as opposed to next to it, beware that the liquid handling will be compromised due to the physical dimensions of the robot. Channel 8 of the LiHa will not be able to reach the first row of a plate in the first site of an MP4 pos carrier and Channel 1 of the LiHa will not be able to reach the last row of a plate in the fourth site of a MP4 pos carrier. In this case, liquid handling commands will need to take such limitations into consideration.
4. Depending on the installed EVOware version, it may not be possible to define variables for grid numbers in Transfer Labware commands. This is circumvented by joining two 9-site Hotel Carriers to a single 18-site hotel carrier. In this carrier the first nine sites will have exactly the same *X*-Offsets as in a conventional 9-site Hotel carrier, whereas the next nine sites will have increased *X*-Offsets by ~128 mm.
5. Labware transfers are trained via carrier definitions. All such training is to be made using a single location as a reference point and by using a single plate as a reference labware.
6. The sanitization protocol washes the tips with 10 mL in the waste and cleaner using the Fast-Wash module. This is followed with two cycles of aspirating and dispensing 900 μL of 0.5 M NaOH to a single trough and using a custom liquid class. Finally, the tips are then washed again with 10 mL in the waste and cleaner using the Fast-Wash module and then with 1 mL in the waste and cleaner while not using the Fast-Wash module so as to regenerate the air gap.
7. The custom liquid class used for the sanitization has an 8 mm offset in the aspiration so as to submerge the tips further in the sanitization solution.
8. The sanitization protocol is effective in cleaning the tips for most crude and purified proteinaceous solutions. It needs to be tested in a new laboratory for its effectiveness by liquid handling a sample, applying the sanitization protocol, liquid handling a buffer or DI water and testing the lastly liquid handled solution for its content in contaminants via a sensitive assay (e.g., ELISA).
9. The number of steps in a gradient will affect the total number of collected fractions and prepared elution buffers and also the volumes of the collected fractions. For example, a 10 CV gradient with 100 steps will result in steps with a size of 0.1 of a CV. In the case of both 0.2 and 0.6 mL RoboColumns, such

fractions are too small to be measured reliably in a plate reader with full area collection plates and offer too little volume for further analytical steps. Maintain fraction volumes of at least 100 μL to obtain separations with a sufficient number of steps and with enough volume for further analysis. Conversely, in the case of small number of steps, do not select them in a fashion giving fraction volumes greater than the volumetric capacity of a collection plate (typically ~0.3 mL for most 96 well full area microplates) as this will cause the plates to flood and will lead to cross contamination of samples and robot contamination.

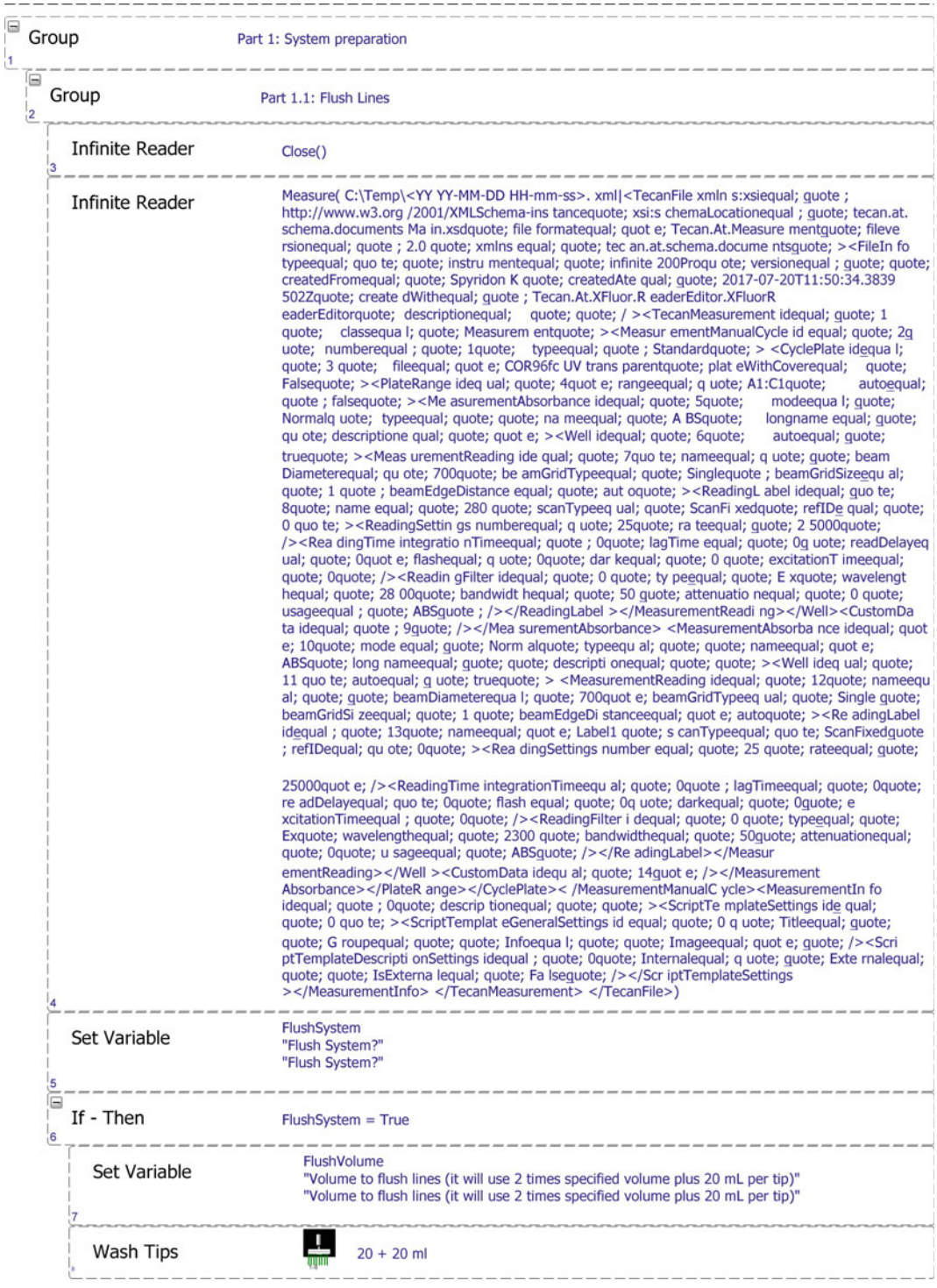
10. To measure fractions in the UV spectrum, use UV transparent plates (Corning Inc., NY, USA).
11. To determine fraction volumes, also measure each collected fraction at 900 and 990 nm and determine volumes, and then pathlength (by dividing the volume by the cross sectional surface area of the wells in a collection plate), as described in [15].
12. When preparing buffers ensure to prepare them at a volume that includes at least 150 μL of excess for 96-well deep square well plates (Fisher Scientific, Loughborough, UK) and an additional of 150 μL for the aliquoting of the buffers in plates to blank the measured fractions.
13. When defining gradients in the HT scale, define them on a basis of mM CV^{-1} or %B CV^{-1} and not as mM or %B per unit time, since, when scaling down or up using RoboColumns this takes place on a constant residence time basis. In this case, the flowrate is adjusted so as to match the residence time, as opposed to the linear velocity that is typically implemented with conventional chromatography.
14. Include at least ~500 μL of excess to account for dead volumes in the wells of 48-well deep well square plates (Elkay Ltd., Hampshire, UK).
15. Residence times of up to 4 min and 12 min for the 0.2 mL and 0.6 mL RoboColumns respectively are possible with the provided liquid classes; On EVO robots it is not possible to implement different liquid classes per channel and hence RoboColumn screening experiments are grouped based on residence times.
16. A probe capable of fitting into wells of 96-well plates needs to be used (~4 mm shaft diameter).

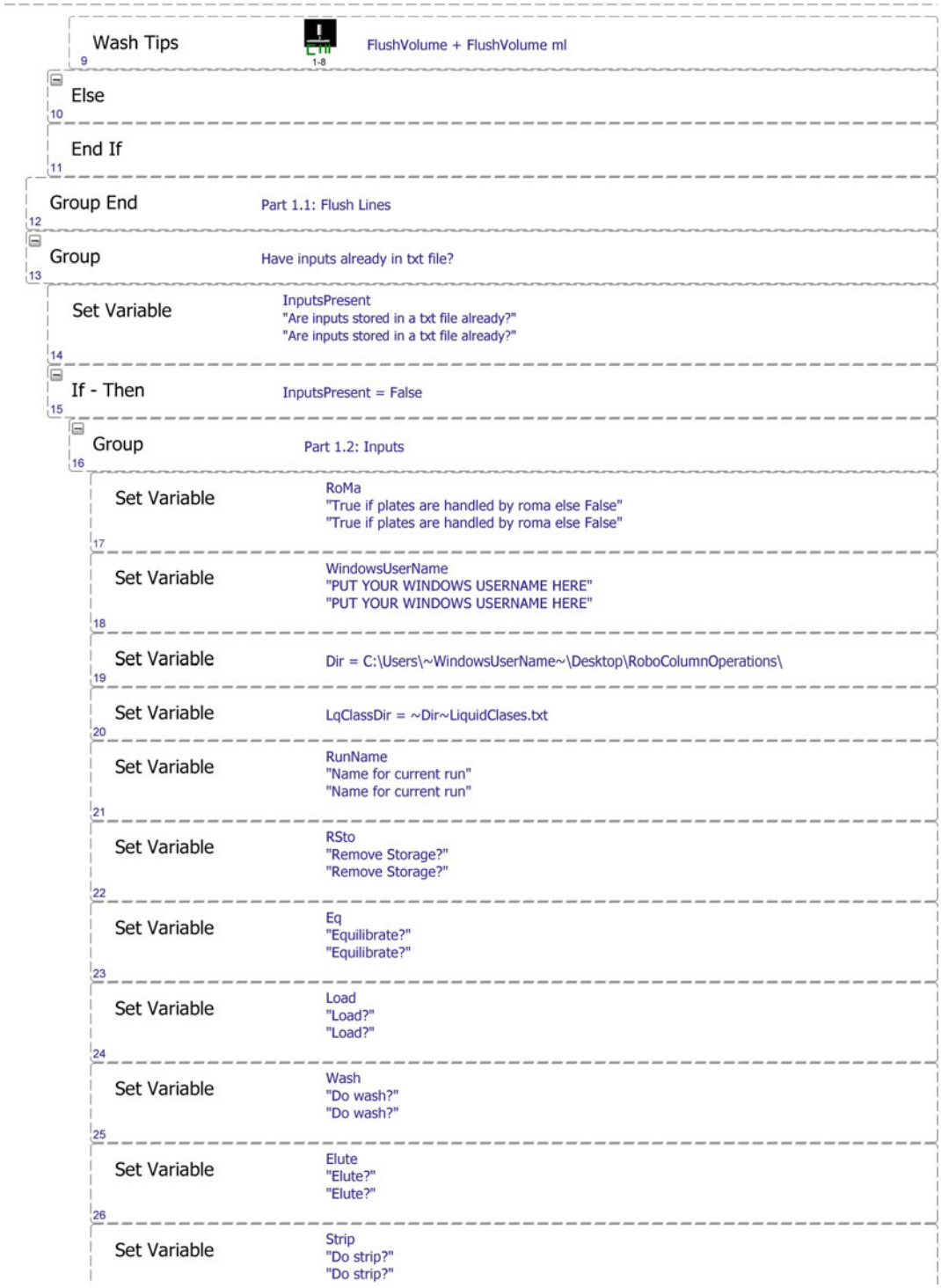
5. Appendix 1: EVOware Script for Preparation of Gradient Elution Buffers

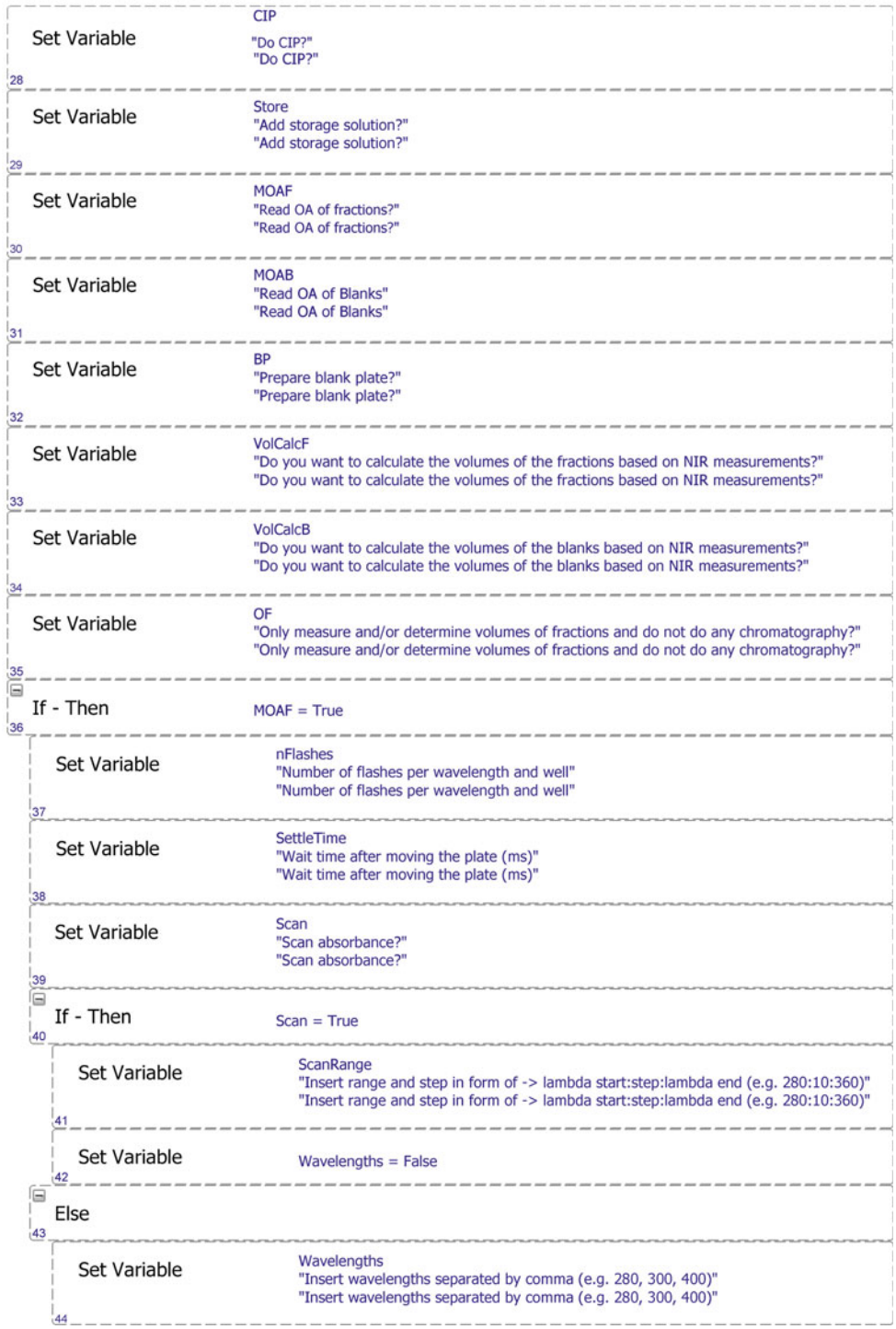

21 Dispense
1-8
SanVol µl Tip Sanitization
"NaOH" (Col. 1, Rows 1-8)
22 End Loop
"Sanitization"
23 Wash Tips
1-8
SanIn + SanOut ml
24 Else
25 End If
26 Group End
Sanitization
27 Group
ElPlatePrep
28 Set Variable
DirName
"Where do you have the scripts"
"Where do you have the scripts"
29 Worklist
1-8
Load Worklist "~DirName~\BufferScript.gwl"
Water free dispense
30 Worklist
Execute loaded worklist(s)
31 Group End
ElPlatePrep
32 User Prompt
"Mix the plates!"
sound : no



6. Appendix 2: EVOware Script for Performing RoboColumn Chromatography Using 0.6 mL Columns




Wash Tips
1-8
FlushVolume + FlushVolume ml
9
Else
10
End If
11
Group End
Part 1.1: Flush Lines
12
Group
Have inputs already in txt file?
13
Set Variable
InputsPresent
"Are inputs stored in a txt file already?"
"Are inputs stored in a txt file already?"
14
If - Then
InputsPresent = False
15
Group
Part 1.2: Inputs
16
Set Variable
RoMa
"True if plates are handled by roma else False"
"True if plates are handled by roma else False"
17
Set Variable
WindowsUserName
"PUT YOUR WINDOWS USERNAME HERE"
"PUT YOUR WINDOWS USERNAME HERE"
18
Set Variable
Dir = C:\Users\~WindowsUserName~\Desktop\RoboColumnOperations\
19
Set Variable
LqClassDir = ~Dir~LiquidClases.txt
20
Set Variable
RunName
"Name for current run"
"Name for current run"
21
Set Variable
RSto
"Remove Storage?"
"Remove Storage?"
22
Set Variable
Eq
"Equilibrate?"
"Equilibrate?"
23
Set Variable
Load
"Load?"
"Load?"
24
Set Variable
Wash
"Do wash?"
"Do wash?"
25
Set Variable
Elute
"Elute?"
"Elute?"
26
Set Variable
Strip
"Do strip?"
"Do strip?"


Set Variable
CIP
"Do CIP?"
"Do CIP?"
28
Set Variable
Store
"Add storage solution?"
"Add storage solution?"
29
Set Variable
MOAF
"Read OA of fractions?"
"Read OA of fractions?"
30
Set Variable
MOAB
"Read OA of Blanks"
"Read OA of Blanks"
31
Set Variable
BP
"Prepare blank plate?"
"Prepare blank plate?"
32
Set Variable
VolCalcF
"Do you want to calculate the volumes of the fractions based on NIR measurements?"
"Do you want to calculate the volumes of the fractions based on NIR measurements?"
33
Set Variable
VolCalcB
"Do you want to calculate the volumes of the blanks based on NIR measurements?"
"Do you want to calculate the volumes of the blanks based on NIR measurements?"
34
Set Variable
OF
"Only measure and/or determine volumes of fractions and do not do any chromatography?"
"Only measure and/or determine volumes of fractions and do not do any chromatography?"
35
If - Then
MOAF = True
36
Set Variable
nFlashes
"Number of flashes per wavelength and well"
"Number of flashes per wavelength and well"
37
Set Variable
SettleTime
"Wait time after moving the plate (ms)"
"Wait time after moving the plate (ms)"
38
Set Variable
Scan
"Scan absorbance?"
"Scan absorbance?"
39
If - Then
Scan = True
40
Set Variable
ScanRange
"Insert range and step in form of -> lambda start:step:lambda end (e.g. 280:10:360)"
"Insert range and step in form of -> lambda start:step:lambda end (e.g. 280:10:360)"
41
Set Variable
Wavelengths = False
42
Else
43
Set Variable
Wavelengths
"Insert wavelengths separated by comma (e.g. 280, 300, 400)"
"Insert wavelengths separated by comma (e.g. 280, 300, 400)"
44


Set Variable ScanRange = False
45
End If
46
If - Then MOAB = True
47
Set Variable MOAFB = Both
48
Else
49
Set Variable MOAFB = F
50
End If
51
Else
52
If - Then MOAB = True
53
Set Variable nFlashes "Number of flashes per wavelength and well" "Number of flashes per wavelength and well"
54
Set Variable SettleTime "Wait time after moving the plate (ms)" "Wait time after moving the plate (ms)"
55
Set Variable Scan "Scan absorbance?" "Scan absorbance?"
56
If - Then Scan = True
57
Set Variable ScanRange "Insert range and step in form of -> lambda start:step:lambda end (e.g. 280:10:360)" "Insert range and step in form of -> lambda start:step:lambda end (e.g. 280:10:360)"
58
Set Variable Wavelengths = False
59
Else
60
Set Variable Wavelengths "Insert wavelengths separated by comma (e.g. 280, 300, 400)" "Insert wavelengths separated by comma (e.g. 280, 300, 400)"
61
Set Variable ScanRange = False
62
End If
63
Set Variable MOAFB = B
64
Else

```
66  Set Variable    ScanRange = False
67  Set Variable    Wavelengths = False
68  Set Variable    nFlashes = 0
69  Set Variable    SettleTime = 0
70  Set Variable    MOAFB = Neither
71  End If
72  End If
73  Set Variable    EqualNF
                    "In case of varying number of fractions per column, force all columns to have equal number
                    of fractions? (WARNING IF SET TO FALSE THEN SOME COLUMNS WILL WAIT AND IN
                    EXTREME CASES THEY MIGHT GET DRY)"
                    "In case of varying number of fractions per column, force all columns to have equal number
                    of fractions? (WARNING IF SET TO FALSE THEN SOME COLUMNS WILL WAIT AND IN
                    EXTREME CASES THEY MIGHT GET DRY)"
74  Set Variable    ElBPN = ElPlate
75  Set Variable    ElBPT = 96 Well DeepWell square
76  Set Variable    TotalEPC = 4
77  Set Variable    TotalPC = 18
78  Set Variable    RPN = Reagents
79  Set Variable    TeShuttleN = FPlate
80  Set Variable    TeShuttleT = 96 Well Microplate
81  Set Variable    HPN = FBPlate
82  Set Variable    HPT = 96 Well Microplate
83  Set Variable    TipPrepare
                    "Sanitize tips at the beginning of the run?"
                    "Sanitize tips at the beginning of the run?"
84  Set Variable    nTips = 8
85  Set Variable    SanVol
                    "Volume for NaOH sanitization (uL)"
                    "Volume for NaOH sanitization (uL)"
    Set Variable    SanWaste
                    "Inside Sanitization wash (mL)"
                    "Inside Sanitization wash (mL)"
```

87	Set Variable	SanCleaner "Outside sanitization wash (mL)" "Outside sanitization wash (mL)"
88	Set Variable	nC "How many columns are you running (1-8)?" "How many columns are you running (1-8)?", 1 - 8
89	Group	Initialize CVs
90	Set Variable	LCV1 = 0
91	Set Variable	WCV1 = 0
92	Set Variable	ElCV1 = 0
93	Set Variable	StrCV1 = 0
94	Set Variable	LCV2 = 0
95	Set Variable	WCV2 = 0
96	Set Variable	ElCV2 = 0
97	Set Variable	StrCV2 = 0
98	Set Variable	LCV3 = 0
99	Set Variable	WCV3 = 0
100	Set Variable	ElCV3 = 0
101	Set Variable	StrCV3 = 0
102	Set Variable	LCV4 = 0
103	Set Variable	WCV4 = 0
104	Set Variable	ElCV4 = 0
105	Set Variable	StrCV4 = 0
106	Set Variable	LCV5 = 0
107	Set Variable	WCV5 = 0
108	Set Variable	ElCV5 = 0
	Set Variable	StrCV5 = 0


Set Variable LCV6 = 0
110
Set Variable WCV6 = 0
111
Set Variable ElCV6 = 0
112
Set Variable StrCV6 = 0
113
Set Variable LCV7 = 0
114
Set Variable WCV7 = 0
115
Set Variable ElCV7 = 0
116
Set Variable StrCV7 = 0
117
Set Variable LCV8 = 0
118
Set Variable WCV8 = 0
119
Set Variable ElCV8 = 0
120
Set Variable StrCV8 = 0
121
Group End Initialize CVs
122
Group Initialize fraction volumes
123
Set Variable FvL1 = 0
124
Set Variable FvW1 = 0
125
Set Variable FvE1 = 0
126
Set Variable FvS1 = 0
127
Set Variable FvL2 = 0
128
Set Variable FvW2 = 0
129
Set Variable FvE2 = 0
130
Set Variable FvS2 = 0
131
Set Variable FvL3 = 0
132
Set Variable FvW3 = 0

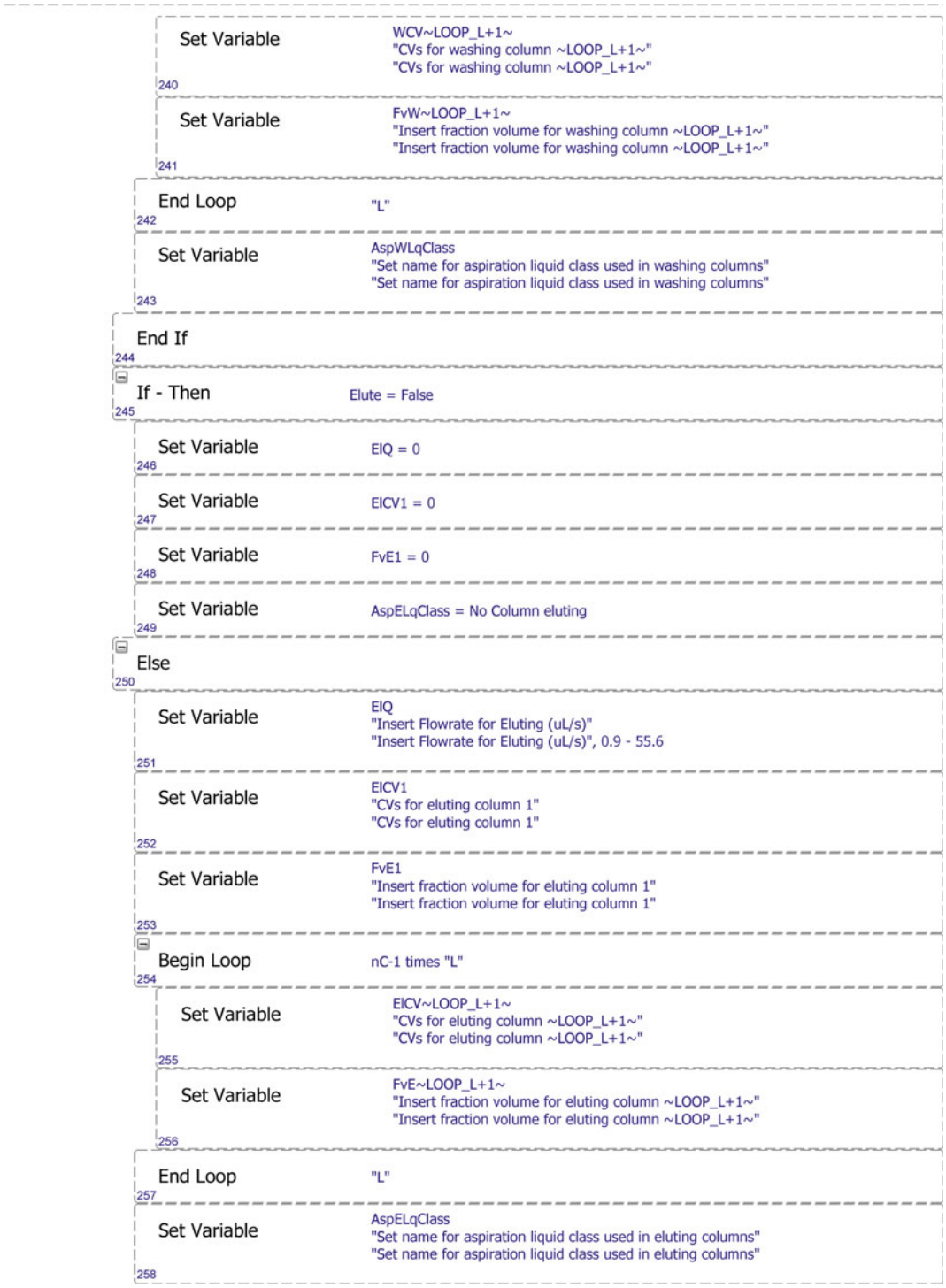
134	Set Variable	FvE3 = 0
135	Set Variable	FvS3 = 0
136	Set Variable	FvL4 = 0
137	Set Variable	FvW4 = 0
138	Set Variable	FvE4 = 0
139	Set Variable	FvS4 = 0
140	Set Variable	FvL5 = 0
141	Set Variable	FvW5 = 0
142	Set Variable	FvE5 = 0
143	Set Variable	FvS5 = 0
144	Set Variable	FvL6 = 0
145	Set Variable	FvW6 = 0
146	Set Variable	FvE6 = 0
147	Set Variable	FvS6 = 0
148	Set Variable	FvL7 = 0
149	Set Variable	FvW7 = 0
150	Set Variable	FvE7 = 0
151	Set Variable	FvS7 = 0
152	Set Variable	FvL8 = 0
153	Set Variable	FvW8 = 0
154	Set Variable	FvE8 = 0
155	Set Variable	FvS8 = 0
156	Group End	Initialize fraction volumes

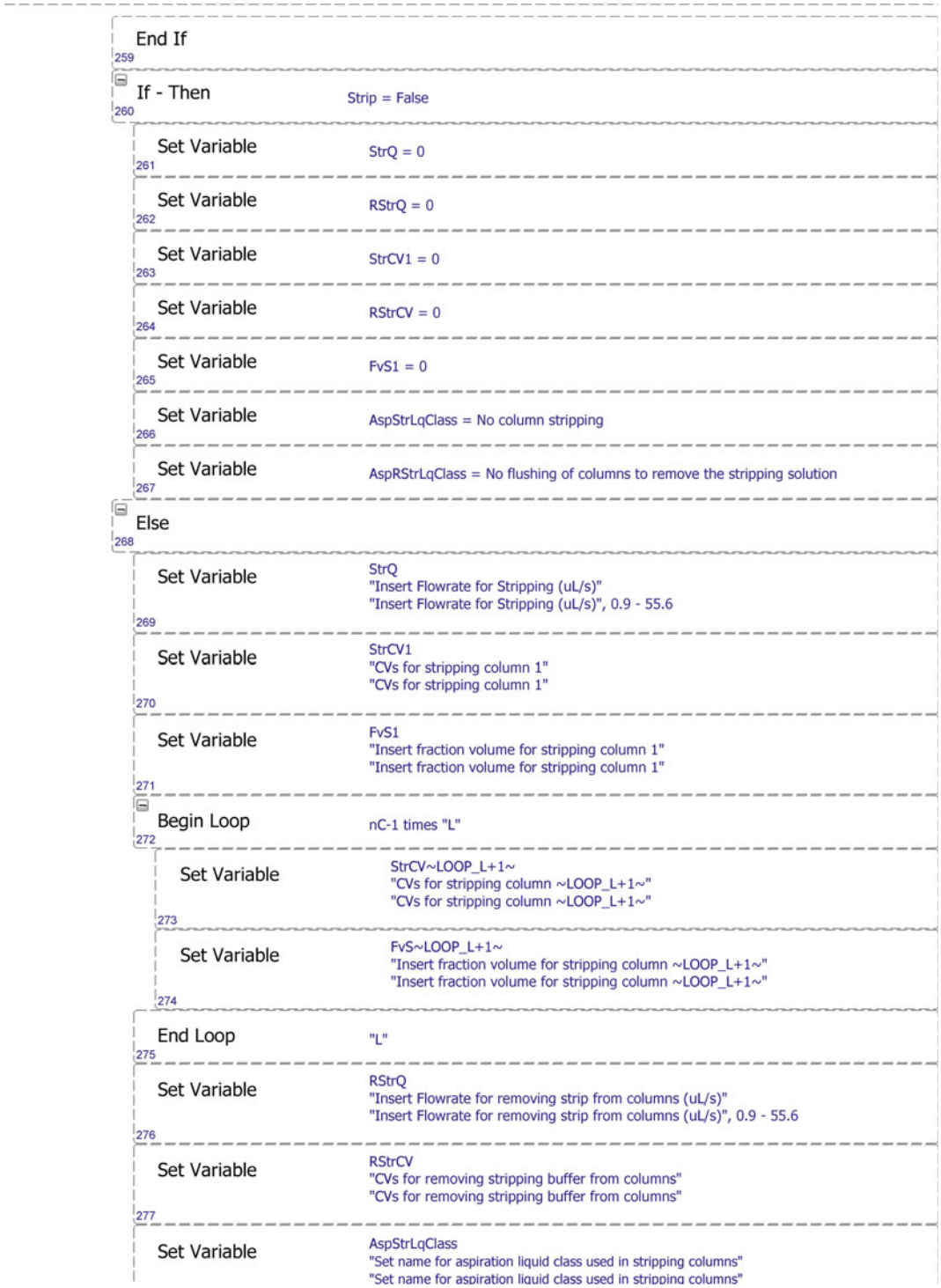

Set Variable
CV
"What is the Column volume(s) (uL)"
"What is the Column volume(s) (uL)"
157
If - Then
CV = 600
158
Set Variable
TeChromN = RoboColumn600
159
Set Variable
TeChromT = Atoll 600 set 24
160
Set Variable
RPT
"Type of Labware for reagent plate (96 or 48 Well DeepWell square)"
"Type of Labware for reagent plate (96 or 48 Well DeepWell square)"
161
Else
162
Set Variable
TeChromN = RoboColumn200
163
Set Variable
TeChromT = Atoll 50/100/200 set 24
164
Set Variable
RPT
"Type of Labware for reagent plate (96 or 48 Well DeepWell square)"
"Type of Labware for reagent plate (96 or 48 Well DeepWell square)"
165
End If
166
Set Variable
EIP_GridStart = 3
167
Set Variable
EIP_SiteStart = 1
168
Set Variable
EIP_nC = 12
169
Set Variable
EIP_nR = 8
170
Set Variable
RP_Grid = 9
171
Set Variable
RP_Site = 3
172
If - Then
RPT = 96 Well DeepWell square
173
Set Variable
RP_nC = 12
174
Else
175
Set Variable
RP_nC = 6
176
End If
177
Set Variable
RP_nR = 8
178


Set Variable RP_EqC = 1
179
Set Variable RP_LC = 2
180
Set Variable RP_WC = 3
181
Set Variable RP_StrC = 4
182
Set Variable RP_CIPC = 5
183
Set Variable RP_StoC = 6
184
Set Variable TeChrom_Grid = 15
185
Set Variable TeChrom_Site = 1
186
Set Variable TeChrom_nC = 12
187
Set Variable TeChrom_nR = 8
188
Set Variable HP_Grid = 9
189
Set Variable HP_Site = 2
190
Set Variable HP_nC = 12
191
Set Variable HP_nR = 8
192
Set Variable
Slope
"Slope of calibration curve of volume (uL*10^13) as y and liquid detection or absorbance result for x (default are for OA)"
"Slope of calibration curve of volume (uL*10^13) as y and liquid detection or absorbance result for x (default are for OA)"
193
Set Variable
Intercept
"Intercept of calibration curve of volume (uL*10^13) as y and liquid detection or absorbance result for x (default are for OA)"
"Intercept of calibration curve of volume (uL*10^13) as y and liquid detection or absorbance result for x (default are for OA)"
194
Set Variable
Alpha
"Cross section surface area of well in ~HPN~ (cm^2)"
"Cross section surface area of well in ~HPN~ (cm^2)"
195
Group Questions for Chromatography
196
If - Then RSto = False
197
Set Variable RStoQ = 0
198
Set Variable RStoCV = 0
199

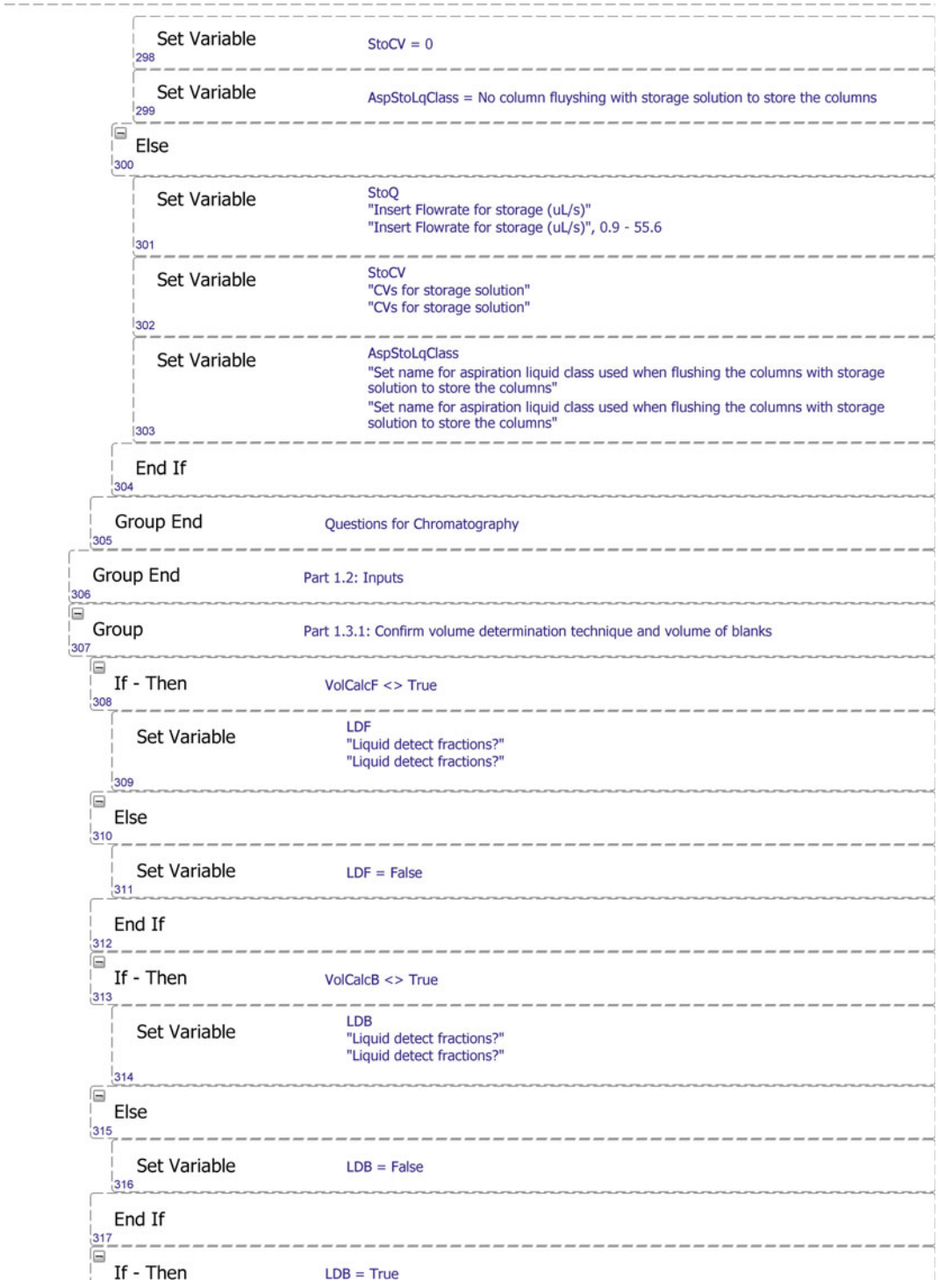

Set Variable
AspRStoLqClass = No column flushing for removal of storage
200
Else
201
Set Variable
RStoQ
"Insert Flowrate fro removing storage (uL/s)"
"Insert Flowrate fro removing storage (uL/s)", 0.9 - 55.6
202
Set Variable
RStoCV
"CVs for removing storage"
"CVs for removing storage"
203
Set Variable
AspRStoLqClass
"Set name for aspiration liquid class used in flushing storage solution from columns"
"Set name for aspiration liquid class used in flushing storage solution from columns"
204
End If
205
If - Then
Eq = False
206
Set Variable
EqQ = 0
207
Set Variable
EqCV = 0
208
Set Variable
AspEqLqClass = No column equilibrating
209
Else
210
Set Variable
EqQ
"Insert Flowrate for equilibration (uL/s)"
"Insert Flowrate for equilibration (uL/s)", 0.9 - 55.6
211
Set Variable
EqCV
"CVs for equilibrating columns"
"CVs for equilibrating columns"
212
Set Variable
AspEqLqClass
"Set name for aspiration liquid class used in column equilibration"
"Set name for aspiration liquid class used in column equilibration"
213
End If
214
If - Then
Load = False
215
Set Variable
LQ = 0
216
Set Variable
LCV1 = 0
217
Set Variable
FvL1 = 0
218
Set Variable
AspLLqClass = No column loading
219
Else


Set Variable
LQ
"Insert Flowrate for loading (uL/s)"
"Insert Flowrate for loading (uL/s)", 0.9 - 55.6
221
Set Variable
LCV1
"CVs for loading column 1"
"CVs for loading column 1"
222
Set Variable
FvL1
"Insert fraction volume for loading column 1"
"Insert fraction volume for loading column 1"
223
Begin Loop
nC-1 times "L"
224
Set Variable
LCV~LOOP_L+1~
"CVs for loading column ~LOOP_L+1~"
"CVs for loading column ~LOOP_L+1~"
225
Set Variable
FvL~LOOP_L+1~
"Insert fraction volume for loading column ~LOOP_L+1~"
"Insert fraction volume for loading column ~LOOP_L+1~"
226
End Loop
"L"
227
Set Variable
AspLLqClass
"Set name for aspiration liquid class used loading columns"
"Set name for aspiration liquid class used loading columns"
228
End If
229
If - Then
Wash = False
230
Set Variable
WQ = 0
231
Set Variable
WCV1 = 0
232
Set Variable
FvW1 = 0
233
Set Variable
AspWLqClass = No column washing
234
Else
235
Set Variable
WQ
"Insert Flowrate for Washing (uL/s)"
"Insert Flowrate for Washing (uL/s)", 0.9 - 55.6
236
Set Variable
WCV1
"CVs for washing column 1"
"CVs for washing column 1"
237
Set Variable
FvW1
"Insert fraction volume for washing column 1"
"Insert fraction volume for washing column 1"
238
Begin Loop
nC-1 times "L"
239

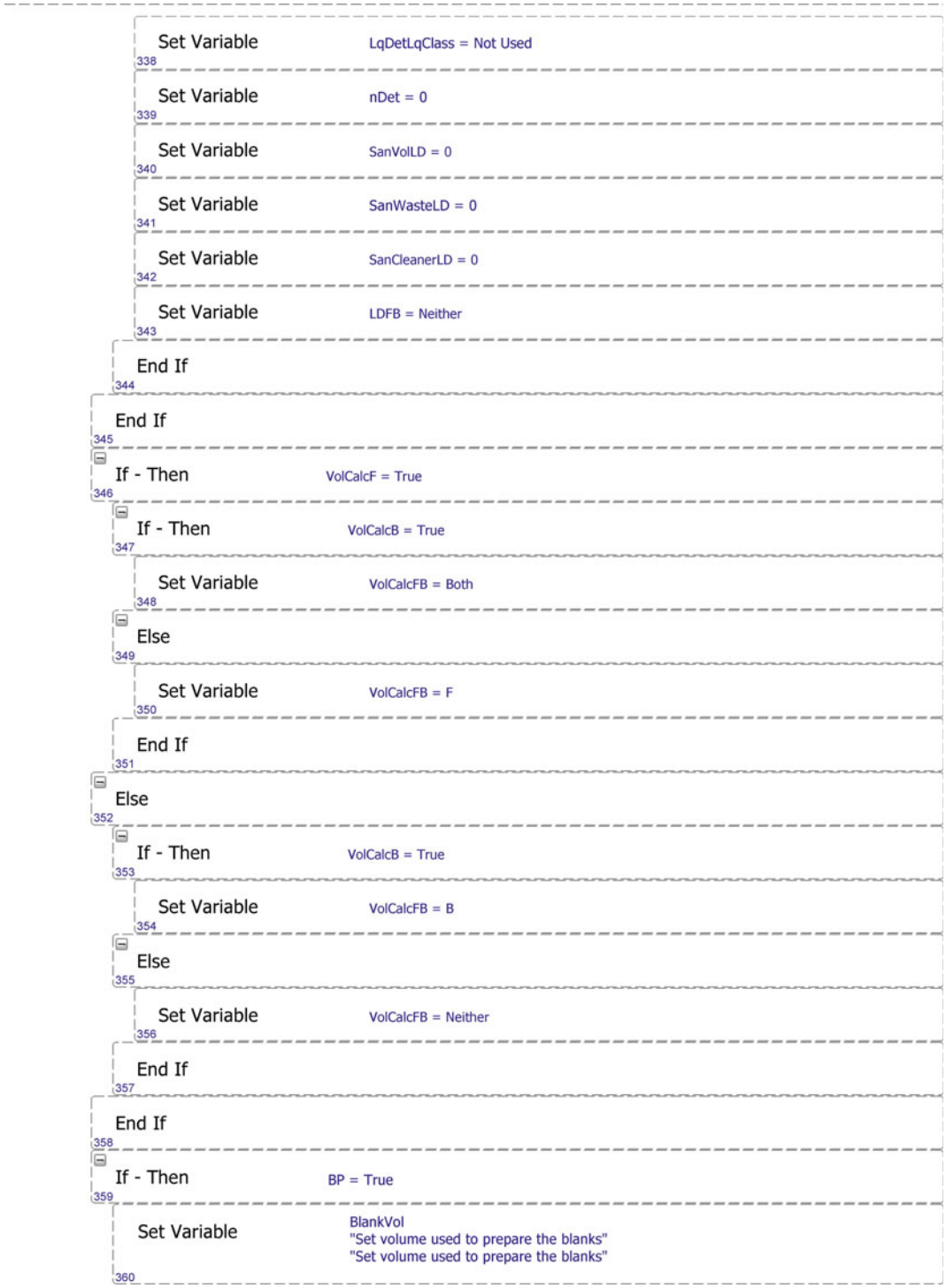

Set Variable
WCV~LOOP_L+1~
"CVs for washing column ~LOOP_L+1~"
"CVs for washing column ~LOOP_L+1~"
240
Set Variable
FvW~LOOP_L+1~
"Insert fraction volume for washing column ~LOOP_L+1~"
"Insert fraction volume for washing column ~LOOP_L+1~"
241
End Loop
"L"
242
Set Variable
AspWLqClass
"Set name for aspiration liquid class used in washing columns"
"Set name for aspiration liquid class used in washing columns"
243
End If
244
If - Then
Elute = False
245
Set Variable
ElQ = 0
246
Set Variable
ElCV1 = 0
247
Set Variable
FvE1 = 0
248
Set Variable
AspELqClass = No Column eluting
249
Else
250
Set Variable
ElQ
"Insert Flowrate for Eluting (uL/s)"
"Insert Flowrate for Eluting (uL/s)", 0.9 - 55.6
251
Set Variable
ElCV1
"CVs for eluting column 1"
"CVs for eluting column 1"
252
Set Variable
FvE1
"Insert fraction volume for eluting column 1"
"Insert fraction volume for eluting column 1"
253
Begin Loop
nC-1 times "L"
254
Set Variable
ElCV~LOOP_L+1~
"CVs for eluting column ~LOOP_L+1~"
"CVs for eluting column ~LOOP_L+1~"
255
Set Variable
FvE~LOOP_L+1~
"Insert fraction volume for eluting column ~LOOP_L+1~"
"Insert fraction volume for eluting column ~LOOP_L+1~"
256
End Loop
"L"
257
Set Variable
AspELqClass
"Set name for aspiration liquid class used in eluting columns"
"Set name for aspiration liquid class used in eluting columns"
258

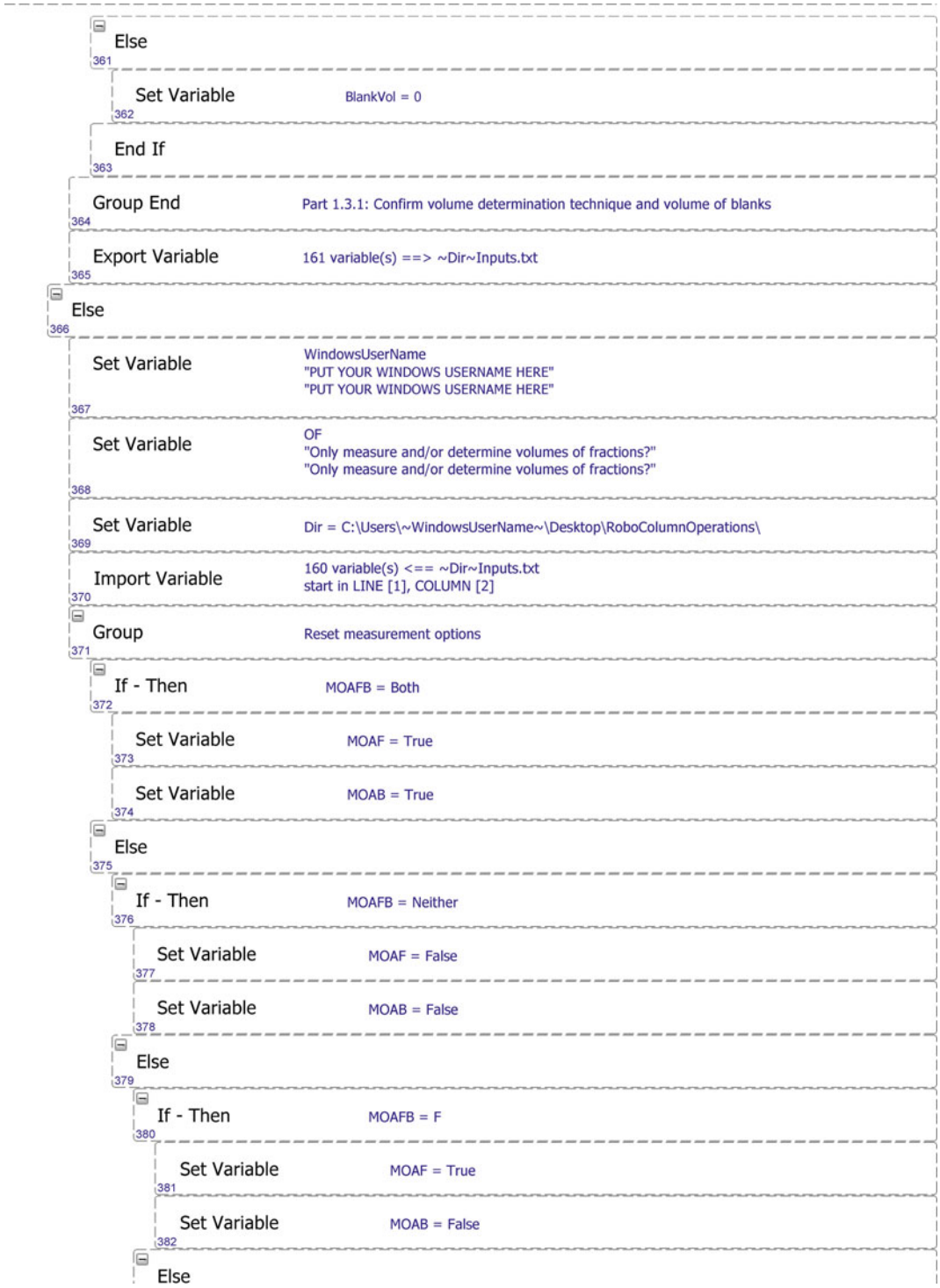

End If
259
If - Then
Strip = False
260
Set Variable
StrQ = 0
261
Set Variable
RStrQ = 0
262
Set Variable
StrCV1 = 0
263
Set Variable
RStrCV = 0
264
Set Variable
FvS1 = 0
265
Set Variable
AspStrLqClass = No column stripping
266
Set Variable
AspRStrLqClass = No flushing of columns to remove the stripping solution
267
Else
268
Set Variable
StrQ
"Insert Flowrate for Stripping (uL/s)"
"Insert Flowrate for Stripping (uL/s)", 0.9 - 55.6
269
Set Variable
StrCV1
"CVs for stripping column 1"
"CVs for stripping column 1"
270
Set Variable
FvS1
"Insert fraction volume for stripping column 1"
"Insert fraction volume for stripping column 1"
271
Begin Loop
nC-1 times "L"
272
Set Variable
StrCV~LOOP_L+1~
"CVs for stripping column ~LOOP_L+1~"
"CVs for stripping column ~LOOP_L+1~"
273
Set Variable
FvS~LOOP_L+1~
"Insert fraction volume for stripping column ~LOOP_L+1~"
"Insert fraction volume for stripping column ~LOOP_L+1~"
274
End Loop
"L"
275
Set Variable
RStrQ
"Insert Flowrate for removing strip from columns (uL/s)"
"Insert Flowrate for removing strip from columns (uL/s)", 0.9 - 55.6
276
Set Variable
RStrCV
"CVs for removing stripping buffer from columns"
"CVs for removing stripping buffer from columns"
277
Set Variable
AspStrLqClass
"Set name for aspiration liquid class used in stripping columns"
"Set name for aspiration liquid class used in stripping columns"

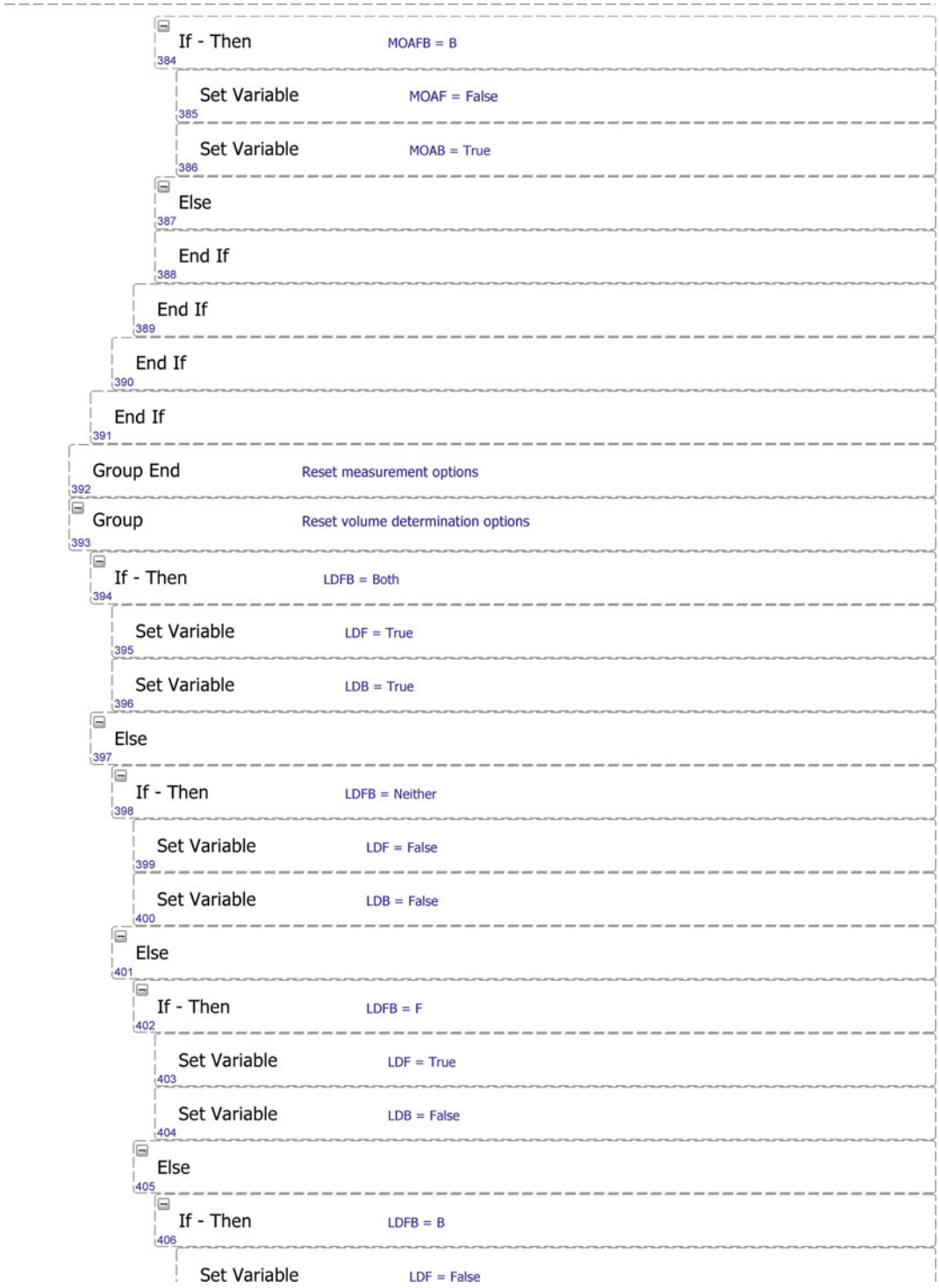
```
Set Variable        AspRStrLqClass
                    "Set name for aspiration liquid class used when flushing columns to remove the strip solution"
                    "Set name for aspiration liquid class used when flushing columns to remove the strip solution"
279
End If
280
If - Then           CIP = False
281
    Set Variable    CIPQ = 0
282
    Set Variable    RCIPQ = 0
283
    Set Variable    CIPCV = 0
284
    Set Variable    RCIPCV = 0
285
    Set Variable    AspCIPLqClass = No column regeneration
286
    Set Variable    AspRCIPLqClass = No column flushing to remove the regeneration solution
287
Else
288
    Set Variable    CIPQ
                    "Insert Flowrate for CIP (uL/s)"
                    "Insert Flowrate for CIP (uL/s)", 0.9 - 55.6
289
    Set Variable    RCIPQ
                    "Insert Flowrate for removing CIP (uL/s)"
                    "Insert Flowrate for removing CIP (uL/s)", 0.9 - 55.6
290
    Set Variable    CIPCV
                    "CVs for CIP solution"
                    "CVs for CIP solution"
291
    Set Variable    RCIPCV
                    "CVs for removing CIP solution"
                    "CVs for removing CIP solution"
292
    Set Variable    AspCIPLqClass
                    "Set name for aspiration liquid class used in regenerating columns"
                    "Set name for aspiration liquid class used in regenerating columns"
293
    Set Variable    AspRCIPLqClass
                    "Set name for aspiration liquid class used when flushing columns to remove the regeneration solution"
                    "Set name for aspiration liquid class used when flushing columns to remove the regeneration solution"
294
End If
295
If - Then           Store = False
296
    Set Variable    StoQ = 0
297
```

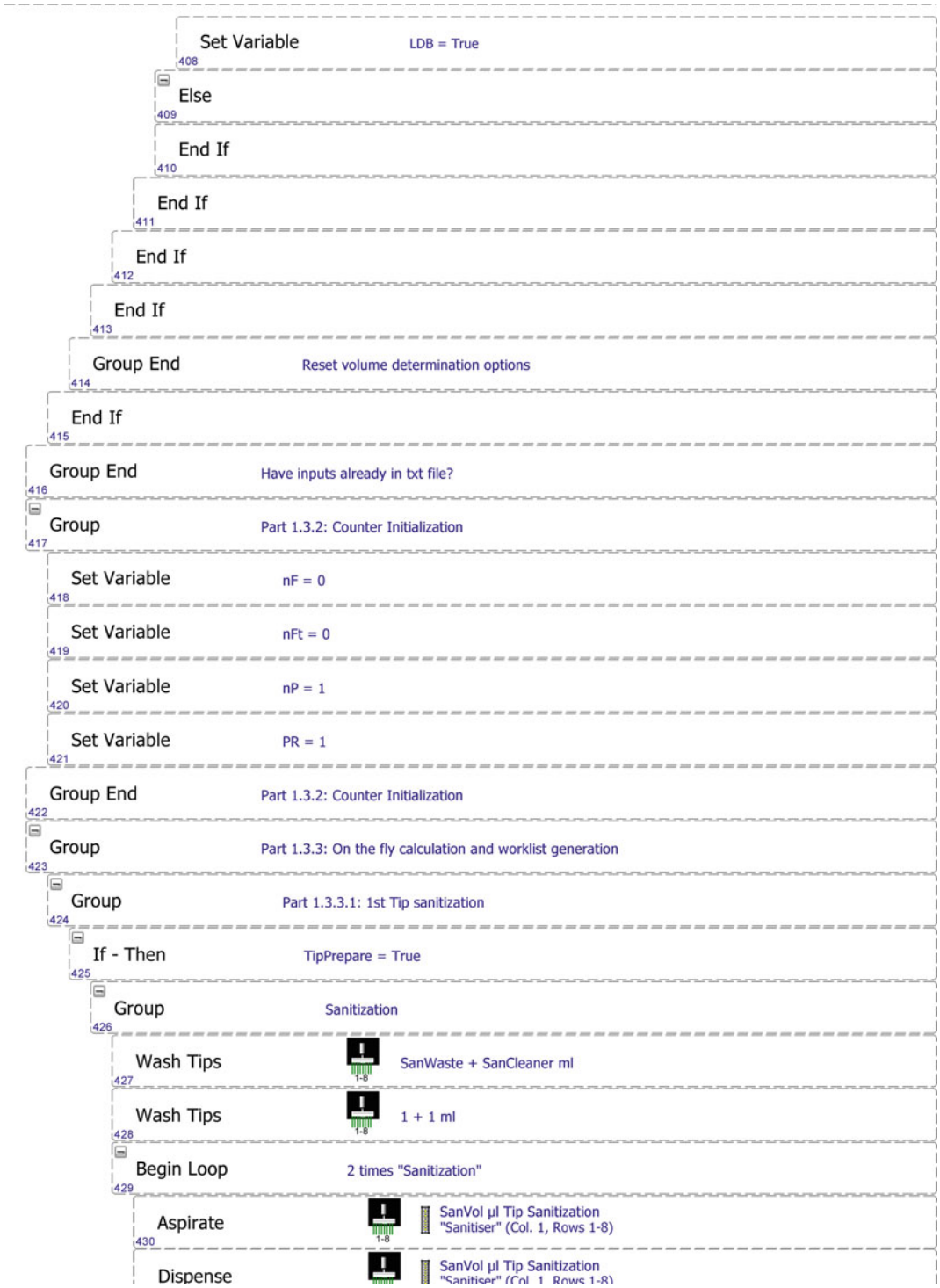

Set Variable
StoCV = 0
298
Set Variable
AspStoLqClass = No column fluyshing with storage solution to store the columns
299
Else
300
Set Variable
StoQ
"Insert Flowrate for storage (uL/s)"
"Insert Flowrate for storage (uL/s)", 0.9 - 55.6
301
Set Variable
StoCV
"CVs for storage solution"
"CVs for storage solution"
302
Set Variable
AspStoLqClass
"Set name for aspiration liquid class used when flushing the columns with storage solution to store the columns"
"Set name for aspiration liquid class used when flushing the columns with storage solution to store the columns"
303
End If
304
Group End
Questions for Chromatography
305
Group End
Part 1.2: Inputs
306
Group
Part 1.3.1: Confirm volume determination technique and volume of blanks
307
If - Then
VolCalcF <> True
308
Set Variable
LDF
"Liquid detect fractions?"
"Liquid detect fractions?"
309
Else
310
Set Variable
LDF = False
311
End If
312
If - Then
VolCalcB <> True
313
Set Variable
LDB
"Liquid detect fractions?"
"Liquid detect fractions?"
314
Else
315
Set Variable
LDB = False
316
End If
317
If - Then
LDB = True


Set Variable
LqDetLqClass
"Set name for of liquid class used when liquid level detection is used"
"Set name for of liquid class used when liquid level detection is used"
319
Set Variable
nDet
"Number of liquid detections per fractions or blanks"
"Number of liquid detections per fractions or blanks"
320
Set Variable
SanVolLD
"Volume for NaOH sanitization during fraction LD (uL)"
"Volume for NaOH sanitization during fraction LD (uL)"
321
Set Variable
SanWasteLD
"Inside Sanitization LD wash (mL)"
"Inside Sanitization LD wash (mL)"
322
Set Variable
SanCleanerLD
"Outside sanitization LD wash (mL)"
"Outside sanitization LD wash (mL)"
323
If - Then
LDF = True
324
Set Variable
LDFB = Both
325
Else
326
Set Variable
LDFB = B
327
End If
328
Else
329
If - Then
LDF = True
330
Set Variable
LqDetLqClass
"Set name for of liquid class used when liquid level detection is used"
"Set name for of liquid class used when liquid level detection is used"
331
Set Variable
nDet
"Number of liquid detections per fractions or blanks"
"Number of liquid detections per fractions or blanks"
332
Set Variable
SanVolLD
"Volume for NaOH sanitization during fraction LD (uL)"
"Volume for NaOH sanitization during fraction LD (uL)"
333
Set Variable
SanWasteLD
"Inside Sanitization LD wash (mL)"
"Inside Sanitization LD wash (mL)"
334
Set Variable
SanCleanerLD
"Outside sanitization LD wash (mL)"
"Outside sanitization LD wash (mL)"
335
Set Variable
LDFB = F
336
Else


Set Variable LqDetLqClass = Not Used
338
Set Variable nDet = 0
339
Set Variable SanVolLD = 0
340
Set Variable SanWasteLD = 0
341
Set Variable SanCleanerLD = 0
342
Set Variable LDFB = Neither
343
End If
344
End If
345
If - Then VolCalcF = True
346
If - Then VolCalcB = True
347
Set Variable VolCalcFB = Both
348
Else
349
Set Variable VolCalcFB = F
350
End If
351
Else
352
If - Then VolCalcB = True
353
Set Variable VolCalcFB = B
354
Else
355
Set Variable VolCalcFB = Neither
356
End If
357
End If
358
If - Then BP = True
359
Set Variable BlankVol
"Set volume used to prepare the blanks"
"Set volume used to prepare the blanks"
360


Else
361
Set Variable BlankVol = 0
362
End If
363
Group End Part 1.3.1: Confirm volume determination technique and volume of blanks
364
Export Variable 161 variable(s) ==> ~Dir~Inputs.txt
365
Else
366
Set Variable WindowsUserName
"PUT YOUR WINDOWS USERNAME HERE"
"PUT YOUR WINDOWS USERNAME HERE"
367
Set Variable OF
"Only measure and/or determine volumes of fractions?"
"Only measure and/or determine volumes of fractions?"
368
Set Variable Dir = C:\Users\~WindowsUserName~\Desktop\RoboColumnOperations\
369
Import Variable 160 variable(s) <== ~Dir~Inputs.txt
start in LINE [1], COLUMN [2]
370
Group Reset measurement options
371
If - Then MOAFB = Both
372
Set Variable MOAF = True
373
Set Variable MOAB = True
374
Else
375
If - Then MOAFB = Neither
376
Set Variable MOAF = False
377
Set Variable MOAB = False
378
Else
379
If - Then MOAFB = F
380
Set Variable MOAF = True
381
Set Variable MOAB = False
382
Else


If - Then MOAFB = B
384
Set Variable MOAF = False
385
Set Variable MOAB = True
386
Else
387
End If
388
End If
389
End If
390
End If
391
Group End Reset measurement options
392
Group Reset volume determination options
393
If - Then LDFB = Both
394
Set Variable LDF = True
395
Set Variable LDB = True
396
Else
397
If - Then LDFB = Neither
398
Set Variable LDF = False
399
Set Variable LDB = False
400
Else
401
If - Then LDFB = F
402
Set Variable LDF = True
403
Set Variable LDB = False
404
Else
405
If - Then LDFB = B
406
Set Variable LDF = False

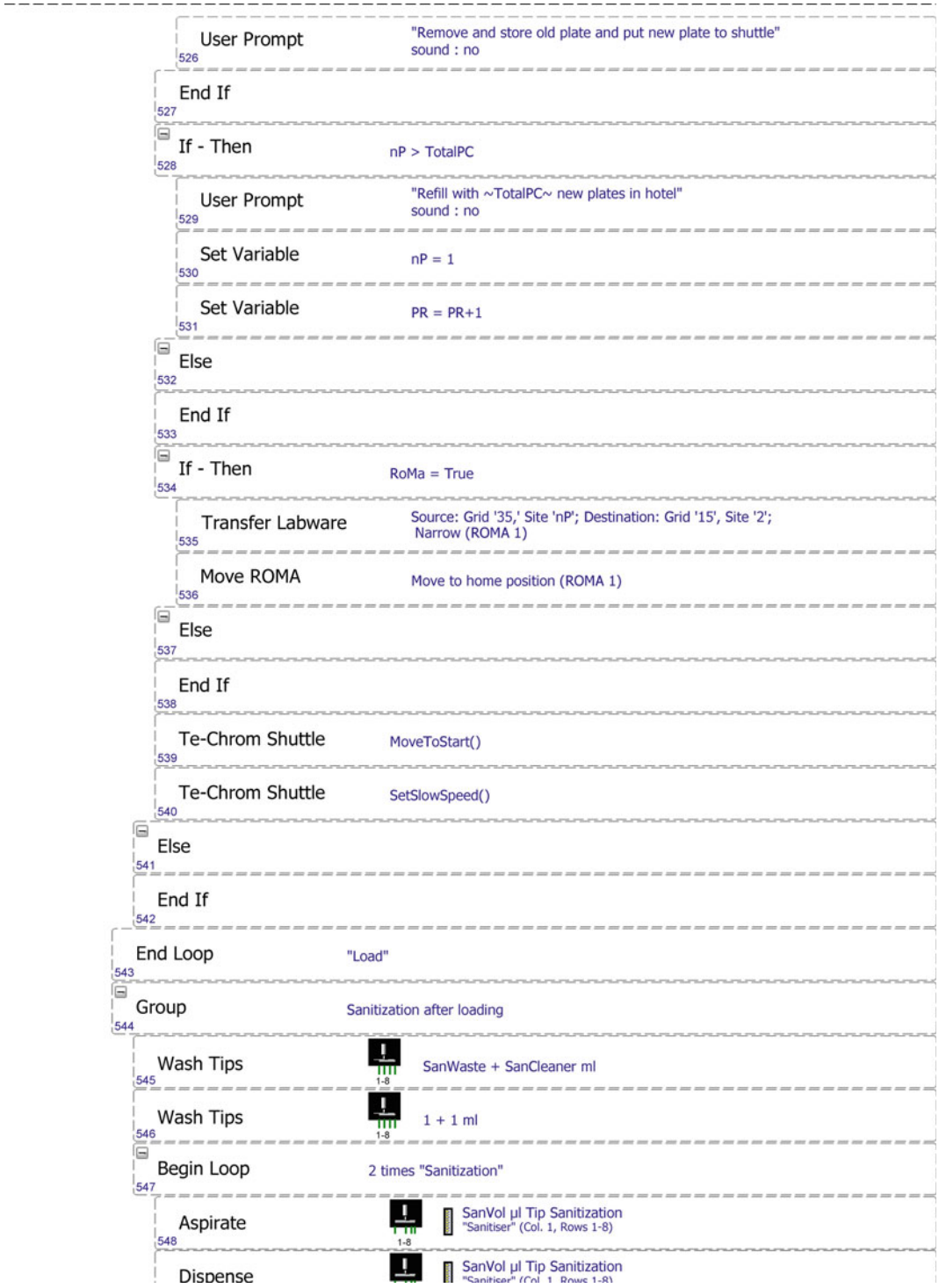

Set Variable LDB = True
408
Else
409
End If
410
End If
411
End If
412
End If
413
Group End Reset volume determination options
414
End If
415
Group End Have inputs already in txt file?
416
Group Part 1.3.2: Counter Initialization
417
Set Variable nF = 0
418
Set Variable nFt = 0
419
Set Variable nP = 1
420
Set Variable PR = 1
421
Group End Part 1.3.2: Counter Initialization
422
Group Part 1.3.3: On the fly calculation and worklist generation
423
Group Part 1.3.3.1: 1st Tip sanitization
424
If - Then TipPrepare = True
425
Group Sanitization
426
Wash Tips SanWaste + SanCleaner ml
427
Wash Tips 1 + 1 ml
428
Begin Loop 2 times "Sanitization"
429
Aspirate SanVol µl Tip Sanitization "Sanitiser" (Col. 1, Rows 1-8)
430
Dispense SanVol µl Tip Sanitization


End Loop "Sanitization"
432
Wash Tips SanWaste + SanCleaner ml
433
Wash Tips 1 + 1 ml
434
Group End Sanitization
435
Else
436
End If
437
Group End Part 1.3.3.1: 1st Tip sanitization
438
Group Part 1.3.3.2: RoboCoumnApplication exe, variable import, user queries
439
Comment ~Dir~RoboColumnApplicationv2.exe
440
Execute Application "C:\Users\Spyridon K\Desktop\RoboColumnOperations\RoboColumnApplicationv3_Robust.exe" wait
441
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [2], COLUMN [2]
442
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [3], COLUMN [2]
443
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [4], COLUMN [2]
444
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [5], COLUMN [2]
445
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [6], COLUMN [2]
446
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [7], COLUMN [2]
447
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [8], COLUMN [2]
448
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [9], COLUMN [2]
449
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [10], COLUMN [2]
450
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [11], COLUMN [2]
451
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [12], COLUMN [2]
452
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [13], COLUMN [2]
453
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt start in LINE [14], COLUMN [2]
454
Import Variable 1 variable(s) <== ~Dir~~RunName~\Calculations and Inputs\CalcsAndInputsTableFormat.txt


If - Then TotalFP+TotalBP > TotalPC
456
User Prompt "The number of total plates is greater than ~TotalPC~; Rework script to reduce the number of factions; else be around to switch plates." sound : no
457
Else
458
End If
459
If - Then nEF > TotalEPC*EIP_nC
460
User Prompt "You use a number of elution buffer plates which is greater than ~TotalEPC~. Be around to replenish the elution plates!!" sound : no
461
Else
462
End If
463
User Prompt "You generate ~TotalF~ fractions and you need ~TotalFP~ fraction plates and ~TotalBP~ blank plates, if prepared. Put the fraction plates in the Hotel!" sound : no
464
Group End Part 1.3.3.2: RoboCoumnApplication exe, variable import, user queries
465
Group End Part 1.3.3: On the fly calculation and worklist generation
466
Group Part 1.3.4: Engage waste and move Te-Shuttle to Transfer position
467
If - Then OF = False
468
Infinite Reader Close()
469
Move ROMA Move to home position (ROMA 1)
470
Te-Chrom Shuttle MoveToTransfer()
471
If - Then RoMa = True
472
Transfer Labware Source: Grid '35,' Site '1'; Destination: Grid '15', Site '2'; Narrow (ROMA 1)
473
Move ROMA Move to home position (ROMA 1)
474
Else
475
User Prompt "Put plate to transfer position of Te-chrom" sound : no
476
End If
477

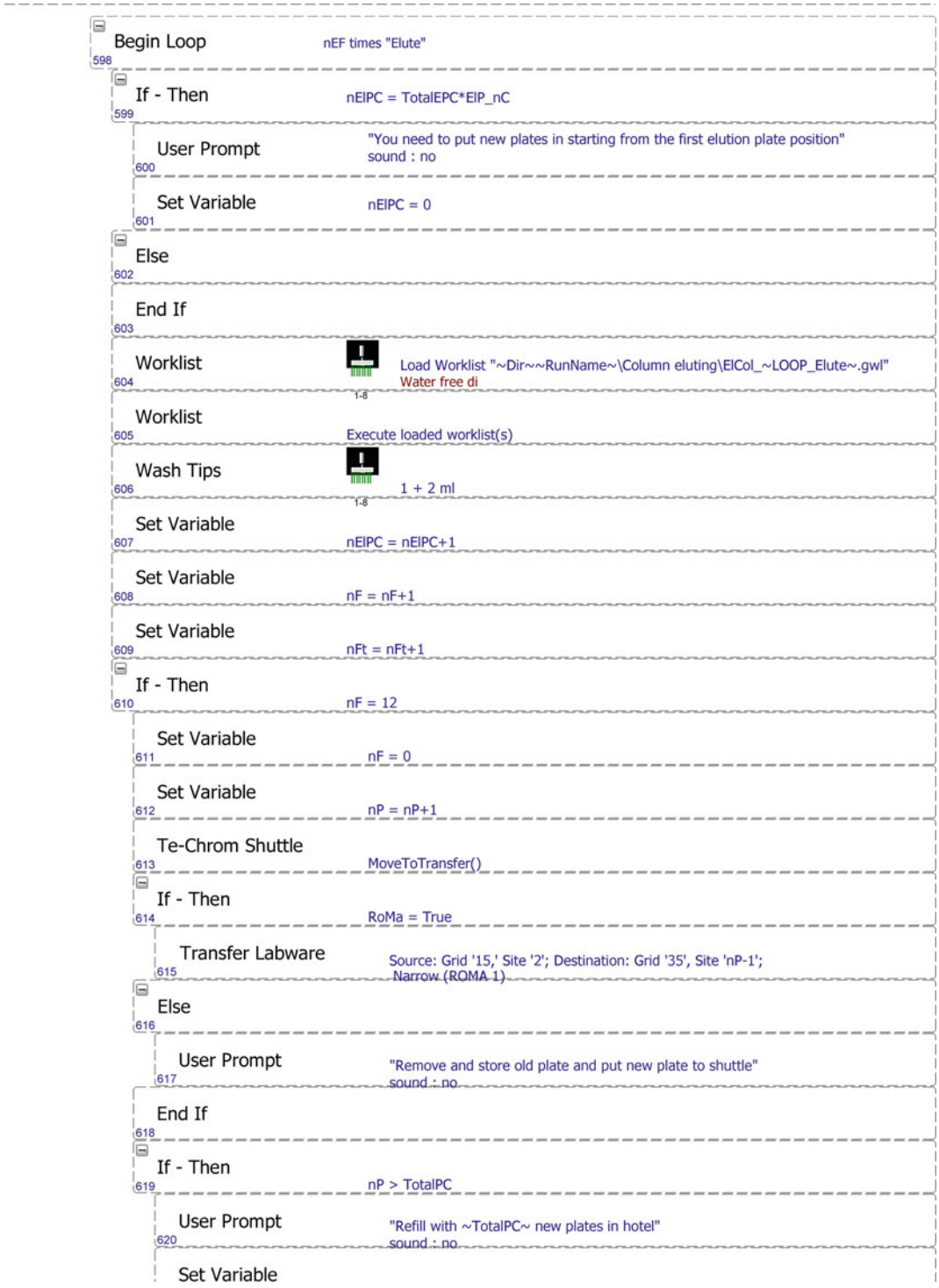

Wash Tips 1-8 1 + 2 ml
478
Else
479
End If
480
Group End Part 1.3.4: Engage waste and move Te-Shuttle to Transfer position
481
Group End Part 1: System preparation
482
Group Part 2: Chromatography
483
If - Then OF = False
484
Group Part 2.1: Remove storage solution
485
If - Then RSto = True
486
Begin Loop RStoL times "RmvStorage"
487
Worklist 1-8 Load Worklist "~Dir~~RunName~\Remove storage\RSto_~LOOP_RmvStorage~.gwl"
488
Worklist Execute loaded worklist(s)
489
End Loop "RmvStorage"
490
Wash Tips 1-8 1 + 2 ml
491
Else
492
End If
493
Group End Part 2.1: Remove storage solution
494
Group Part 2.2: Equilibrate columns
495
If - Then Eq = True
496
Begin Loop EqL times "Equilibration"
497
Worklist 1-8 Load Worklist "~Dir~~RunName~\Column equilibration\EqCol_~LOOP_Equilibration~.gwl"
498
Worklist Execute loaded worklist(s)
499
End Loop "Equilibration"
500
Wash Tips 1 + 2 ml


Else
502
End If
503
Group End
Part 2.2: Equilibrate columns
504
Group
Part 2.3: Load columns
505
Group
Part 2.3.1: Disengage waste and move Te-Shuttle to Start position
506
Move ROMA
Move to home position (ROMA 1)
507
Te-Chrom Shuttle
MoveToStart()
508
Te-Chrom Shuttle
SetSlowSpeed()
509
Group End
Part 2.3.1: Disengage waste and move Te-Shuttle to Start position
510
Group
Part 2.3.2: Dispense load into columns
511
If - Then
Load = True
512
Begin Loop
nLF times "Load"
513
Worklist
1-8
Load Worklist "~Dir~~RunName~\Column loading\LCol_~LOOP_Load~.gwl"
514
Worklist
Execute loaded worklist(s)
515
Wash Tips
1-8
1 + 2 ml
516
Set Variable
nF = nF+1
517
Set Variable
nFt = nFt+1
518
If - Then
nF = 12
519
Set Variable
nF = 0
520
Set Variable
nP = nP+1
521
Te-Chrom Shuttle
MoveToTransfer()
522
If - Then
RoMa = True
523
Transfer Labware
Source: Grid '15,' Site '2'; Destination: Grid '35', Site 'nP-1';
Narrow (ROMA 1)
524
Else

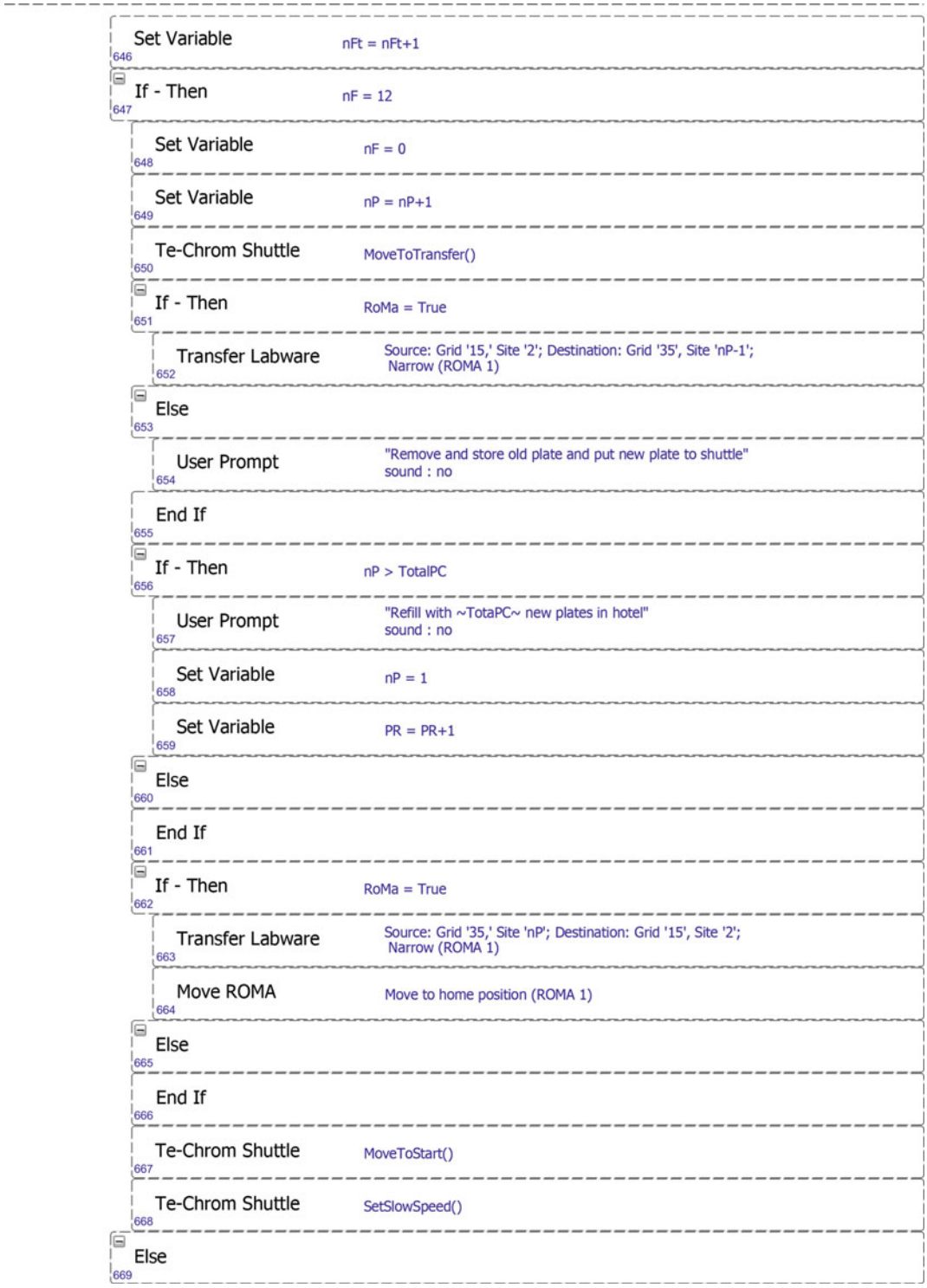

User Prompt "Remove and store old plate and put new plate to shuttle" sound : no
526
End If
527
If - Then nP > TotalPC
528
User Prompt "Refill with ~TotalPC~ new plates in hotel" sound : no
529
Set Variable nP = 1
530
Set Variable PR = PR+1
531
Else
532
End If
533
If - Then RoMa = True
534
Transfer Labware Source: Grid '35,' Site 'nP'; Destination: Grid '15', Site '2'; Narrow (ROMA 1)
535
Move ROMA Move to home position (ROMA 1)
536
Else
537
End If
538
Te-Chrom Shuttle MoveToStart()
539
Te-Chrom Shuttle SetSlowSpeed()
540
Else
541
End If
542
End Loop "Load"
543
Group Sanitization after loading
544
Wash Tips 1-8 SanWaste + SanCleaner ml
545
Wash Tips 1-8 1 + 1 ml
546
Begin Loop 2 times "Sanitization"
547
Aspirate 1-8 SanVol µl Tip Sanitization "Sanitiser" (Col. 1, Rows 1-8)
548
Dispense SanVol µl Tip Sanitization


End Loop "Sanitization"
550
Wash Tips SanWaste + SanCleaner ml
1-8
551
Wash Tips 1 + 1 ml
1-8
552
Group End Sanitization after loading
553
Else
554
End If
555
Group End Part 2.3.2: Dispense load into columns
556
Group End Part 2.3: Load columns
557
Group Part 2.4: wash columns
558
If - Then Wash = True
559
Begin Loop nWF times "Wash"
560
Worklist Load Worklist "~Dir~~RunName~\Column washing\WCol_~LOOP_Wash~.gwl" Water free
1-8
561
Worklist Execute loaded worklist(s)
562
Wash Tips 1 + 2 ml
1-8
563
Set Variable nF = nF+1
564
Set Variable nFt = nFt+1
565
If - Then nF = 12
566
Set Variable nF = 0
567
Set Variable nP = nP+1
568
Te-Chrom Shuttle MoveToTransfer()
569
If - Then RoMa = True
570
Transfer Labware Source: Grid '15,' Site '2'; Destination: Grid '35', Site 'nP-1'; Narrow (ROMA 1)
571
Else
572
User Prompt "Remove and store old plate and put new plate to shuttle"

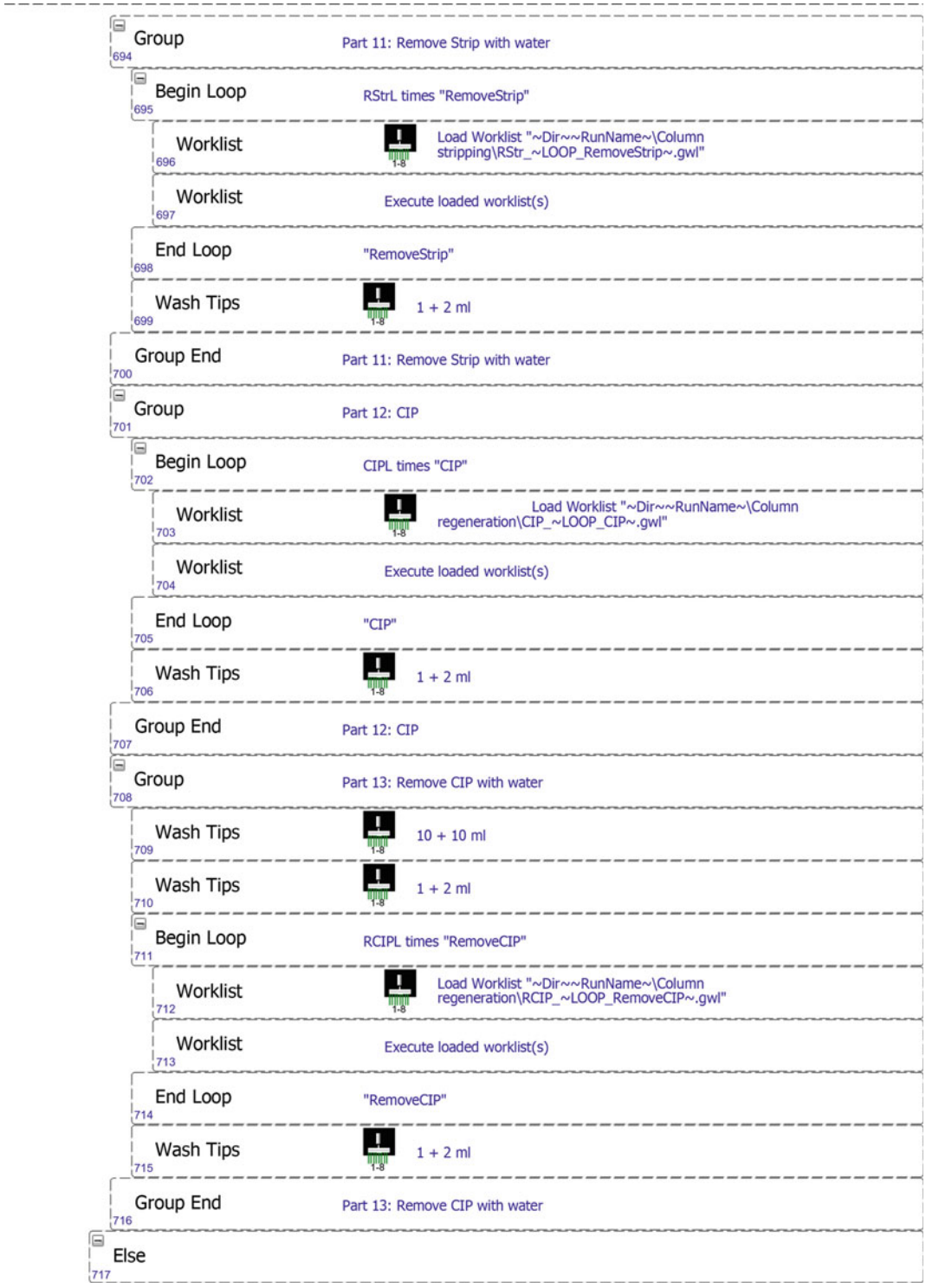

End If
574
If - Then
nP > TotalPC
575
User Prompt
"Refill with ~TotalPC~ new plates in hotel"
sound : no
576
Set Variable
nP = 1
577
Set Variable
PR = PR+1
578
Else
579
End If
580
If - Then
RoMa = True
581
Transfer Labware
Source: Grid '35,' Site 'nP'; Destination: Grid '15', Site '2';
Narrow (ROMA 1)
582
Move ROMA
Move to home position (ROMA 1)
583
Else
584
End If
585
Te-Chrom Shuttle
MoveToStart()
586
Te-Chrom Shuttle
SetSlowSpeed()
587
Else
588
End If
589
End Loop
"Wash"
590
Wash Tips
1-8
1 + 2 ml
591
Else
592
End If
593
Group End
Part 2.4: wash columns
594
Group
Part 2.5: Elute columns
595
If - Then
Elute = True
596
Set Variable
nElPC = 0

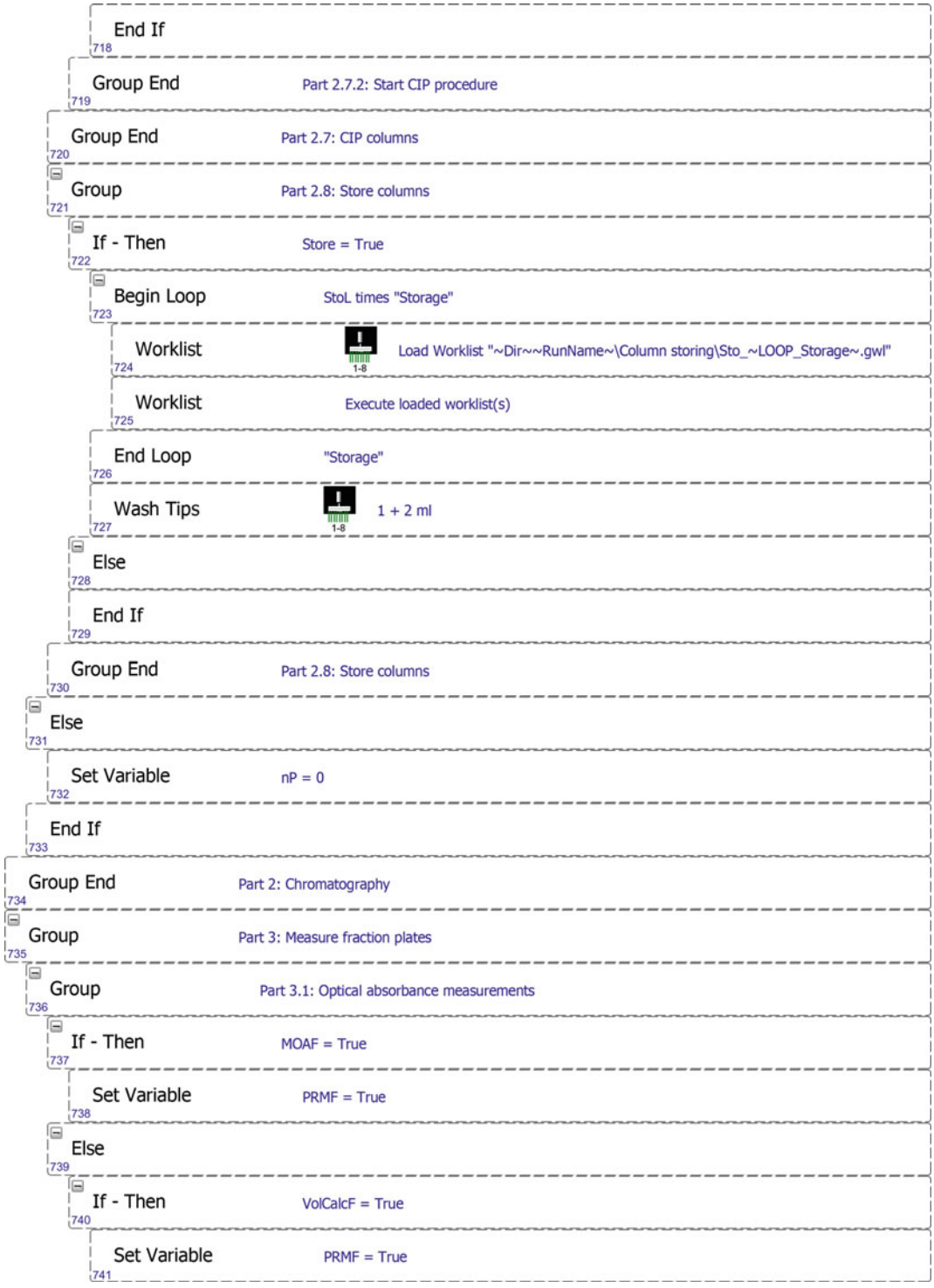

Begin Loop nEF times "Elute"
598
If - Then nElPC = TotalEPC*ElP_nC
599
User Prompt "You need to put new plates in starting from the first elution plate position"
sound : no
600
Set Variable nElPC = 0
601
Else
602
End If
603
Worklist Load Worklist "~Dir~~RunName~\Column eluting\ElCol_~LOOP_Elute~.gwl"
Water free di
604
1-8
Worklist Execute loaded worklist(s)
605
Wash Tips 1 + 2 ml
606
1-8
Set Variable nElPC = nElPC+1
607
Set Variable nF = nF+1
608
Set Variable nFt = nFt+1
609
If - Then nF = 12
610
Set Variable nF = 0
611
Set Variable nP = nP+1
612
Te-Chrom Shuttle MoveToTransfer()
613
If - Then RoMa = True
614
Transfer Labware Source: Grid '15,' Site '2'; Destination: Grid '35', Site 'nP-1';
Narrow (ROMA 1)
615
Else
616
User Prompt "Remove and store old plate and put new plate to shuttle"
sound : no
617
End If
618
If - Then nP > TotalPC
619
User Prompt "Refill with ~TotalPC~ new plates in hotel"
sound : no
620
Set Variable

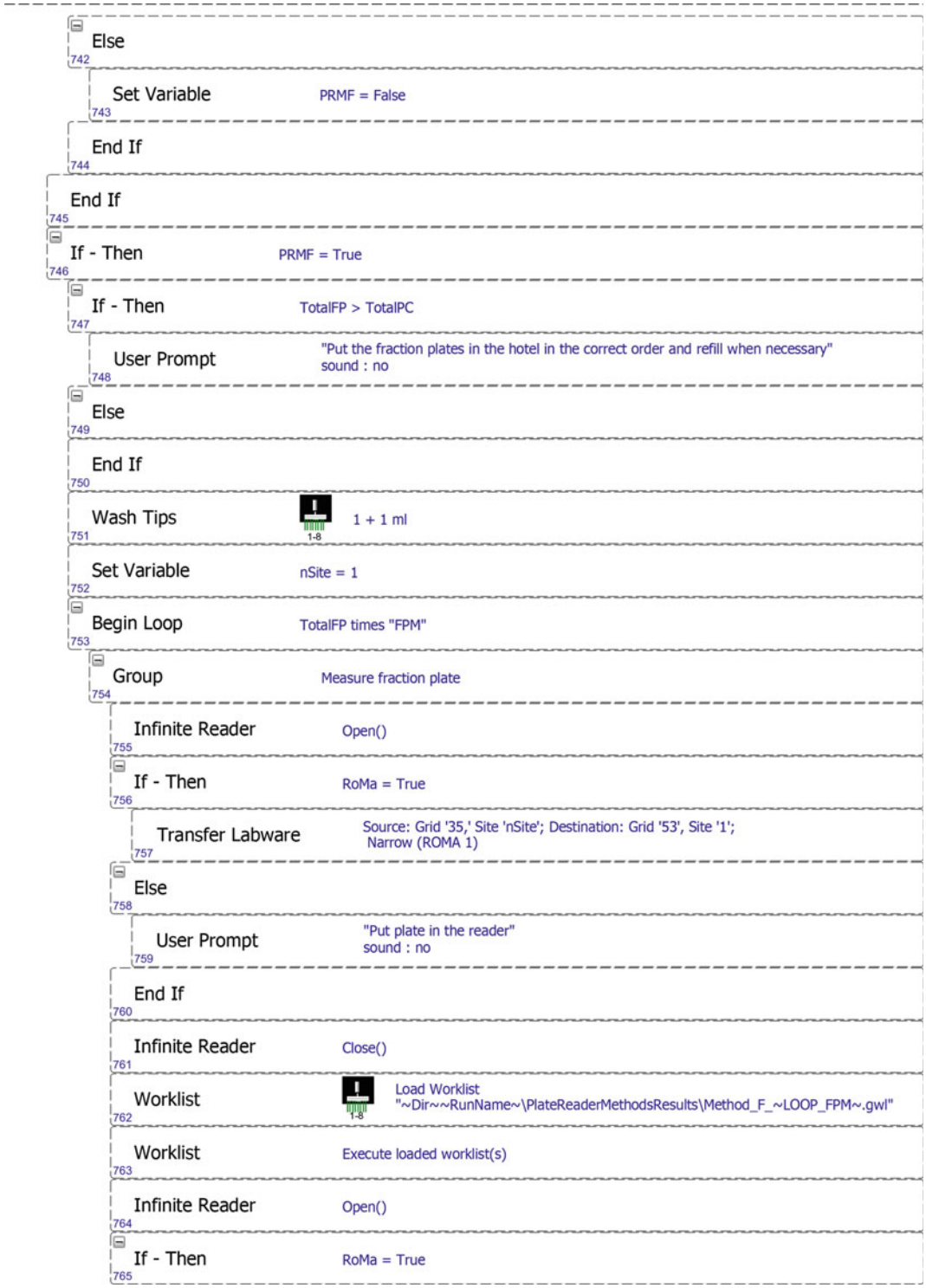

Set Variable
622
PR = PR+1
Else
623
End If
624
If - Then
625
RoMa = True
Transfer Labware
626
Source: Grid '35,' Site 'nP'; Destination: Grid '15', Site '2';
Narrow (ROMA 1)
Move ROMA
627
Move to home position (ROMA 1)
Else
628
End If
629
Te-Chrom Shuttle
630
MoveToStart()
Te-Chrom Shuttle
631
SetSlowSpeed()
Else
632
End If
633
End Loop
634
"Elute"
Wash Tips
635
1-8
1 + 2 ml
Else
636
End If
637
Group End
638
Part 2.5: Elute columns
Group
639
Part 2.6: Strip columns
If - Then
640
Strip = True
Begin Loop
641
nSF times "Strip"
Worklist
642
1-8
Load Worklist "~Dir~~RunName~\Column stripping\StrCol_~LOOP_Strip~.gwl"
Worklist
643
Execute loaded worklist(s)
Wash Tips
644
1-8
1 + 2 ml
Set Variable
nF = nF+1

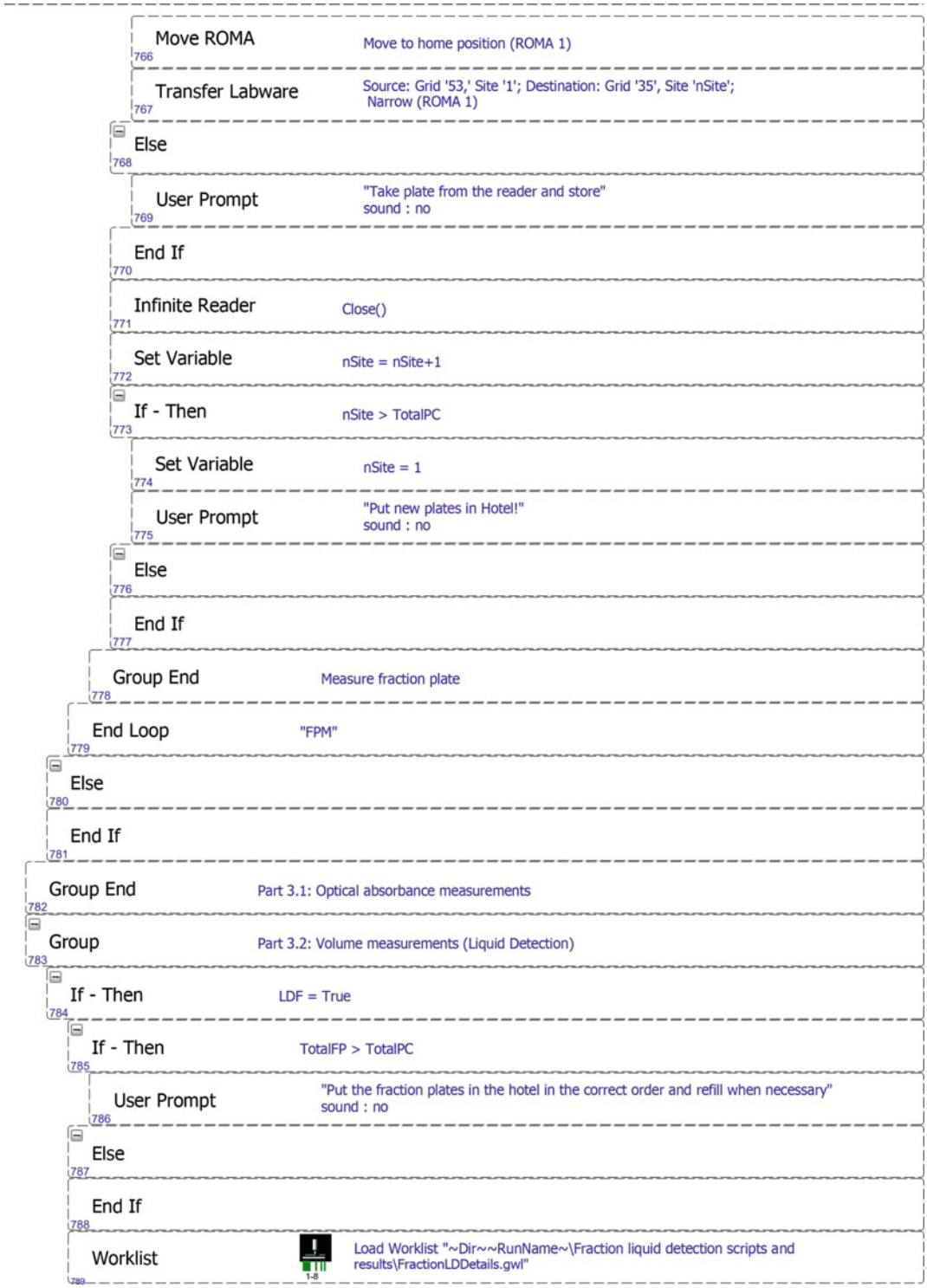

Set Variable nFt = nFt+1
646
If - Then nF = 12
647
Set Variable nF = 0
648
Set Variable nP = nP+1
649
Te-Chrom Shuttle MoveToTransfer()
650
If - Then RoMa = True
651
Transfer Labware Source: Grid '15,' Site '2'; Destination: Grid '35', Site 'nP-1';
Narrow (ROMA 1)
652
Else
653
User Prompt "Remove and store old plate and put new plate to shuttle"
sound : no
654
End If
655
If - Then nP > TotalPC
656
User Prompt "Refill with ~TotaPC~ new plates in hotel"
sound : no
657
Set Variable nP = 1
658
Set Variable PR = PR+1
659
Else
660
End If
661
If - Then RoMa = True
662
Transfer Labware Source: Grid '35,' Site 'nP'; Destination: Grid '15', Site '2';
Narrow (ROMA 1)
663
Move ROMA Move to home position (ROMA 1)
664
Else
665
End If
666
Te-Chrom Shuttle MoveToStart()
667
Te-Chrom Shuttle SetSlowSpeed()
668
Else
669


End If
670
End Loop "Strip"
671
Wash Tips 1-8 1 + 2 ml
672
Else
673
End If
674
Group End Part 2.6: Strip columns
675
Group Part 2.7: CIP columns
676
Group Part 2.7.1: Store last fraction plate and engage waste
677
Te-Chrom Shuttle MoveToTransfer()
678
If - Then RoMa = True
679
Transfer Labware Source: Grid '15,' Site '2'; Destination: Grid '35', Site 'nP';
Narrow (ROMA 1)
680
Move ROMA Move to home position (ROMA 1)
681
Else
682
User Prompt "Remove and store plate"
sound : no
683
End If
684
If - Then LM = 1
685
Set Variable nP = nP-1
686
Else
687
End If
688
Wash Tips 1-8 1 + 2 ml
689
Move ROMA Move to home position (ROMA 1)
690
Group End Part 2.7.1: Store last fraction plate and engage waste
691
Group Part 2.7.2: Start CIP procedure
692
If - Then CIP = True
693


Group
694
Part 11: Remove Strip with water
Begin Loop
695
RStrL times "RemoveStrip"
Worklist
696
Load Worklist "~Dir~~RunName~\Column stripping\RStr_~LOOP_RemoveStrip~.gwl"
Worklist
697
Execute loaded worklist(s)
End Loop
698
"RemoveStrip"
Wash Tips
699
1 + 2 ml
Group End
700
Part 11: Remove Strip with water
Group
701
Part 12: CIP
Begin Loop
702
CIPL times "CIP"
Worklist
703
Load Worklist "~Dir~~RunName~\Column regeneration\CIP_~LOOP_CIP~.gwl"
Worklist
704
Execute loaded worklist(s)
End Loop
705
"CIP"
Wash Tips
706
1 + 2 ml
Group End
707
Part 12: CIP
Group
708
Part 13: Remove CIP with water
Wash Tips
709
10 + 10 ml
Wash Tips
710
1 + 2 ml
Begin Loop
711
RCIPL times "RemoveCIP"
Worklist
712
Load Worklist "~Dir~~RunName~\Column regeneration\RCIP_~LOOP_RemoveCIP~.gwl"
Worklist
713
Execute loaded worklist(s)
End Loop
714
"RemoveCIP"
Wash Tips
715
1 + 2 ml
Group End
716
Part 13: Remove CIP with water
Else
717


End If
718
Group End
Part 2.7.2: Start CIP procedure
719
Group End
Part 2.7: CIP columns
720
Group
Part 2.8: Store columns
721
If - Then
Store = True
722
Begin Loop
StoL times "Storage"
723
Worklist
1-8
Load Worklist "~Dir~~RunName~\Column storing\Sto_~LOOP_Storage~.gwl"
724
Worklist
Execute loaded worklist(s)
725
End Loop
"Storage"
726
Wash Tips
1-8
1 + 2 ml
727
Else
728
End If
729
Group End
Part 2.8: Store columns
730
Else
731
Set Variable
nP = 0
732
End If
733
Group End
Part 2: Chromatography
734
Group
Part 3: Measure fraction plates
735
Group
Part 3.1: Optical absorbance measurements
736
If - Then
MOAF = True
737
Set Variable
PRMF = True
738
Else
739
If - Then
VolCalcF = True
740
Set Variable
PRMF = True
741


Else
742
Set Variable PRMF = False
743
End If
744
End If
745
If - Then PRMF = True
746
If - Then TotalFP > TotalPC
747
User Prompt "Put the fraction plates in the hotel in the correct order and refill when necessary" sound : no
748
Else
749
End If
750
Wash Tips 1-8 1 + 1 ml
751
Set Variable nSite = 1
752
Begin Loop TotalFP times "FPM"
753
Group Measure fraction plate
754
Infinite Reader Open()
755
If - Then RoMa = True
756
Transfer Labware Source: Grid '35,' Site 'nSite'; Destination: Grid '53', Site '1'; Narrow (ROMA 1)
757
Else
758
User Prompt "Put plate in the reader" sound : no
759
End If
760
Infinite Reader Close()
761
Worklist 1-8 Load Worklist "~Dir~~RunName~\PlateReaderMethodsResults\Method_F_~LOOP_FPM~.gwl"
762
Worklist Execute loaded worklist(s)
763
Infinite Reader Open()
764
If - Then RoMa = True
765

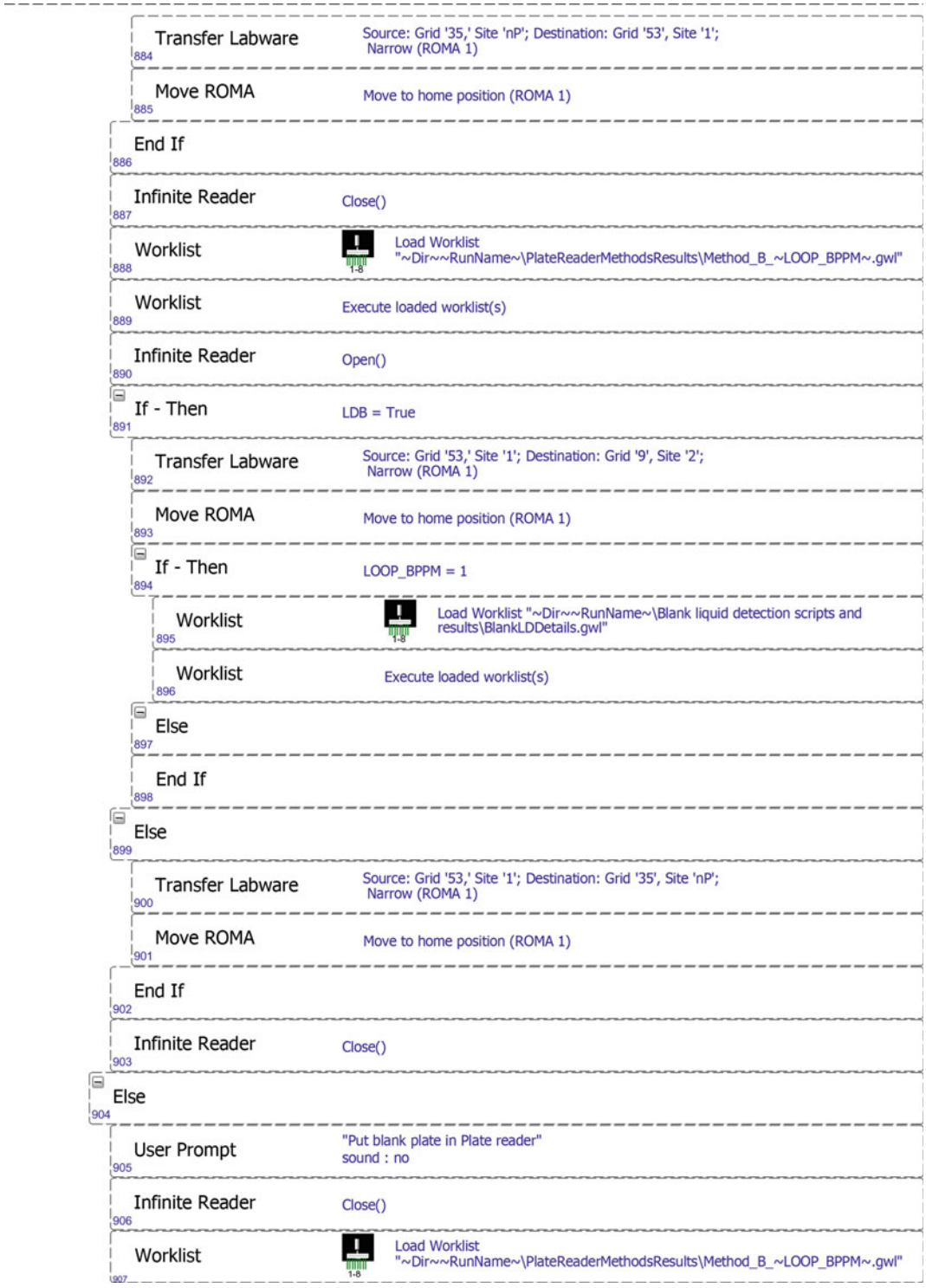

Move ROMA
766
Move to home position (ROMA 1)
Transfer Labware
767
Source: Grid '53,' Site '1'; Destination: Grid '35', Site 'nSite';
Narrow (ROMA 1)
Else
768
User Prompt
769
"Take plate from the reader and store"
sound : no
End If
770
Infinite Reader
771
Close()
Set Variable
772
nSite = nSite+1
If - Then
773
nSite > TotalPC
Set Variable
774
nSite = 1
User Prompt
775
"Put new plates in Hotel!"
sound : no
Else
776
End If
777
Group End
778
Measure fraction plate
End Loop
779
"FPM"
Else
780
End If
781
Group End
782
Part 3.1: Optical absorbance measurements
Group
783
Part 3.2: Volume measurements (Liquid Detection)
If - Then
784
LDF = True
If - Then
785
TotalFP > TotalPC
User Prompt
786
"Put the fraction plates in the hotel in the correct order and refill when necessary"
sound : no
Else
787
End If
788
Worklist
1-8
789
Load Worklist "~Dir~~RunName~\Fraction liquid detection scripts and
results\FractionLDDetails.gwl"

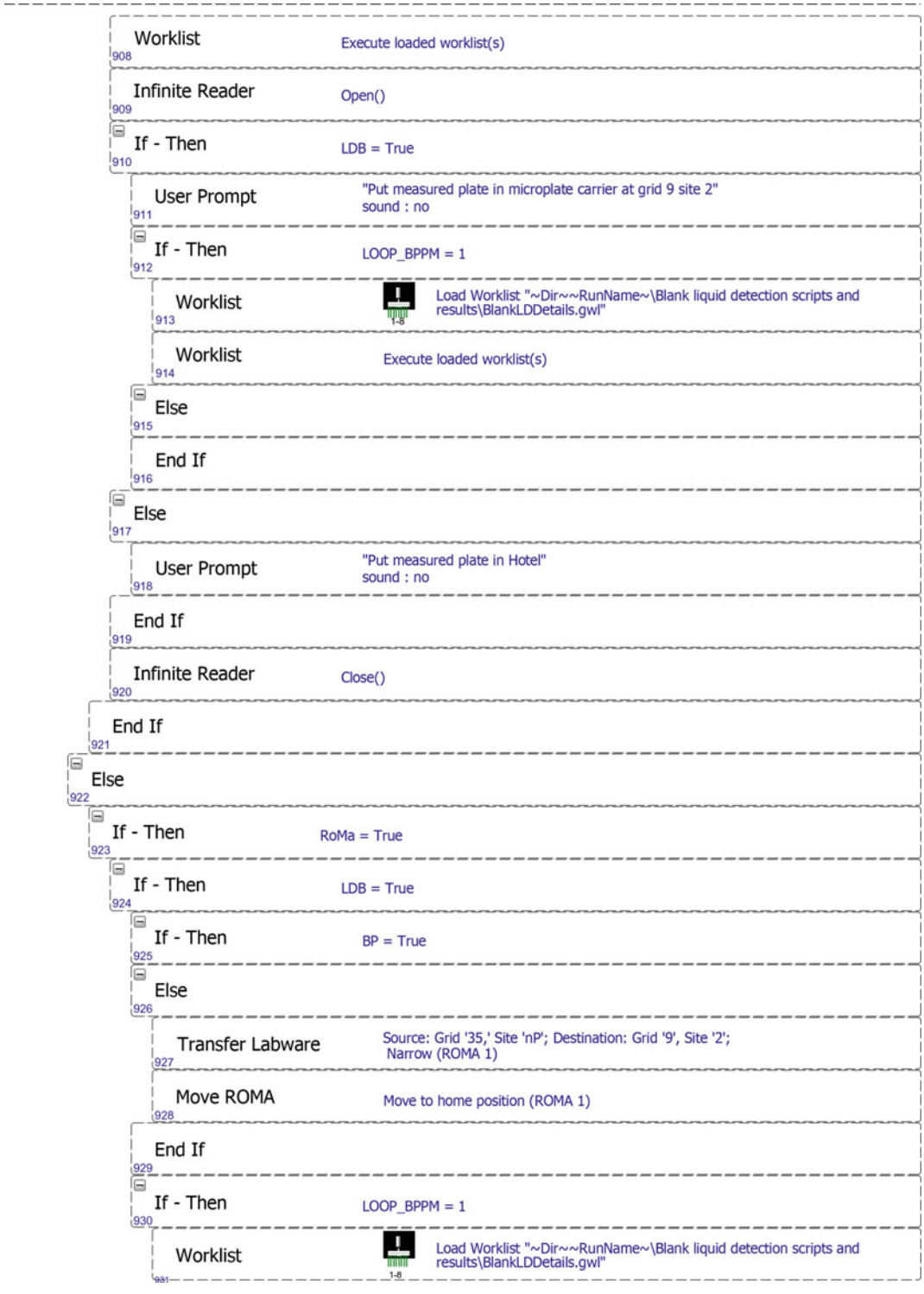

Worklist
Execute loaded worklist(s)
790
Set Variable
nSite = 1
791
Begin Loop
TotalFP times "FPM"
792
If - Then
RoMa = True
793
Transfer Labware
Source: Grid '35,' Site 'nSite'; Destination: Grid '9', Site '2';
Narrow (ROMA 1)
794
Move ROMA
Move to home position (ROMA 1)
795
Else
796
User Prompt
"Put a plate in grid 9 site 2"
sound : no
797
End If
798
Set Variable
nPlateLDLoops = FractionPlate~LOOP_FPM~NoLoops
799
Begin Loop
nPlateLDLoops times "LDloop"
800
Begin Loop
nDet times "LDrep"
801
Set Variable
nPlateLDLoopsnSubLoop = Plate~LOOP_FPM~Loop~LOOP_LDLoop~NoSubloops
802
Begin Loop
nPlateLDLoopsnSubLoop times "LDsubloop"
803
Set Variable
TipNumber =
Plate~LOOP_FPM~Loop~LOOP_LDLoop~Subloop~LOOP_LDsubloop~NoTips
804
Begin Loop
TipNumber times "LDset"
805
Set Variable
TipID = Plate~LOOP_F PM~Loop~LOOP_LDLoop ~Subloop~LOOP_LDsub
loop~Tip~LOOP_LDset ~TipID
806
Set Variable
detected_volume_~TipID~ = 0
807
End Loop
"LDset"
808
Worklist
1-8
Load Worklist "~Dir ~~RunName~\Fraction liquid detection s cripts and
results\ LqDetScriptFraction plate~LOOP_FPM~Loop
~LOOP_LDloop~Subloo p~LOOP_LDsubloop~.g wl"
809
Worklist
Execute loaded worklist(s)
810
Begin Loop
TipNumber times "LDexport"
811
Set Variable
TipID = Plate~LOOP_F PM~Loop~LOOP_LDLoop ~Subloop~LOOP_LDsub
loop~Tip~LOOP_LDexp ort~TipID
812

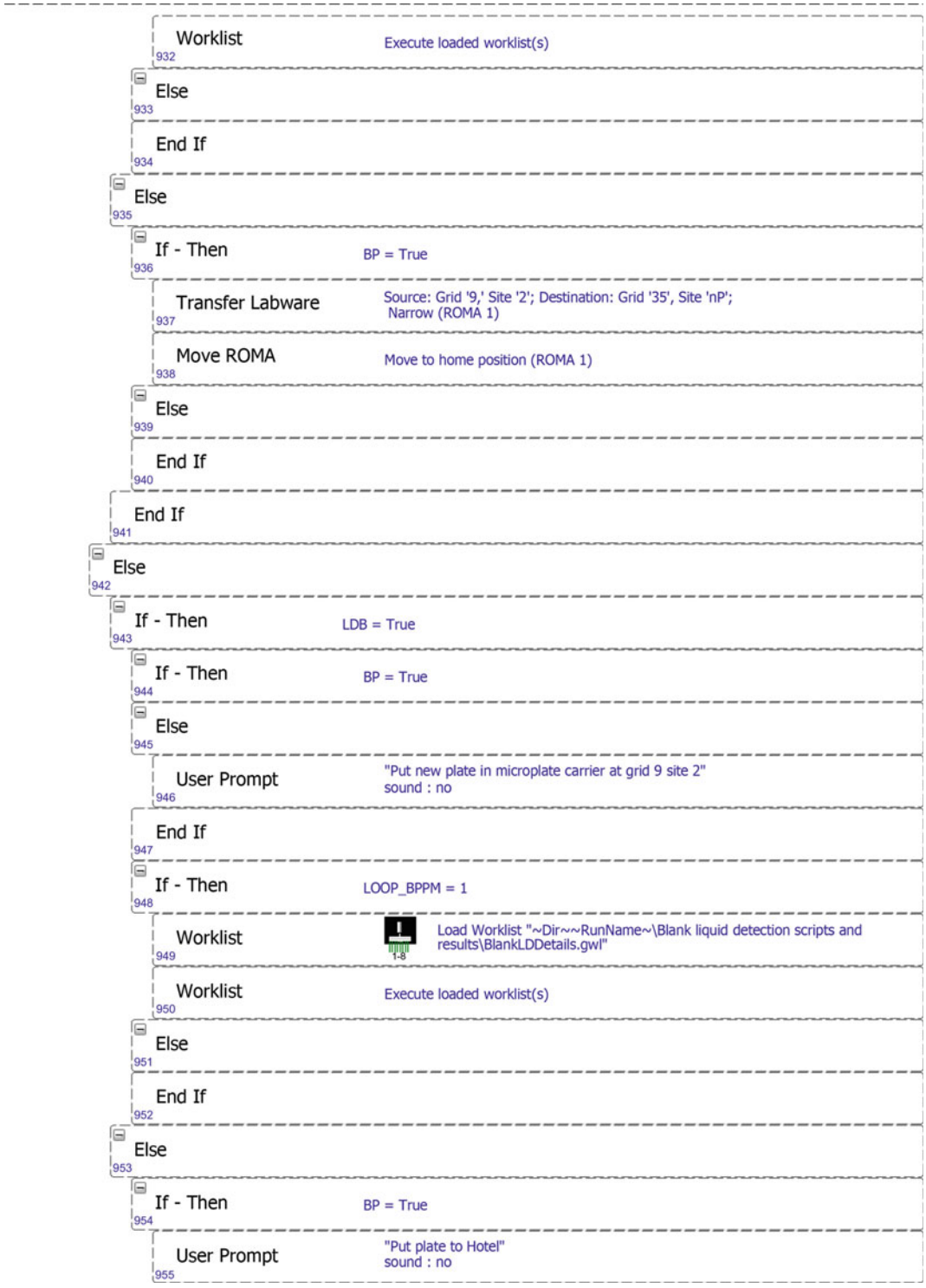

Set Variable
813
VDE = detected_volume_~TipID~
Export Variable
814
1 variable(s) ==> ~Dir~~RunName~\Fraction liquid detection scripts and results\LqDetResult_Fraction_plate_~LOOP_FPM~.txt
End Loop
815
"LDexport"
End Loop
816
"LDsubloop"
End Loop
817
"LDrep"
Group
818
Sanitization
Wash Tips
819
1-8
SanWasteLD + SanCleanerLD ml
Wash Tips
820
1-8
1 + 1 ml
Begin Loop
821
2 times "Sanitization"
Aspirate
822
1-8
SanVolLD µl Tip Sanitization "Sanitiser" (Col. 1, Rows 1-8)
Dispense
823
1-8
SanVolLD µl Tip Sanitization "Sanitiser" (Col. 1, Rows 1-8)
End Loop
824
"Sanitization"
Wash Tips
825
1-8
SanWasteLD + SanCleanerLD ml
Wash Tips
826
1-8
1 + 1 ml
Group End
827
Sanitization
End Loop
828
"LDloop"
If - Then
829
RoMa = True
Transfer Labware
830
Source: Grid '9,' Site '2'; Destination: Grid '35', Site 'nSite'; Narrow (ROMA 1)
Move ROMA
831
Move to home position (ROMA 1)
Else
832
User Prompt
833
"Take plate out of grid 9 site 2"
sound : no
End If
834
Set Variable
835
nSite = nSite+1
If - Then
836
nSite > TotalPC

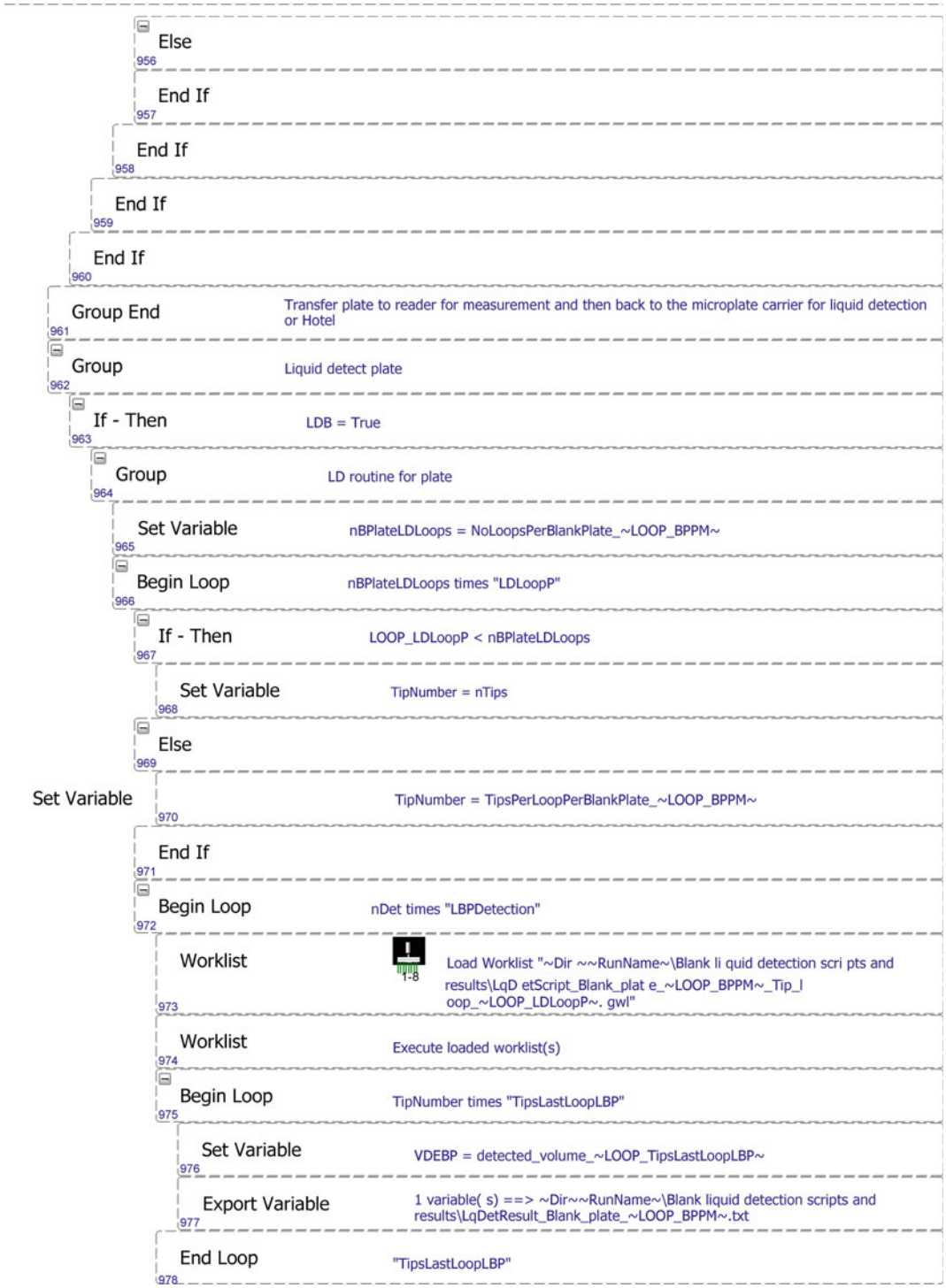

User Prompt
"Put new fraction plates in hoptel"
sound : no
837
Set Variable
nSite = 1
838
Else
839
End If
840
End Loop
"FPM"
841
Else
842
End If
843
Group End
Part 3.2: Volume measurements (Liquid Detection)
844
Group End
Part 3: Measure fraction plates
845
Group
Part 4: Preparation,optical absorbance measurement, volume determination of blanks
846
Wash Tips
1 + 2 ml
847
If - Then
nEF > TotalEPC*EIP_nC
848
User Prompt
"Put the elution buffer plates in the deck using their correct order (i.e. start from EIPlate1 to EIPlate N). "
sound : no
849
Else
850
End If
851
Set Variable
nP = nP+1
852
Begin Loop
TotalBP times "BPPM"
853
Group
Prepare blank plate
854
If - Then
BP = True
855
If - Then
RoMa = True
856
Transfer Labware
Source: Grid '35,' Site 'nP'; Destination: Grid '9', Site '2';
Narrow (ROMA 1)
857
Move ROMA
Move to home position (ROMA 1)
858
Else
859

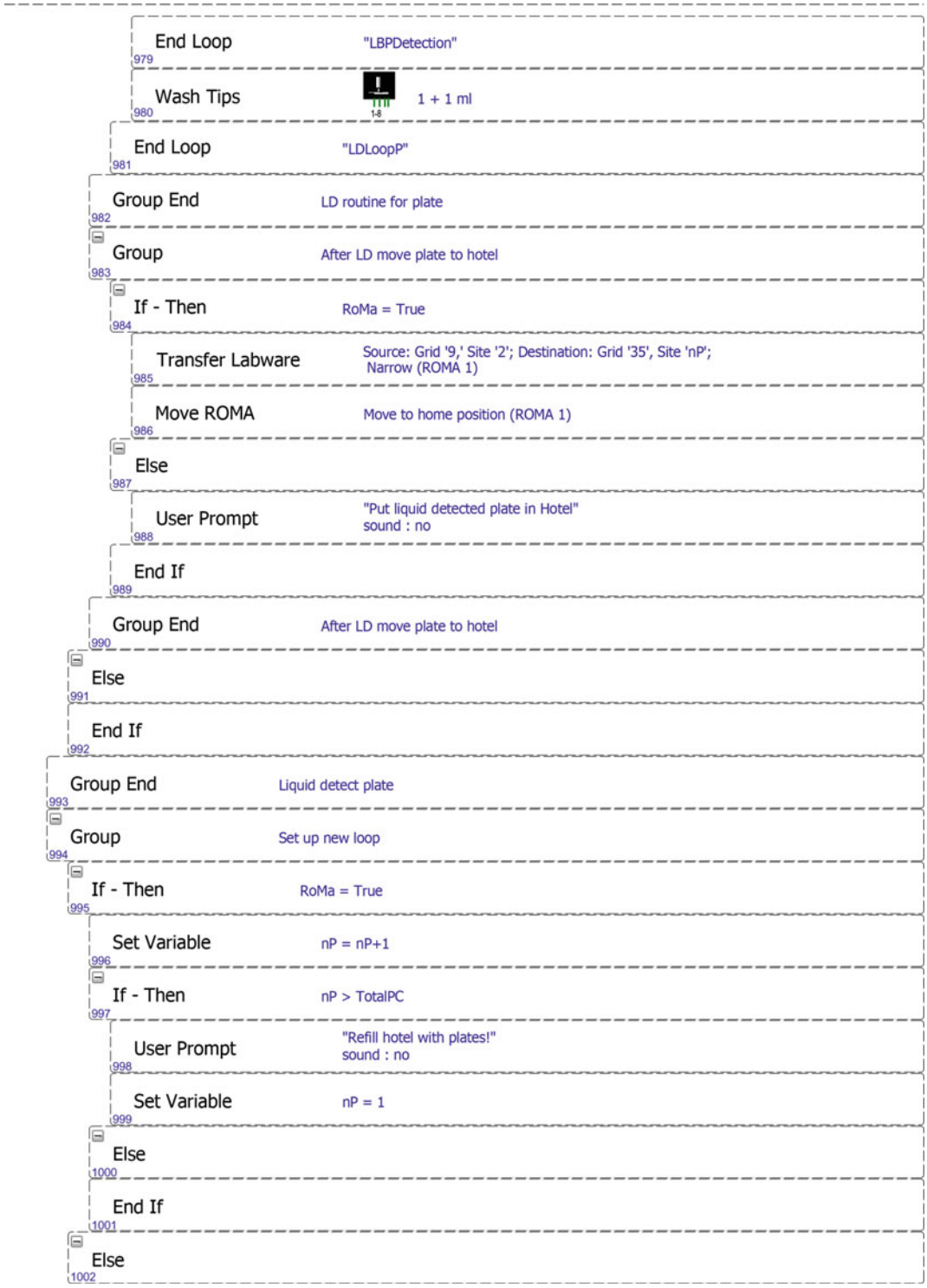

User Prompt
860
"Put new plate in microplate carrier at grid 9 site 2"
sound : no
End If
861
Worklist
862
1-8
Load Worklist "~Dir~~RunName~\Blank preparation\BlankPrepScriptPlate_~LOOP_BPPM~.gwl"
Worklist
863
Execute loaded worklist(s)
Else
864
End If
865
Group End
866
Prepare blank plate
Group
867
Transfer plate to reader for measurement and then back to the microplate carrier for liquid detection or Hotel
If - Then
868
MOAB = True
Set Variable
869
PRMB = True
Else
870
If - Then
871
VolCalcB = True
Set Variable
872
PRMB = True
Else
873
Set Variable
874
PRMB = False
End If
875
End If
876
If - Then
877
PRMB = True
Infinite Reader
878
Open()
If - Then
879
RoMa = True
If - Then
880
BP = True
Transfer Labware
881
Source: Grid '9,' Site '2'; Destination: Grid '53', Site '1';
Narrow (ROMA 1)
Move ROMA
882
Move to home position (ROMA 1)
Else
883

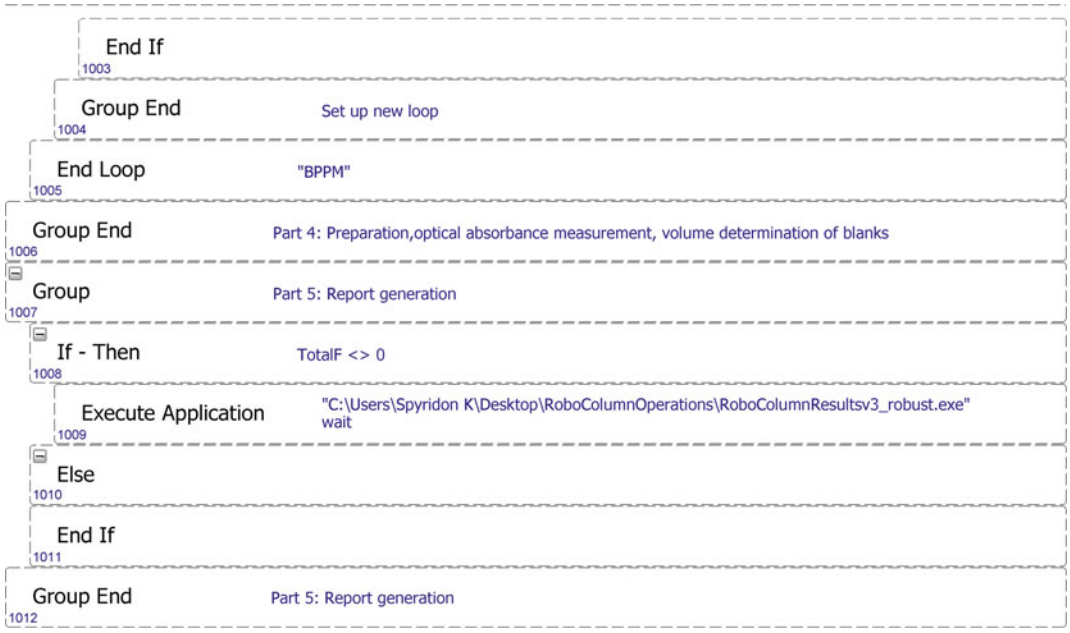

Transfer Labware
884
Source: Grid '35,' Site 'nP'; Destination: Grid '53', Site '1';
Narrow (ROMA 1)
Move ROMA
885
Move to home position (ROMA 1)
End If
886
Infinite Reader
887
Close()
Worklist
888
Load Worklist
"~Dir~~RunName~\PlateReaderMethodsResults\Method_B_~LOOP_BPPM~.gwl"
Worklist
889
Execute loaded worklist(s)
Infinite Reader
890
Open()
If - Then
891
LDB = True
Transfer Labware
892
Source: Grid '53,' Site '1'; Destination: Grid '9', Site '2';
Narrow (ROMA 1)
Move ROMA
893
Move to home position (ROMA 1)
If - Then
894
LOOP_BPPM = 1
Worklist
895
Load Worklist "~Dir~~RunName~\Blank liquid detection scripts and
results\BlankLDDetails.gwl"
Worklist
896
Execute loaded worklist(s)
Else
897
End If
898
Else
899
Transfer Labware
900
Source: Grid '53,' Site '1'; Destination: Grid '35', Site 'nP';
Narrow (ROMA 1)
Move ROMA
901
Move to home position (ROMA 1)
End If
902
Infinite Reader
903
Close()
Else
904
User Prompt
905
"Put blank plate in Plate reader"
sound : no
Infinite Reader
906
Close()
Worklist
907
Load Worklist
"~Dir~~RunName~\PlateReaderMethodsResults\Method_B_~LOOP_BPPM~.gwl"

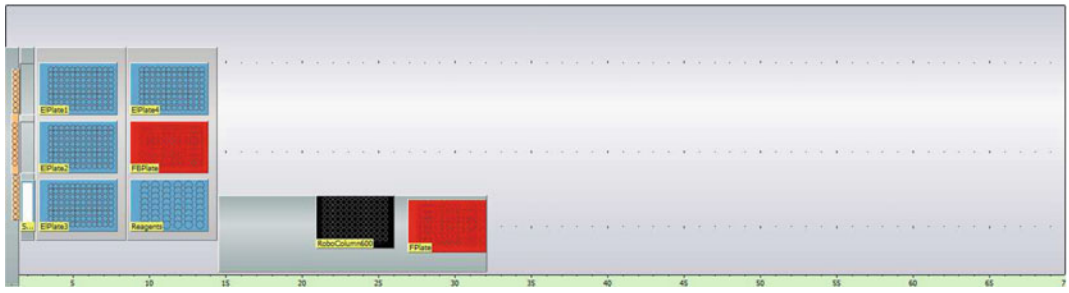

Worklist
908
Execute loaded worklist(s)
Infinite Reader
909
Open()
If - Then
910
LDB = True
User Prompt
911
"Put measured plate in microplate carrier at grid 9 site 2"
sound : no
If - Then
912
LOOP_BPPM = 1
Worklist
913
1-8
Load Worklist "~Dir~~RunName~\Blank liquid detection scripts and results\BlankLDDetails.gwl"
Worklist
914
Execute loaded worklist(s)
Else
915
End If
916
Else
917
User Prompt
918
"Put measured plate in Hotel"
sound : no
End If
919
Infinite Reader
920
Close()
End If
921
Else
922
If - Then
923
RoMa = True
If - Then
924
LDB = True
If - Then
925
BP = True
Else
926
Transfer Labware
927
Source: Grid '35,' Site 'nP'; Destination: Grid '9', Site '2';
Narrow (ROMA 1)
Move ROMA
928
Move to home position (ROMA 1)
End If
929
If - Then
930
LOOP_BPPM = 1
Worklist
931
1-8
Load Worklist "~Dir~~RunName~\Blank liquid detection scripts and results\BlankLDDetails.gwl"


Worklist
932
Execute loaded worklist(s)
Else
933
End If
934
Else
935
If - Then
936
BP = True
Transfer Labware
937
Source: Grid '9,' Site '2'; Destination: Grid '35', Site 'nP';
Narrow (ROMA 1)
Move ROMA
938
Move to home position (ROMA 1)
Else
939
End If
940
End If
941
Else
942
If - Then
943
LDB = True
If - Then
944
BP = True
Else
945
User Prompt
946
"Put new plate in microplate carrier at grid 9 site 2"
sound : no
End If
947
If - Then
948
LOOP_BPPM = 1
Worklist
949
1-8
Load Worklist "~Dir~~RunName~\Blank liquid detection scripts and
results\BlankLDDetails.gwl"
Worklist
950
Execute loaded worklist(s)
Else
951
End If
952
Else
953
If - Then
954
BP = True
User Prompt
955
"Put plate to Hotel"
sound : no


Else
956
End If
957
End If
958
End If
959
End If
960
Group End
Transfer plate to reader for measurement and then back to the microplate carrier for liquid detection or Hotel
961
Group
Liquid detect plate
962
If - Then
LDB = True
963
Group
LD routine for plate
964
Set Variable
nBPlateLDLoops = NoLoopsPerBlankPlate_~LOOP_BPPM~
965
Begin Loop
nBPlateLDLoops times "LDLoopP"
966
If - Then
LOOP_LDLoopP < nBPlateLDLoops
967
Set Variable
TipNumber = nTips
968
Else
969
Set Variable
TipNumber = TipsPerLoopPerBlankPlate_~LOOP_BPPM~
970
End If
971
Begin Loop
nDet times "LBPDetection"
972
Worklist
1-8
Load Worklist "~Dir ~~RunName~\Blank li quid detection scri pts and results\LqD etScript_Blank_plat e_~LOOP_BPPM~_Tip_l oop_~LOOP_LDLoopP~. gwl"
973
Worklist
Execute loaded worklist(s)
974
Begin Loop
TipNumber times "TipsLastLoopLBP"
975
Set Variable
VDEBP = detected_volume_~LOOP_TipsLastLoopLBP~
976
Export Variable
1 variable(s) ==> ~Dir~~RunName~\Blank liquid detection scripts and results\LqDetResult_Blank_plate_~LOOP_BPPM~.txt
977
End Loop
"TipsLastLoopLBP"
978


End Loop "LBPDetection"
979
Wash Tips 1-8 1 + 1 ml
980
End Loop "LDLoopP"
981
Group End LD routine for plate
982
Group After LD move plate to hotel
983
If - Then RoMa = True
984
Transfer Labware Source: Grid '9,' Site '2'; Destination: Grid '35', Site 'nP'; Narrow (ROMA 1)
985
Move ROMA Move to home position (ROMA 1)
986
Else
987
User Prompt "Put liquid detected plate in Hotel" sound : no
988
End If
989
Group End After LD move plate to hotel
990
Else
991
End If
992
Group End Liquid detect plate
993
Group Set up new loop
994
If - Then RoMa = True
995
Set Variable nP = nP+1
996
If - Then nP > TotalPC
997
User Prompt "Refill hotel with plates!" sound : no
998
Set Variable nP = 1
999
Else
1000
End If
1001
Else
1002





References

1. Bhambure R, Rathore A (2013) Chromatography process development in the quality by design paradigm I: establishing a high-throughput process development platform as a tool for estimating "characterization space" for an ion exchange chromatography step. Biotechnol Prog 29:403–414
2. Daniels C, Rodriguez J, Lim E et al (2016) An integrated robotic system for high-throughput process development of cell and virus culture conditions: application to biosafety level 2 live virus vaccines. Eng Life Sci 16:202–209
3. Wenger MD, Dephillips P, Price CE et al (2007) An automated microscale chromatographic purification of virus-like particles as a strategy for process development. Biotechnol Appl Biochem 47:131–139
4. Konstantinidis S, Welsh JP, Roush DJ et al (2016) Application of simplex-based experimental optimization to challenging bioprocess development problems: case studies in downstream processing. Biotechnol Prog 32:404–419
5. Konstantinidis S, Welsh JP, Titchener-Hooker NJ et al (2018) Data-driven multi-objective optimization via grid compatible simplex technique and desirability approach for challenging high throughput chromatography applications. Biotechnol Prog 34:1393–1406

6. Treier K, Hansen S, Richter C et al (2012) High-throughput methods for miniaturization and automation of monoclonal antibody purification processes. Biotechnol Prog 28:723–732
7. Welsh JP, Petroff MG, Rowicki P et al (2014) A practical strategy for using miniature chromatography columns in a standardized high-throughput workflow for purification development of monoclonal antibodies. Biotechnol Prog 30:626–635
8. Feliciano J, Berrill A, Ahnfelt M et al (2016) Evaluating high-throughput scale-down chromatography platforms for increased process understanding. Eng Life Sci 16:169–178
9. Łącki KM (2014) High throughput process development in biomanufacturing. Curr Opin Chem Eng 6:25–32
10. Konstantinidis S, Goh HY, Martin Bufájer JM et al (2018) Flexible and accessible automated operation of miniature chromatography columns on a liquid handling station. Biotechnol J 13:1700390
11. Li X, De Roo G, Burgers K et al (2012) Self-packed filter plates: a good alternative for pre-packed filter plates for developing purification processes for therapeutic proteins. Biotechnol J 7:1269–1276
12. Jacob SI, Khogeer B, Bampos N et al (2017) Development and application of synthetic affinity ligands for the purification of ferritin-based influenza antigens. Bioconjug Chem 28:1931–1943
13. El Khoury G, Wang Y, Wang D et al (2013) Design, synthesis, and assessment of a de novo affinity adsorbent for the purification of recombinant human erythropoietin. Biotechnol Bioeng 110:3063–3069
14. Khoury GE, Khogeer B, Chen C et al (2015) Bespoke affinity ligands for the purification of therapeutic proteins. Pharmaceut Bioprocess 3:139–152
15. Mcgown EL, Hafeman DG (1998) Multichannel pipettor performance verified by measuring pathlength of reagent dispensed into a microplate. Anal Biochem 258:155–157

Chapter 10

Production of Zika Virus Virus-Like Particles

Atichat Kuadkitkan, Suwipa Ramphan, Suchin Worawichawong, Wannapa Sornjai, Nitwara Wikan, and Duncan R. Smith

Abstract

Zika virus (ZIKV) is a mosquito-transmitted virus that has caused major outbreaks of disease around the world over the last few years. The infectious ZIKV consists of a structural protein outer shell surrounding a nucleocapsid. Virus-like particles (VLP) consist of the outer structural protein shell, but without the nucleocapsid, and are hence noninfectious. VLP, however, are structurally equivalent to the native virus and thus present a similar antigenic profile. These properties make them good candidates for vaccine development. ZIKV VLP can be generated on a laboratory scale by cloning the relevant structural proteins into a eukaryotic expression vector and transfecting the construct into mammalian cells. The secreted VLP can be harvested from the culture medium and purified by sucrose cushion ultracentrifugation. Validation of the VLP is achieved through western blotting and electron microscopy.

Key words Flavivirus, Structural proteins, Eukaryotic expression vector, Transfection, Centrifugation, Zika, Virus-like particles, Vaccine

1 Introduction

Mosquito transmitted viruses impose a significant public health burden in many tropical and subtropical countries [1]. While human pathogenic mosquito transmitted viruses belong to several families, the largest health burden worldwide results from viruses belonging to the family *Flaviviridae*, genus *Flavivirus*. A total of 53 viral species are recognized in this genus [2], and the genus includes viruses such as dengue virus, yellow fever virus, Japanese encephalitis virus, and West Nile virus. Over the last few years, one member of the genus *Flavivirus*, Zika virus (ZIKV), has garnered considerable public attention as a result of the rapid emergence of this virus and its transmission around much of the world. ZIKV was first isolated in 1947 [3], although following the original identification of ZIKV there were only a handful of reported cases of human infection from Africa and Asia over the next 60 years (as reviewed elsewhere [4]). A large outbreak of ZIKV infections in

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French Polynesia in 2013 [5] was followed by the rapid spread of the virus to South, Central and North America where transmission was associated with millions of cases of infection [6]. The majority of cases of ZIKV infection are asymptomatic, but infection can be associated with a range of manifestations including rash, fever, headache, and muscle and joint pain [7]. While ZIKV infection is normally self-limiting and is often resolved without medical intervention, in some cases more serious consequences can arise, including Guillain–Barre syndrome in adults and major birth defects in the fetuses of woman infected while pregnant [8]. The significant effects of ZIKV on fetuses of women who become infected while pregnant is perhaps the single most important public health aspect of ZIKV infection and will shape future vaccine development for this virus.

Vaccine development to protect against infection with members of the genus *Flavivirus* has been uneven. While there are excellent vaccines against yellow fever virus [9] and Japanese encephalitis virus [10], vaccine development against dengue virus has been much slower and recently the first commercial dengue vaccine (Dengvaxia) has been associated with adverse effects in certain vaccinees [11]. A number of approaches toward vaccine development are available. The most successful approaches to date have been the development of live attenuated vaccines, as used for yellow fever virus and Japanese encephalitis virus vaccines and whole inactivated viruses as used previously in some Japanese encephalitis vaccines [10]. However, both of these approaches have some drawbacks, including potential reversion to wild type for live attenuated viruses and underinactivation for whole inactivated virus vaccines. In particular, neither type of vaccine approach is desired for use with ZIKV given the association with neonatal birth defects. Virus-like particles (VLP) are an attractive approach to vaccine development for *Flaviviruses* [12]. VLP consist of the structural proteins of the virus in a native form but without the associated genetic element. The lack of a genetic element makes VLP a safer choice than either live attenuated or inactivated vaccines. This protocol can be used to prepare laboratory scale preparations of purified ZIKV VLP for vaccine development through expression of the VLP in a mammalian cell culture system and subsequent purification through buoyant density centrifugation.

2 Materials

2.1 Zika VLP Plasmid Construction and Preparation

1. The sequence of *capsid* (*C*), *premembrane/membrane* (*prM/M*), and *envelope* (*E*) genes of a Cambodian isolate of Zika virus (isolate FSS13025) Genbank number AFD30972.1 was taken from the NCBI database and commercially synthesized (*see* **Note 1**).

2. The forward and reverse primers are *NheI*-19CprME-Zika-Fw (5′-GAGCTAGCCACCATGGGAAGAGGGACCGATACAA GC-3′) and 19CprME-Zika-*EcoRI*-Rw (5′-CGGAATTCTTA TGCGGACACTGCGGTGGACAGAAAA-3′), respectively. The plasmid construction has been briefly described in a previous study [13].
3. Phusion High-Fidelity DNA polymerase DreamTaq DNA polymerase.
4. 10 mM dNTPs.
5. T4 DNA ligase enzyme.
6. 50× TAE buffer: 2 M Tris–HCl, 1 M acetic acid, 0.1 M EDTA. Weigh 242 g of Tris base and 37.2 g of ethylenediaminetetraacetic acid (EDTA) disodium salt, dihydrate ($EDTA \cdot Na_2 \cdot 2H_2O$, MW 372.24 g/mol) into a glass beaker and dissolve in 500 mL of deionized water using a magnetic stirrer. After all the chemicals have completely dissolved, add 57.1 mL of glacial acetic acid (*see* **Note 2**) to the solution and adjust the volume to 1 L with deionized water. The stock buffer can be kept at room temperature.
7. 1× TAE buffer: add 20 mL 50× TAE buffer to a 1 L measuring cylinder and add deionized water to 1 L.
8. 1% agarose gel in 1× TAE buffer. Weigh 1 g of agarose (molecular biology grade) and add 100 mL of 1× TAE buffer into a glass bottle then heat the agar in a microwave oven (*see* **Note 3**) until it is completely melted. Let the melted agarose cool down to 42 °C followed by pouring into a gel setting chamber and leave at room temperature for 30 min before use.
9. 0.5 μg/mL ethidium bromide (EthBr) DNA staining solution. Take 15 μL of 10 mg/mL stock ethidium bromide solution in water and mix with 300 mL of deionized water in a plastic chamber (*see* **Note 4**).
10. 100 mg/mL (w/v) ampicillin stock. Weigh 1 g of ampicillin powder into a glass beaker and dissolve in 8 mL of deionized water using a magnetic stirrer. After the ampicillin has completely dissolved, add deionized water to a final volume of 10 mL. Then filter the ampicillin stock solution through a 0.22 μm PES membrane filter and aliquot into 1.5 mL tubes for storage at −20 °C.
11. LB broth. Weigh 4 g of tryptone powder, 4 g of sodium chloride and 2 g of yeast extract into a glass bottle and add 400 mL of deionized water and mix thoroughly before autoclaving. Add 200 μL of stock ampicillin to a final concentration of 50 μg/mL before use (*see* **Note 5**) and keep the LB-ampicillin broth at 4 °C.

12. LB agar. Weigh 4 g of tryptone powder, 4 g of sodium chloride, 2 g of yeast extract, and 8 g of bacteriological agar into a glass bottle, add 400 mL of deionized water, then mix thoroughly before autoclaving. After sterilization, let the melted agar solution cool down to 42 °C and then add 400 μL of stock ampicillin to a final concentration of 100 μg/mL (*see* **Note 6**). Then pour 10 mL of the melted agar into a Sterilin single-use plastic plate; let the agar set and keep at 4 °C.
13. 0.1 M $CaCl_2$. Weigh 5.88 g of $CaCl_2$ powder into a glass bottle and add 400 mL of deionized water; then mix thoroughly before autoclaving and keep at 4 °C.
14. 1 M $CaCl_2$. Weigh 58.81 g of $CaCl_2$ powder into a glass bottle and add 400 mL of deionized water; then mix thoroughly before autoclaving and keep at 4 °C.
15. 15% glycerol with 0.1 M $CaCl_2$. Measure 60 mL of 100% glycerol into a 500 mL measuring cylinder, add 40 mL of 1 M $CaCl_2$ and 300 mL of deionized water, then mix thoroughly before transferring into a glass bottle glass followed by autoclaving and storage at 4 °C.
16. 0.22 μm polyethersulfone (PES) membrane filter.
17. Sterilin Single-use plastic plates.
18. Gel electrophoresis chamber.
19. Microwave oven.
20. FavorPrep™ GEL/PCR Purification Mini Kit.
21. FavorPrep™ Plasmid Extraction Mini Kit.
22. NanoDrop™ 2000 spectrophotometer.
23. 1.7 mL microcentrifuge tubes.
24. 0.2 mL nuclease-free microcentrifuge tubes.
25. Microcentrifuge capable of centrifuging 0.2 mL and 1.7 mL microcentrifuge tubes.
26. Methylene blue.
27. *NheI* FastDigest restriction enzyme with provided buffer.
28. *EcoRI* FastDigest restriction enzyme with provided buffer.
29. *Escherichia coli* (*E. coli*) strain DH5α.
30. Autoclaved (sterile) toothpicks.
31. 250 mL bacterial culture flask.
32. Corning 50 mL centrifuge tubes.
33. Shaking incubator.

2.2 Zika VLP Expression

1. 2.5 M calcium chloride. Weigh 36.755 g of calcium chloride ($CaCl_2 \cdot 2H_2O$) into a glass beaker and dissolve in 80 mL of deionized water using a magnetic stirrer. After the $CaCl_2$ has completely dissolved, adjusted to a final volume of 100 mL with deionized water. Filter the solution through a 0.22 μm PES membrane filter and aliquot into 0.65 mL tubes for storage at 4 °C.
2. 2× HBS: 274 mM sodium chloride, 10 mM potassium chloride, 1.4 mM disodium hydrogen phosphate, 42 mM HEPES, 11 mM glucose. Weigh 8 g of sodium chloride (NaCl), 0.38 g of potassium chloride (KCl), 0.1 g of disodium hydrogen phosphate (Na_2HPO_4), 5 g of HEPES and 1 g of glucose ($C_6H_{12}O_6$) into a glass beaker and dissolve with 400 mL of deionized water using a magnetic stirrer. After all the chemicals have completely dissolved, adjusted the final volume to 500 mL with deionized water using a measuring cylinder. Filter the solution through a 0.22 μm PES membrane filter and aliquot into 1.5 mL tubes for storage at 4 °C.
3. Opti-MEM® media (*see* **Note 7**). Pour 13.6 g of Opti-MEM® media powder into a beaker and dissolve it with 600 mL deionized water using a magnetic stirrer. In parallel, weigh 2.4 g of sodium hydrogen carbonate ($NaHCO_3$) into a glass beaker and dissolve it with 100 mL of deionized water using a magnetic stirrer. After both solutions have dissolved completely, mix the sodium hydrogen carbonate solution with the Opti-MEM® media. Adjust the final volume to 1 L with deionized water. Filter the solution through a 0.22 μm PES membrane filter and aliquot into 500 mL bottles and store at 4 °C.
4. 1× Dulbecco's Modified Eagle's Medium (DMEM). Pour 13.5 g of DMEM media powder into a beaker and dissolve it with 600 mL deionized water using a magnetic stirrer. In parallel, weigh 3.7 g of sodium hydrogen carbonate ($NaHCO_3$) into a glass beaker and dissolve it with 100 mL of deionized water using a magnetic stirrer. After both solutions have dissolved completely, mix the sodium hydrogen carbonate solution with the DMEM media. Adjust pH to 7.2 with concentrated hydrochloric acid (HCl) (*see* **Note 8**). Adjust the final volume to 1 L with deionized water. Filter the solution through a 0.22 μm PES membrane filter and aliquot into 500 mL bottles and store at 4 °C.
5. HEK293T/17 cells cultured in 1× DMEM supplemented with 10% fetal bovine serum in an incubator with 5% CO_2 at 37 °C.
6. 0.22 μm nitrocellulose membrane.

2.3 Zika VLP Detection by Western Blot

1. 1.5 M Tris-HCl pH 8.8. Weigh 27.26 g of Tris base into a glass beaker and dissolve with 80 mL of deionized water using a magnetic stirrer until completely dissolved. Adjust the pH to 8.8 using concentrated HCl (*see* **Note 8**). Transfer the solution into a measuring cylinder then add deionized water to make the final volume of 150 mL and store at room temperature.
2. 1 M Tris–HCl pH 6.8. Weigh 12.1 g of Tris base into a glass beaker and dissolve with 50 mL of deionized water using a magnetic stirrer until completely dissolved. Adjust the pH to 6.8 using concentrated HCl (*see* **Note 8**). Transfer the solution into a measuring cylinder then add deionized water to adjust the final volume to 100 mL and store at room temperature.
3. 10% SDS (w/v). Weigh 10 g of sodium dodecyl sulfate (SDS) (*see* **Note 9**) into a glass beaker and dissolve with deionized water using a magnetic stirrer. The solution can be heated to 68 °C to improve the solubility. After the SDS has dissolved completely, adjust the volume to 100 mL with deionized water and mix thoroughly. The solution can be kept at room temperature.
4. 10% ammonium persulfate (APS) (w/v). Weigh 0.1 g of APS into a 1.5 mL tube and dissolve with 1 mL of deionized water by vigorously vortex. The solution should be freshly prepared and can be kept at 4 °C for 1 week.
5. 40% acrylamide–bis solution (29.1:0.9).
6. *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED).
7. 5× nonreducing sample buffer: 0.3 M Tris–HCl pH 6.8, 5% SDS, 50% glycerol, 0.015% bromophenol blue. Weigh 1.5 g of SDS into a glass beaker and dissolve with 9 mL of 1 M Tris–HCl pH 6.8 using a magnetic stirrer. The solution can be heated to 68 °C to improve the solubility. Then add 15 mL of 100% glycerol into the solution and continue mixing. In parallel, weigh 4.5 mg of bromophenol blue into a 5 mL tube and dissolve with 2 mL of deionized water by vortexing. After the solution is completely solubilized, add the bromophenol blue solution into the Tris–HCl/SDS/glycerol solution and adjust the volume to 30 mL with deionized water and mix thoroughly. The solution can be kept at room temperature.
8. 10× SDS-PAGE running buffer: 0.25 M Tris–HCl pH 8.3, 1.92 M glycine, 1% SDS. Weigh 30.2 g of Tris base, 141.4 of glycine and 10 g of SDS into a glass beaker and dissolve with 500 mL of deionized water using a magnetic stirrer. The pH of the buffer should be 8.3, and pH adjustment is not normally required. After all the chemicals have dissolved completely, adjust the volume to 1 L with deionized water and mix thoroughly. The solution can be kept at room temperature.

9. 1× SDS-PAGE running buffer: 25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% SDS. Measure 100 mL of 10× SDS-PAGE buffer into a 1 L measuring cylinder, add 900 mL of deionized water, and then mix thoroughly.
10. 10× transfer buffer: 250 mM Tris–HCl, 2 M glycine. Weigh 30.0 g of Tris-base and 144.1 g of glycine into a glass beaker and dissolve with 500 mL of deionized water using a magnetic stirrer. After all the chemicals have dissolved completely, adjust the volume to 1 L with deionized water and mix thoroughly. The solution can be kept at room temperature.
11. Methanol (analytical grade).
12. 1× transfer buffer: 25 mM Tris–HCl, 200 mM glycine, 20% methanol. Measure 700 mL of deionized water into a 1 L measuring cylinder, then add 100 mL of 10× transfer buffer followed by 200 mL of methanol and then mix thoroughly. This solution must be freshly prepared and cooled to 4 °C before use.
13. 10× TBS buffer: 200 mM Tris–HCl, 1.4 M NaCl. Weigh 24.2 g of Tris base and 80 g of NaCl into a glass beaker and dissolve with 800 mL of deionized water using a magnetic stirrer. After the chemicals have dissolved completely, adjust the pH to 7.6 with concentrated HCl (*see* **Note 6**) followed by adjusting the volume to 1 L with deionized water and mixing thoroughly. The solution can be kept at room temperature.
14. 0.1% Ponceau S (w/v) in 5% (v/v) acetic acid.
15. Plastic box with a lid.
16. 1× TBS-T: 20 mM Tris–HCl, 140 mM NaCl, 0.05% Tween 20. Measure 100 mL of 10× TBS buffer into a 1 L measuring cylinder, then add 900 mL of deionized water and 500 μL of Tween 20 and mix thoroughly.
17. 5% skim milk: 20 mM Tris–HCl, 140 mM NaCl, 0.05% Tween 20, 5% dry skim milk. Weigh 5 g of skim milk powder into a glass beaker and dissolve with 100 mL of 1× TBS-T buffer using a magnetic stirrer. The solution can be kept for 1 week at 4 °C.
18. Precision Plus Protein™ All Blue Prestained Protein Standards.
19. Immobilon® Forte Western HRP Substrate.
20. Azure C400 imaging system.
21. 100% isopropanol.
22. Mouse monoclonal antibody HB112 (*see* **Note 10**).
23. Goat anti-mouse IgG horseradish peroxidase (HRP) conjugated secondary antibody (*see* **Note 11**).
24. 3MM Chr cellulose chromatography paper.

2.4 Zika VLP Purification by Discontinuous Sucrose Gradient Centrifugation

1. 0.22 μm PES membrane filter.
2. 100 kDa cutoff column.
3. 1 M Tris–HCl pH 7.5. Weigh 12.11 g of Tris base into a glass beaker and dissolve with 60 mL of deionized water using a magnetic stirrer. After the Tris base has dissolved completely, adjust the pH to 7.5 with concentrated HCl (*see* **Note 8**). Transfer the solution into a measuring cylinder and adjust the volume to 100 mL with deionized water and then mix thoroughly. The solution can be kept at room temperature.
4. 2.5 M NaCl. Weigh 29.22 g of NaCl into a glass beaker and dissolve with 150 mL of deionized water using a magnetic stirrer. After the chemical has completely dissolved, transfer the solution into a measuring cylinder and adjust the volume to 200 mL with deionized water and then mix thoroughly. The solution can be kept at room temperature.
5. 0.5 M EDTA. Weigh 186.12 g of EDTA · Na_2 · $2H_2O$ into a glass beaker and dissolve with 800 mL of deionized water. Adjust pH to 8.0 with sodium hydroxide (NaOH) (*see* **Note 12**). After the chemical has dissolved completely, transfer the solution into a measuring cylinder and adjust the final volume to 1 L with deionized water and then sterilize by autoclaving. The solution can be kept at room temperature.
6. TNE buffer: 10 mM Tris–HCl, 140 mM NaCl, 1 mM EDTA. Measure 10 mL of 1 M Tris–HCl pH 7.5 solution, 56 mL of 2.5 M NaCl solution and 2 mL of 0.5 M EDTA solution into a measuring cylinder. Adjust the final volume to 1 L with deionized water and then sterilize by autoclaving. The solution should be kept at 4 °C.
7. 10% sucrose (w/v). Weigh 10 g of sucrose into a glass beaker and dissolve in 80 mL of TNE buffer using a magnetic stirrer. After the sucrose has dissolved completely, transfer the solution into a measuring cylinder and adjust the final volume to 100 mL with TNE buffer and then sterilize by autoclaving. The solution should be kept at 4 °C.
8. 30% sucrose (w/v). Weigh 30 g of sucrose into a glass beaker and dissolve in 80 mL of TNE buffer using a magnetic stirrer. After the sucrose has completely dissolved, transfer the solution into a measuring cylinder and adjust the final volume to 100 mL with TNE buffer, then sterilize by autoclaving. The solution should be kept at 4 °C.
9. 60% sucrose (w/v). Weigh 60 g of sucrose into a glass beaker and dissolve in 60 mL of TNE buffer using a magnetic stirrer. After the sucrose has dissolved completely, transfer the solution into a measuring cylinder and adjust the final volume to 100 mL with TNE buffer, then sterilize by autoclaving. The solution should be kept at 4 °C.

10. Ultra-Clear Tubes $^{9}/_{16} \times 3^{1}/_{2}$ in. (14 × 89 mm) (Beckman Coulter, Brea, CA).
11. Tomy MX-301 centrifuge.
12. Beckman Optima XL-A centrifuge, SW 41Ti rotor.

2.5 Zika VLP Detection by Transmission Electron Microscopy

1. Formvar-coated grids with evaporated carbon film on 400 mesh nickel grid.
2. 2% Uranyl acetate. Weigh 0.4 g of uranyl acetate into a brown glass bottle then add 10 mL of methanol and 10 mL of deionized water (*see* **Note 13**). Warm the solution at 70 °C for 40 min then store at 4 °C. The solution should be filtered through two layers of 8 μm filter paper (Whatman® paper Grade 40 Ashless) before use.
3. Glass funnel.
4. Dumont Tweezer, style N3 Dumont Tweezer, style N7.
5. Clear glass microscope slides.
6. Quorum SC7620 Sputter Coater.
7. Transmission electron microscope (TEM).
8. Whatman® paper Grade 40 Ashless.

3 Method

Perform all procedures at room temperature, except where stated otherwise.

3.1 Construction of Zika VLP Expression Plasmid

Good aseptic technique is required for this experiment to avoid contamination between samples. Importantly, working with recombinant plasmid and bacteria must be undertaken carefully to avoid spread to the environment.

1. The 19 amino acids at the C-terminal end of the *capsid* (*C*) along with the *premembrane/membrane* (*prM/M*) and *envelope* (*E*) gene of Zika virus will be amplified.
   (a) Step 1: Add the following components into a 0.2 mL nuclease-free microcentrifuge tube (prepare the reaction on ice (*see* **Note 14**)):

13 μL	5× HF buffer (*see* **Note 15**)
1.625 μL	*NheI*-19CprME-Zika-Fw forward primer (10 μM)
1.625 μL	19CprME-Zika-*EcoRI*-Rw reverse primer (10 μM)
1.3 μL	10 mM dNTPs
0.65 μL	Phusion DNA polymerase
Up to 50 μL	nuclease-free water

(b) Step 2: Take 10 μL from the master mix as a negative control.

(c) Step 3: Add 1 μL template plasmid (dilution 1:100).

(d) Step 4: Mix and spin down the PCR solution in a 0.2 mL microcentrifuge tube.

(e) Step 5: Perform the PCR condition using the following conditions: denature template at 98 °C for 30 s, followed by 25–30 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 2 min. Then incubate at 72 °C for 5 min to complete amplification. The negative control (no template) should be subjected to PCR in parallel with the experimental sample.

2. Examine the PCR product by duplicate electrophoresis through 1% agarose gels in 1× TAE buffer, followed by staining one gel with EthBr (*see* **Note 4**) and one with methylene blue (*see* **Note 16**).

3. Excise the PCR product band from the methylene blue stained gel and perform gel purification using the FavorPrep™ GEL/PCR Purification Mini Kit following the manufacturer's protocol.

4. Digest the purified PCR product and pcDNA™ 3.1(+) plasmid vector with restriction enzymes to get sticky end overhangs as follows:

(a) Step 1: Add the following components into a nuclease-free microcentrifuge tube (prepare the reaction on ice (*see* **Note 14**)):

1 μL	10× Fast Digest buffer (*see* **Note 17**)
0.5 μL	*NheI* Fast Digest enzyme
0.5 μL	*EcoRI* Fast digest enzyme
X μL	purified PCR product OR plasmid vector (5 μg of plasmid vector)
Up to 10 μL	nuclease-free water

(b) Step 2: Mix the constituents and briefly spin down the reaction in a microcentrifuge.

(c) Step 3: Incubate at 37 °C for 1 h.

5. Examine the digested PCR product and plasmid vector by electrophoresis through a 1% agarose gel in 1× TAE buffer and perform gel purification on the product bands as described above (**step 2**).

6. Measure the concentration of the digested PCR product and plasmid vector using a NanoDrop™ 2000 spectrophotometer.
7. Ligate the digested PCR product into the purified digested pcDNA™ 3.1(+) plasmid vector at a ratio of 1:3 (vector–insert) as follows:
   (a) Step 1: Add the following components into a nuclease-free microcentrifuge tube (prepare the reaction on ice (*see* **Note 14**)):

2 μL	10× T4 ligase buffer
X μL	*insert*
X μL	*vector (50 ng)*
0.2 μL	T4 DNA ligase enzyme (5 U/μL)
Up to 20 μL	nuclease-free water

   (b) Step 2: Mix the components and briefly spin down the reaction in a microcentrifuge.
   (c) Step 3: Incubate at 16 °C overnight.
8. Prepare competent *E. coli* strain DH5α as follows (*see* **Note 18**):
   (a) Step 1: Steak *E. coli* strain DH5α onto an LB agar plate and incubate at 37 °C for 16 h.
   (b) Step 2: Pick a separated single colony using a sterile toothpick or loop and inoculate into 5 mL LB broth and incubate at 37 °C with vigorous shaking at 220 rpm for 8–12 h to generate a bacterial starter culture.
   (c) Step 3: Transfer 1 mL of bacterial starter culture into 50 mL LB broth in a 250 mL bacterial culture flask and incubate at 37 °C with vigorous shaking at 220 rpm for 2–3 h.
   (d) Step 4: Monitor the bacterial number by measuring the optical density (OD) at 600 nm (OD_{600}) every 20 min until $OD_{600} = 0.3–0.4$ (this OD_{600} value is reached in the mid log phase of *E. coli* strain DH5α growth).
   (e) Step 5: When the desired OD_{600} is reached, cool the bacterial culture down by placing the growth flask on ice for 10 min.
   (f) Step 6: Pellet the bacteria aseptically by transferring the bacterial culture into a 50 mL centrifugation tube and centrifuge at 3000 × *g* for 10 min at 4 °C.
   (g) Step 7: Discard the culture media and resuspend the bacterial pellet in 10 mL of chilled 0.1 M $CaCl_2$ and incubate on ice for 10 min.

(h) Step 8: Remove $CaCl_2$ by centrifugation at 3000 × *g* for 10 min at 4 °C and resuspend cells in 2 mL 15% glycerol in 0.1 M $CaCl_2$.

(i) Step 9: Aliquot 100 μL competent cells into each 1.7 mL microcentrifuge tube and immediately soak in liquid nitrogen.

(j) Step 10: Store the competent cells at −80 °C until use.

9. Transform the recombinant plasmid into competent *Escherichia coli* (*E. coli*) strain DH5α as follows:

(a) Step 1: Add 10 μL of ligation reaction mixture to 100 μL of competent cells.

(b) Step 2: Incubate on ice for 10 min.

(c) Step 3: Immediately incubate at 42 °C for 45 s (heat shock).

(d) Step 4: Immediately transfer the tube onto ice and incubate for 5 min.

(e) Step 5: Add 900 μL of LB broth (without antibiotic) to the competent cell mixture.

(f) Step 6: Incubate the transformants at 37 °C for 1 h with shaking at 220 rpm.

(g) Step 7: Spread the transformants on the LB agar plates containing 100 μg/mL ampicillin, allow to dry, and then incubate inverted at 37 °C for 16 h.

(h) Step 8: Make a master plate by randomly picking isolated transformants using a sterile toothpick and streaking the transformants in an orderly manner (*see* Fig. 1) onto a new LB agar plate containing 100 μg/mL ampicillin, then incubate inverted at 37 °C for 16 h.

10. Screen for positive clones that contain the recombinant plasmid by colony PCR as follows:

(a) Step 1: Add the following components into a 0.2 mL nuclease-free microcentrifuge tube (prepare the reactions on ice (*see* **Note 14**)):

1 μL	10× DreamTaq buffer
0.25 μL	*NheI*-19CprME-Zika-Fw forward primer (10 μM)
0.25 μL	19CprME-Zika-*EcoRI*-Rw reverse primer (10 μM)
0.3 μL	10 mM dNTPs
0.5 μL	DreamTaq DNA polymerase
–	Template colony (*see* **Note 19**)
Up to 10 μL	nuclease-free water

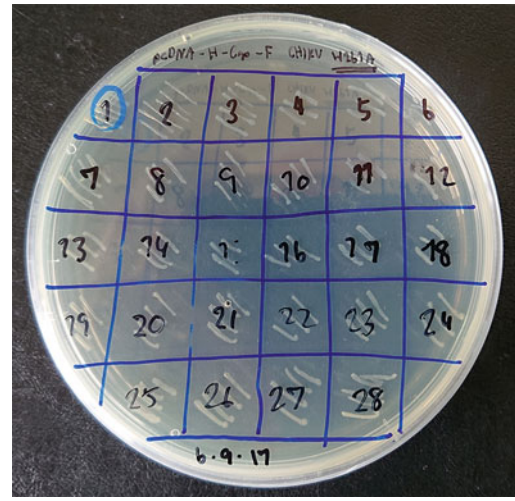

Fig. 1 Colony PCR screening. An ordered array of putative transformants. Each grid square contains one randomly selected transformant that will be screened by colony PCR

(b) Step 2: If screening multiple colonies, prepare a reaction master mix by multiplying by the number of colonies to be screened plus negative and positive controls (Example: 19 + 1 + 1 = 21 + 10% volume pipetting error).

(c) Step 3: Spin down and aliquot the master mix in a 0.2 mL microcentrifuge tube.

(d) Step 4: Perform the PCR reaction as follows, denature the template at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min. Then incubate at 72 °C for 5 min to complete the reaction. Negative control (no template colony) and positive control (an aliquot of the original purified PCR product) should be run in parallel.

11. Examine the PCR products by gel electrophoresis with 1% agarose in 1× TAE buffer.
12. Select a positive colony containing the recombinant plasmid and inoculate in 5 mL LB broth containing 100 μg/mL ampicillin. Then incubate at 37 °C with constant agitation at 220 rpm for 16 h.
13. Extract the plasmid using FavorPrep™ Plasmid Extraction Mini Kit by following the standard manufacturer protocol and measure the concentration using a NanoDrop spectrophotometer.
14. Dilute the plasmid to 100 ng/μL using nuclease-free water.
15. Perform restriction enzyme digestion to determine the correct clone as follows.

(a) Step 1: Add the following components into a nuclease-free microcentrifuge tube (prepare the reaction on ice):

1 μL	10× FastDigest green buffer (*see* **Note 17**)
1 μL	*plasmid (100 ng/μL)*
0.5 μL	*NheI Fast Digest enzyme*
0.5 μL	*EcoRI Fast Digest enzyme*
Up to 10 μL	nuclease-free water

(b) Step 2: If screening multiple colonies, prepare a reaction master mix by multiplying by the number of plasmids to be screened plus a negative control (plasmid vector) (Example 10 + 1 = 11 + 10% volume pipetting error).

(c) Step 3: Aliquot 9 μL of the master mix into a microcentrifuge tube followed by adding 1 μL of each diluted recombinant plasmid into each tube, mix and briefly spin down the reaction.

(d) Step 4: Incubate at 37 °C for 1 h.

16. Examine the digested plasmid by gel electrophoresis. It is necessary to load the undigested plasmid onto the gel in parallel with the digested plasmid.
17. Select the correct clone and sequence (*see* **Note 20**).

3.2 Expression of Zika VLP in HEK293T/17 Cells (See Note 21)

1. Seed the HEK293T/17 cells at a density to allow cells to reach 80% confluency within 24 h in a 100 mm cell culture dish in a total volume of 10 mL (*see* **Note 22**).
2. Warm all solutions to room temperature including the plasmid solution.
3. Before transfection, remove the DMEM culture media from the cells and then replace with 6 mL of prewarm at 37 °C Opti-MEM® media.
4. Transfect the Zika VLP expression plasmid into the cells as follows (a no plasmid and an only plasmid vector transfection as mock and transfection controls are required):

(a) Step 1: Dilute 9 μg of Zika VLP expression plasmid in a final volume of 360 μL sterile deionized water in a nuclease-free microcentrifuge tube.

(b) Step 2: Add 360 μL of 2× HBS buffer into the plasmid mixture and then vortex.

(c) Step 3: Add 36 μL of 2.5 M $CaCl_2$ solution into the mixture followed by immediate vigorous vortexing.

(d) Step 4: Briefly spin down the solution inside the microcentrifuge tube.

(e) Step 5: Incubate the plasmid–calcium phosphate complex at room temperature for 20 min with no movement.

(f) Step 6: Gently drop the plasmid-calcium phosphate complex solution onto the surface of the media (*see* **Note 23**) and gently rock the cell culture dish 2–3 times.

5. Incubate the transfected and control cells under 5% CO_2 at 37 °C for 3 days.

3.3 Zika VLP Collection and Purification by Discontinuous Sucrose Gradients

1. Collect and pool (*see* **Note 22**) the Zika VLP-enriched Opti-MEM® media (~40 mL) into a 50 mL centrifuge tube and centrifuge at 1000 × *g* for 5 min to remove floating cells. Collect the media from the mock transfection and treat in parallel.
2. Filter the Zika VLP-enriched media through a 0.22 μm filter (*see* **Note 24**).
3. Transfer the Zika VLP-enriched media into a 100 kDa cut off column and centrifuge at 5000 × *g* for 10 min at 4 °C to concentrate the Zika VLP. The final volume will be about 1.5 mL of concentrated Zika VLP in Opti-MEM® media. Reserve 200 μL for subsequent western blot analysis (Subheading 3.4).
4. Add 4 mL of 60% sucrose into an Ultra-Clear Tube.
5. Gently overlay 4 mL of 30% sucrose onto the 60% sucrose in the Ultra-Clear Tube (Avoid disruption of the surface between the two layers) (*see* **Note 25**).
6. Mix 1.3 mL of concentrated Zika VLP in Opti-MEM® media with 2 mL of 10% sucrose then gently overlay onto the two layers of 30% and 60% sucrose in the Ultra-Clear Tube, again avoiding disruption between the surface layers.
7. Gently put the Ultra-Clear Tube containing the three layers into an ultracentrifugation bucket and fill with 10% sucrose onto the upper layer until full and close the lid (*see* **Note 26**).
8. Perform the centrifugation at 134,434 × *g* for 2 h at 4 °C with no brake (*see* **Note 27**).
9. Collect the visible band (concentrated Zika VLP) between the 30% and 60% sucrose layers by side puncture and put into a new Ultra-Clear Tube (Fig. 2).
10. Add TNE buffer into the collected visible band in the Ultra-Clear Tube until full then mix by inverting the tube and centrifuge at 134,434 × *g* for 1 h at 4 °C with brake.
11. Discard the TNE buffer by pouring from the tube until no excess liquid is left, the Zika VLP can be observed as a white opaque pellet. Then add 50 μL of new TNE buffer onto the

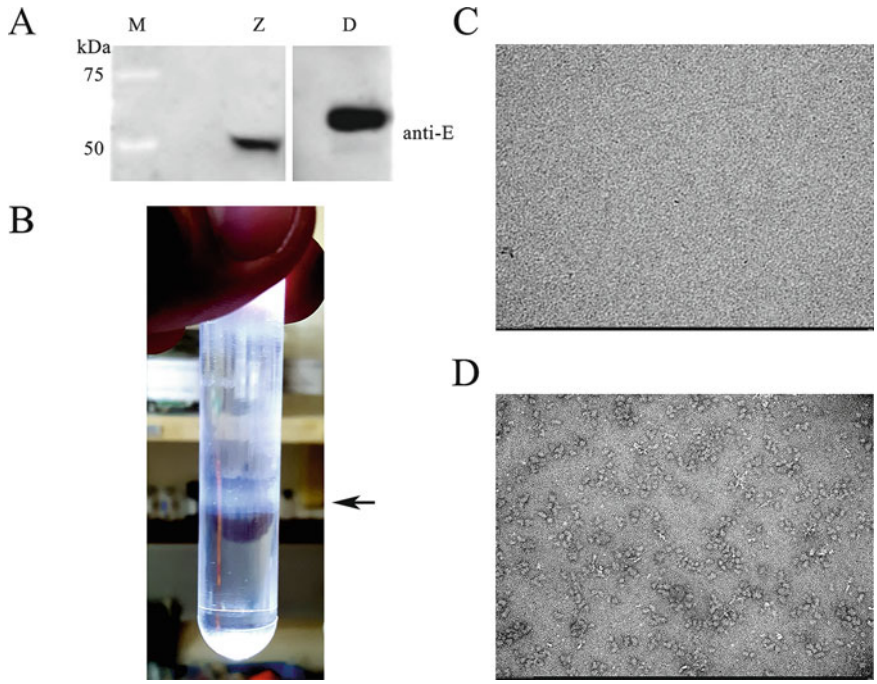

Fig. 2 ZIKV VLP purification and analysis. Zika VLP enriched media was concentrated and (**a**) a small portion was examined by western blot analysis using a pan-specific anti-flavivirus envelope protein monoclonal antibody. A control lane of purified dengue virus is also shown; *M* marker, *Z* Zika, *D* dengue. (**b**) Zika VLP enriched media was subjected to discontinuous sucrose gradient centrifugation. Zika VLP band at the interface of the 30% and 60% sucrose cushions (arrowed). The ZIKV VLP were collected by side puncture of the tube. (**c**, **d**) Examination of a TNE buffer control and purified Zika VLP, respectively

Zika VLP pellet and gently pipette to dissolve the pellet followed by aliquoting and storage at 4 °C (*see* **Note 28**).

3.4 Detection of Zika VLP by Western Blot

Acrylamide solution can cause acute toxicity to the skin, eyes, respiratory and reproductive organs, and is a probable neurotoxin and human carcinogen. Work with acrylamide must be undertaken carefully, and personal protective equipment (PPE) such as a lab coat, gloves and goggles are required when handling acrylamide. The toxicity is significantly reduced when acrylamide is polymerized, but some unpolymerized solution might remain, with the associated potential risk. The following protocol is for one SDS-PAGE gel (8.3 cm × 7.3 cm × 1.5 mm)

1. Clean the glass plates with 70% ethanol and assemble the glass plate sandwich to the gel casting component. Mark the level between separating and stacking gels with a marker pen.
2. Prepare a 10% separating gel by mixing 4.275 mL of water, 2.25 mL of 40% acrylamide, 2.25 mL of 1.5 M Tris–HCl pH 8.8, 90 μL of 10% SDS, and 135 μL of 10% APS in a 25 mL glass beaker and mix by swirling.

3. Add 10 μL of TEMED into the acrylamide mixture, immediately mix by swirling and pour the acrylamide gel solution into the glass plate sandwich. Overlay the top of the acrylamide gel surface with 1 mL of 100% isopropanol and let stand for 10 min for gel polymerization.
4. Remove the 100% isopropanol by pouring and use tissue paper to absorb the excess isopropanol.
5. Prepare a 5% stacking gel by mixing 1.446 mL of water, 250 μL of 40% acrylamide, 254 μL of 1 M Tris–HCl pH 6.8, 20 μL of 10% SDS, and 30 μL of 10% APS in a 25 mL glass beaker and mix by swirling.
6. Add 5 μL of TEMED into the acrylamide mixture and immediately mix by swirling. Pour the acrylamide gel solution on top of the separating gel in the glass plate sandwich, insert the comb to the gel, and then let the gel polymerize for 10 min.
7. Remove the glass plate sandwich from casting component and assemble in the core electrophoresis apparatus. Add 1× running buffer to the top and bottom reservoirs and make sure that the bottom of the gel is submerged in the running buffer and there is no buffer leakage.
8. To prepare the samples for protein loading, mix 44 μL of the reserved filtered Zika VLP in Opti-MEM® media or media from the mock transfection (*see* Subheading 3.3, **step 1**) with 11 μL of 5× nonreducing sample buffer. Boil samples for 5 min and immediately place on ice before a short spin down and load 50 μL into each well of the acrylamide gel. Load 4 μL of molecular weight protein marker into one well as a reference for protein size.
9. Assemble the lid and power supply to the gel chamber. Run the gel at a constant 100 V until the marker dye front (bromophenol blue) has reached the end of the gel (approximately 2 h).
10. Turn off the power and remove the acrylamide gel from the running chamber and transfer the separated protein on to nitrocellulose membrane by blotting. Soak all components required for transfer in 1× transfer buffer before assembly of the blotting cassette. Place the transfer system fiber pad onto the dark side of the blotting cassette followed by two layers of chromatography paper, the acrylamide gel, a 0.22 μm nitrocellulose membrane, two layers of chromatography paper and a fiber pad, respectively. All components should be soaked in 1× transfer buffer before assembly of the transfer sandwich. Place the blotting cassette into the protein blotting transfer chamber and then pour 1× transfer buffer to cover the blotting cassette. Assemble the lid and power supply and transfer with a constant 300 mA for 1 h.

11. Remove the nitrocellulose membrane containing the transferred proteins from the blotting cassette and examine the transferred protein by staining with Ponceau S solution for 10 s and wash with deionized water for 3 times.
12. Block the membrane with 5 mL of 5% skim milk for 1 h at room temperature and then incubate the membrane with primary antibody HB112 at a 1:500 dilution overnight.
13. Wash the membrane 3 times with TBS-T for 5 min each time. Incubate the membrane with a HRP-conjugated goat anti-mouse IgG secondary antibody at a 1:5000 dilution for 1 h at room temperature.
14. Wash the membrane 3 times with TBS-T for 5 min each time before developing the signal by incubating the membrane with an HRP substrate for 5 min in the dark (follow the manufacturer's protocol).
15. Observe the signal using an Azure C400 signal detector or other suitable detection equipment (Fig. 2).

3.5 Detection of Zika VLP by Electron Microscopy

1. Place a formvar-carbon film nickel grid onto a glass slide and attach a small area of the grid to the slide using Scotch Brand masking tape. Put the grid into a Quorum SC7620 Sputter Coat machine and glow discharge at 18 mA for 120 s.
2. Directly drop 3 μL of purified Zika VLP in TNE buffer onto the grid and incubate at room temperature for 1 min. Remove the excess purified Zika VLP solution from the grid by applying filter paper at the edge of the grid to absorb excess solution.
3. Directly drop 3 μL of 2% uranyl acetate onto the grid and incubate at room temperature for 2 min (*see* **Note 13**). Remove the excess uranyl acetate by filter paper and let the grid dry for 2 min at room temperature.
4. Wash the grid 1 time with deionized water followed by removing the excess water with filter paper and dry for 5 min at room temperature.
5. Examine the Zika VLP under a transmission electron microscope (Fig. 2).

4 Notes

1. We purchased a commercially synthesized construct with codon optimization for mammalian expression in vector pUC57, as described in a previous publication [13]. pUC57 is a standard prokaryotic cloning vector and the insert requires transfer to a mammalian expression vector (in this case the pcDNA™ 3.1(+) plasmid vector). Expression of the insert in

the pcDNA™ 3.1(+) plasmid vector is under control of the cytomegalovirus (CMV) enhancer promoter.

2. Glacial acetic acid is skin corrosive and can cause serious eye damage. Working with glacial acetic acid requires personal protective equipment (PPE) and all handling to be performed in a fume hood.
3. Loosen the lid to prevent the accumulation of steam inside the bottle and wear heat protective gloves to prevent scalding.
4. Ethidium bromide is a carcinogen which causes DNA mutation; the user must wear PPE and work carefully. Disposal of used ethidium bromide solution should be in accordance with institutional regulations. Stock ethidium bromide should be stored at room temperature, protected from the light.
5. Cool LB broth after autoclaving to room temperature or 4 °C before adding ampicillin to prevent degradation of ampicillin from the heat. Due to the E protein of flaviviruses being toxic to bacterial cells, a reduction of ampicillin from the normal concentration (100 μg/mL) to 50 μg/mL is useful for bacterial growth.
6. After adding ampicillin, immediately mix the solution by swirling and quickly pour the agar before it sets.
7. Opti-MEM media is required to remove the requirement of adding fetal bovine serum (FBS) to the cell growth media, as FBS can significantly affect the subsequent purification and detection of the VLP.
8. Concentrated HCl is a strong corrosive substance that can cause serious skin, eye, and respiratory damage. The solution preparation must be performed in a fume hood with PPE.
9. SDS is acutely toxic via oral and dermal exposure and by inhalation and can cause skin and eye irritation. The user must wear a face mask to prevent inhalation of SDS while weighing the powder. Some protocols recommend weighing SDS inside a fume hood; however, the user should be aware this will cause significant air currents that may distribute SDS around the working environment.
10. This protocol uses a panspecific anti-flavivirus envelope protein monoclonal antibody that is purified in house from hybridoma HB112 [14]. Both monoclonal and polyclonal antibodies to Zika E protein are commercially available.
11. Other suitable secondary antibodies such as a HRP-conjugated rabbit anti-mouse IgG polyclonal antibody can be substituted as desired.
12. NaOH is a strong corrosive reagent that can cause skin and eye irritation. PPE is required.

13. Do not breath directly onto the uranyl acetate solution, as this can cause turbidity in the solution. This solution will be used for negative staining for electron microscopy.
14. To prevent premature enzyme activity.
15. There are two types of buffer for Phusion DNA polymerase. For this experiment, use only the 5× HF buffer.
16. Methylene blue is used to stain the DNA band on the preparative gel to reduce DNA damage.
17. Restriction enzymes are normally supplied with the appropriate buffer.
18. Competent cell preparation requires aseptic technique to avoid contamination.
19. A colony (or part of the master plate streak) can be used directly as a template in PCR by using a toothpick to touch the colony (or streak) and then swirling the toothpick in the PCR solution, followed by starting the reaction.
20. Plasmid verification should be undertaken by sequencing the entire insert using an in house or commercial sequencing service.
21. Transfection into HEK293T/17 cells is effective using the calcium phosphate method.
22. The protocol describes the conditions for one transfection. In practice, we generated seven transfections per preparation and pool the media.
23. To avoid cells detaching from the culture plate.
24. To remove cell debris and other small components from the culture media. Filtration must be performed gently to avoid membrane shearing.
25. Gently release each concentration of sucrose onto the wall of the UC tube to make sure the layers do not mix.
26. The 10% sucrose must fill to the top of the tube with no air bubbles inside the tube to prevent the tube from cracking. Balancing the UC tube with a counter weight is essential.
27. Do not set the brake for this step to prevent band disruption.
28. For determination of Zika VLP by EM, the purified VLP must be stored at 4 °C. If stored at −20 °C, there might be damage to the VLP structure by ice crystal formation.

Acknowledgments

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References

1. Weaver SC, Barrett AD (2004) Transmission cycles, host range, evolution and emergence of arboviral disease. Nat Rev Microbiol 2 (10):789–801
2. King AM, Adams MJ, Lefkowitz EJ, Carstens EB (2012) Virus taxonomy. In: IXth report of the International Committee on Taxonomy of Viruses, vol 9. Academic Press, San Diego, CA
3. Dick G, Kitchen S, Haddow A (1952) Zika virus (I). Isolations and serological specificity. Trans R Soc Trop Med Hyg 46(5):509–520
4. Wikan N, Smith DR (2016) Zika virus: history of a newly emerging arbovirus. Lancet Infect Dis 16(7):E119–E126
5. Cao-Lormeau VM, Roche C, Teissier A, Robin E, Berry AL, Mallet HP, Sall AA, Musso D (2014) Zika virus, French Polynesia, South Pacific, 2013. Emerg Infect Dis 20 (6):1085–1086
6. Esposito DLA, de Moraes JB, Antonio Lopes da Fonseca B (2018) Current priorities in the Zika response. Immunology 153(4):435–442
7. Musso D, Gubler DJ (2016) Zika virus. Clin Microbiol Rev 29(3):487–524
8. Krauer F, Riesen M, Reveiz L, Oladapo OT, Martinez-Vega R, Porgo TV, Haefliger A, Broutet NJ, Low N, Group WHOZCW (2017) Zika virus infection as a cause of congenital brain abnormalities and Guillain-Barre syndrome: systematic review. PLoS Med 14(1): e1002203
9. Barrett AD, Teuwen DE (2009) Yellow fever vaccine - how does it work and why do rare cases of serious adverse events take place? Curr Opin Immunol 21(3):308–313
10. Yun SI, Lee YM (2014) Japanese encephalitis: the virus and vaccines. Hum Vaccin Immunother 10(2):263–279
11. Halstead SB (2017) Dengvaxia sensitizes seronegatives to vaccine enhanced disease regardless of age. Vaccine 35(47):6355–6358
12. Krol E, Brzuska G, Szewczyk B (2019) Production and biomedical application of flavivirus-like particles. Trends Biotechnol 37:1202
13. Wikan N, Suputtamongkol Y, Yoksan S, Smith DR, Auewarakul P (2016) Immunological evidence of Zika virus transmission in Thailand. Asian Pac J Trop Med 9(2):136–139
14. Henchal EA, Gentry MK, McCown JM, Brandt WE (1982) Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am J Trop Med Hyg 31 (4):830–836

Chapter 11

Biosynthesis of Glycoconjugate Virus-like Particles (VLPs)

Kathryn K. Oi, Tom A. Kloter, and Timothy G. Keys

Abstract

The outermost surface of bacterial pathogens consists primarily of complex carbohydrate structures—polysaccharides, glycolipids, and glycoproteins. To raise a long-lasting and effective immune response against carbohydrate antigens, they generally require covalent attachment to an immunogenic carrier protein—a so-called glycoconjugate vaccine. One hurdle to the development of glycoconjugate vaccines is that carbohydrate antigens remain inaccessible to recombinant production. Thus, the carbohydrate antigen is typically purified from the pathogen and then chemically conjugated to an immunogenic protein. Recent developments in the field of bacterial glycoengineering have opened the opportunity for total recombinant production of glycoconjugate vaccines. In this method, we describe the production of proteinaceous, virus-like particles (VLPs) bearing the conserved N-glycan of *Actinobacillus pleuropneumoniae*, the causative agent of porcine pleuropneumoniae.

Key words *Actinobacillus pleuropneumoniae*, N-glycosylation, Virus-like particle, Glycoconjugate, Vaccine

1 Introduction

The rise of antibiotic-resistant bacterial pathogens threatens the global health system. The root of this problem lies in our reliance on antibiotics to control and prevent infection in clinical and agricultural settings. Vaccines play an important role in reducing the demand for antibiotics; however, the design, production, and distribution of effective and economically viable vaccines still presents significant technical challenges. Simple methods for producing effective antibacterial vaccines at large-scale and low-cost are urgently needed.

Actinobacillus pleuropneumoniae is the causative bacterial agent of porcine pleuropneumonia—a disease underlying widespread antibiotic use and economic losses in the pork industry. A range of attenuated, inactivated, and subunit vaccines has been developed against *A. pleuropneumoniae* [1]. However, due to their variable surface structures (including lipopolysaccharide, capsular polysaccharides, and outer membrane proteins), none of the available

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vaccines has succeeded in providing broad protection against infection with different serotypes and biotypes of *A. pleuropneumoniae*. The most important component of current vaccines are the Apx toxins; neutralizing antibodies raised against these major virulence factors are effective in reducing the burden of disease but do not prevent infection or spread of the pathogen. The recent discovery of a highly conserved protein N-glycosylation system in *A. pleuropneumoniae* represents a new target antigen with the promise to confer broad protection [2–4].

The cytoplasmic N-glycosylation system of *A. pleuropneumoniae* modifies asparagine residues of autotransporter adhesins with short dextran oligosaccharides. The N-glycan biosynthetic pathway consists of two enzymes. The first is an asparagine glucosyltransferase (NGT) that transfers a single glucose in β-linkage onto asparagine residues in the Asn-X-Ser/Thr sequon (X ≠ Pro). The second is an α1,6-glucosyltransferase (α6GlcT) that synthesizes short, linear glucose oligosaccharides (oligoGlc) on the priming glucose [3]. Two proteins modified by this system have been identified. They are autotransporter adhesins with a high-density of target sequons [5], suggesting that they are likely to be modified at multiple sites with the dextran oligosaccharide. Given that the NGT and α6GlcT appear to be constitutively expressed, that both genes for this glycosylation system are absolutely conserved in all available genome sequences, and that NGT activity contributes to adhesion to lung epithelial cells, the dextran oligosaccharide may be an important and unvarying feature of the *A. pleuropneumoniae* surface [4]. In this chapter, we outline a simple method for the recombinant production of glycoconjugate virus-like particle (VLP) vaccines presenting 180 copies of this conserved N-glycan structure on their surface.

The N-glycosylation system of *A. pleuropneumoniae* has been functionally reconstituted in *Escherichia coli* [5]. Recombinant proteins can be targeted for glycosylation by incorporation of the Asn-X-Ser/Thr sequon on a flexible loop or tag [6]. The VLP scaffold that we chose for presenting the N-glycan is formed by the coat protein from the ssRNA phage AP205 (AP205cp) [7, 8]. A unique feature of the AP205 VLP is that the N- and C-termini of the coat protein are exposed on the surface of the capsid and are tolerant to genetic fusions, enabling the display of diverse peptide and protein antigens [9]. To generate an AP205 VLP presenting the asparagine (N)-linked dextran antigen, we genetically fused a short peptide tag including a single glycosylation site to the C-terminus of the AP205cp and coexpressed this construct with NGT and α6GlcT (Fig. 1). Assembly into complete AP205 VLPs is verified by native agarose gel electrophoresis and transmission electron microscopy. Glycosylation of the coat protein is verified by intact protein mass spectrometry and by gel shift assays.

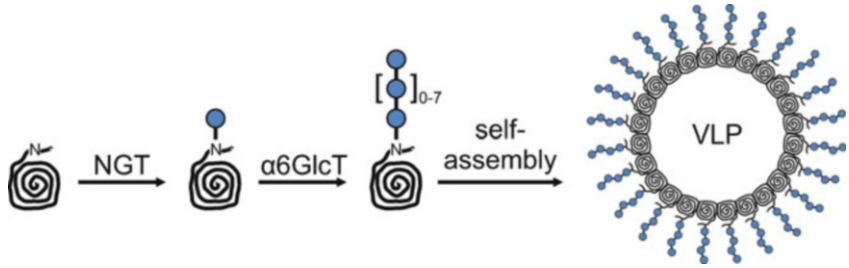

Fig. 1 Schematic of glycoconjugate VLP biosynthesis. The asparagine (N) residue of each coat protein is modified with a single β-linked glucose (blue circle) by the asparagine glucosyltransferase (NGT). The priming N-linked glucose is then extended by the α1,6-glucosyltransferase (α6GlcT) with up to eight further glucose residues. Coat proteins self-assemble into 180mer icosahedral VLPs. Note that the sequence of events is unclear, it is likely that glycosylation continues after particle assembly

2 Materials

2.1 Expression of Glycoconjugate AP205 VLPs

1. Antibiotics: Antibiotic stock solutions are listed in Table 1. For strains carrying one or two plasmids, the stocks are used at 1000-fold dilution in culture medium. Antibiotics are used at 2000-fold dilution for cultivation of strains carrying three plasmids.
2. Inducing agent: A 1 M stock of isopropyl β-D-1-thiogalacto-pyranoside (IPTG) is prepared in water and sterile filtered prior to use. The stock is stable for at least 1 year at −20 °C. Protein expression is induced by addition of 1 mM IPTG to the culture medium.
3. Plasmids: Plasmids used in this study are listed in Table 2.
4. Luria-Bertani (LB) medium: 0.5% (w/v) Bacto yeast extract, 1.0% (w/v) Bacto tryptone, 0.5% (w/v) NaCl. Dissolve in water and autoclaved immediately. Store at room temperature.
5. Terrific Broth (TB) medium: 2.4% (w/v) Bacto yeast extract, 1.2% (w/v) Bacto tryptone, 0.4% (v/v) glycerol, 72 mM K_2HPO_4, 17 mM KH_2PO_4. Dissolve nutrients (Bacto yeast extract, Bacto tryptone, and glycerol) and potassium salts separately in water and autoclave prior to mixing. Store at room temperature.

2.2 Purification of Glycoconjugate AP205 VLPs

1. Lysis Solution: 50 mM Tris–HCl pH 6.8, 150 mM NaCl, 1 mM $MgCl_2$, 0.5 mg/mL lysozyme, 5 μg/mL DNase I.
2. Binding/Wash Buffer: 50 mM Tris–HCl pH 6.8, 150 mM NaCl, 2 mM EDTA.
3. Elution Buffer: 50 mM Tris–HCl pH 6.8, 150 mM NaCl, 2 mM EDTA, 5 mM desthiobiotin.

Table 1
Antibiotics

Antibiotic	Stock	Concentration in media (strains with 1 or 2 plasmids)	Concentration in media (strains with 3 plasmids)
Chloramphenicol (Cm)	30 mg/mL in 50% Ethanol (v/v)	30 μg/mL	15 μg/mL
Kanamycin (Kan)	50 mg/mL in H_2O	50 μg/mL	25 μg/mL
Spectinomycin (Sp)	50 mg/mL in H_2O	50 μg/mL	25 μg/mL

Table 2
Plasmids

Plasmid name	Backbone	Promoter	Protein product (MW)	Antibiotic	Inducing agent
pRSF_AP205cp-GS	pRSF	PT5	AP205cp-GS (16.4 kDa)	Kan	IPTG
pACYC_NGT	pACYC	lacUV5	NGT (72.0 kDa)	Cm	IPTG
pCDF_α6GlcT	pCDF	T7	α6GlcT-6xHis (38.1 kDa)	Sp	IPTG

4. Freezing Buffer: 50 mM Tris–HCl pH 6.8, 150 mM NaCl, 10% glycerol, 2 mM EDTA.
5. 1 L Erlenmeyer flasks.
6. 1 mm electroporation cuvettes (CellProjects).
7. Fritted filtration columns (ISOLUTE).
8. Strep-tactin Superflow beads (IBA).

3 Methods

3.1 Production Strain

1. Glycosylation competent *Escherichia coli*: We use *E. coli* BL21-Gold(DE3) for glycoconjugate VLP production. However, in principle, any *E. coli* strain can be used. To modify target proteins with the glucose oligosaccharide structure requires coexpression of two enzymes (the NGT and α6GlcT) together with the protein substrate. We find that expression of the NGT behind a weak lacUV5 promoter [10] from a medium–low copy plasmid (pACYC_NGT, *see* Table 2) provides sufficient activity to modify 100% of a highly expressed protein substrate. In this method, we include the α6GlcT ORF under the control of a T7 promoter on a separate plasmid (pCDF_α6GlcT). It is also possible to include both NGT and α6GlcT ORFs in a bicistronic arrangement on a single plasmid (*see* **Note 1**).

2. Design of a glycosylation competent VLP: In this protocol, we target the coat protein of the AP205 bacteriophage for glycosylation. This is achieved by genetic fusion of a short polypeptide tag including a glycosylation site (GS) to the C-terminus of the AP205cp. The tag provides a spacer of 9 amino acids between the native C-terminus of the coat protein and the glycosylation site (*see* **Note 2**). The tag includes an extended glycosylation site (ANATA) based on a consensus sequence determined for the NGT [5]. Finally the tag includes a Strep-tag II sequence (WSHPQFEK) for affinity purification [11] (*see* **Note 3**).

3.2 Expression of Glycoconjugate VLPs

Day 1: Transformation

1. For each strain, dilute approximately 50 ng of each plasmid into 2 μL ddH_2O. Mix with 50 μL of electrocompetent *E. coli* BL21-Gold(DE3) and transfer to a cold 1 mm electroporation cuvette on ice. Proceed immediately to electroporation.
   (a) Strain A: pRSF_AP205cp-GS.
   (b) Strain B: pRSF_AP205cp-GS + pACYC_NGT.
   (c) Strain C: pRSF_AP205cp-GS + pACYC_NGT + pCDF_α6GlcT.
2. Electroporate cells using the following parameters: 1800 V, 200 Ω, 25 μF.
3. Directly transfer electroporated cells to 1 mL of LB and allow to recover for 1 h at 37 °C.
4. Pellet cells at 8000 × *g* for 3 min, discard 950 μL of supernatant, resuspend cells in the remaining media.
5. Plate cells on LB agar with the necessary antibiotic(s) and incubate overnight at 37 °C.

Day 2: Precultures

6. Inoculate 10 mL of LB medium with antibiotic(s) with multiple colonies and grow overnight at 37 °C.

Day 3: Protein Expression Cultures

7. Transfer 200 mL of TB medium, containing necessary antibiotic(s), into a 1 L Erlenmeyer flask and inoculate with preculture to an OD_{600} of 0.05.
8. Place the expression culture in 37 °C incubator with shaking at 180 rpm and measure OD_{600} at half-hourly intervals.
9. When OD_{600} reaches 0.6–1.0, cool the cultures to below 28 °C, then induce protein expression by addition of 1 mM IPTG.

10. Following induction, continue incubation at 28 °C, with shaking at 180 rpm overnight (18–22 h) prior to harvest.

Day 4: Harvest Cells

11. Place cultures on ice and measure OD_{600}.
12. Transfer the cooled cultures into centrifuge tubes and spin down the cells at 8000 × *g*, at 4 °C, for 20 min. Discard the supernatant.
13. Resuspend the cells in 40 mL of cold PBS and transfer the cells into new tubes.
14. Spin the cells at 4000 × *g* at 4 °C for 15 min. Discard the supernatant.
15. Determine the approximate wet weights of the cell pellets by weighing against an empty tube.
16. Proceed to cell lysis or freeze the pellets in liquid nitrogen and store them at −20 °C.

3.3 Purification of Glycoconjugate AP205 VLPs

Day 5: Cell lysis by sonication and lysozyme treatment

1. Resuspend the cell pellets in lysis solution (approximately 5 mL per 1 g of pellet).
2. Incubate at 37 °C with mixing for 2 h.
3. Cool the lysates on ice.
4. Disrupt the cells by sonication using a 4 mm tip, total process time of 90 s (10 s pulse, 5 s pause), and amplitude 40 (*see* **Note 4**).
   (a) Take a sample of each lysate as whole cell extract (WCE) for denaturing SDS-PAGE evaluation.
5. Pellet unlysed cells and cellular debris by centrifugation at 25,000 × *g* for 30 min, 4 °C.
6. Transfer supernatant to new tube (pellet can be discarded).
   (a) Take a sample of each supernatant as soluble fraction (S) for denaturing SDS-PAGE evaluation.
7. Proceed to protein purification or freeze lysates in liquid nitrogen and store at −20 °C.

Day 6: Protein purification

8. Preequilibrate 5 mL of Strep-Tactin Superflow beads per sample; wash the beads twice with 10 column volumes (CV) of binding buffer.
9. Spin down beads at 300 × *g* for 2 min and discard supernatant.
10. Add the soluble protein lysates to the Strep-Tactin Superflow beads, and incubate at 25 °C for 60 min on a rotator.

11. Load the beads onto fritted filtration columns attached to a vacuum manifold system.
12. Allow the supernatant to pass through the column under gravity.
    (a) Take a sample of the flow through (FT) for denaturing SDS-PAGE evaluation.
13. Wash beads with 20 CV of wash buffer.
    (b) Take a sample of the wash (W) for denaturing SDS-PAGE evaluation.
14. Elute protein with 4 CV of elution buffer under slow gravity flow (*see* **Note 5**).
    (a) Take a sample of the eluate (E) from each strain for denaturing SDS-PAGE evaluation.
15. Record the absorption spectrum from 200 to 350 nm and determine the concentration of AP205 VLPs taking into account the protein and nucleic acid concentration according to the algorithm of Porterfield and Zlotnick [12].
16. Concentrate and buffer exchange the purified proteins into freezing buffer using 100 kDa MWCO centrifugal filters for an approximate final concentration of 1–2 mg/mL (*see* **Note 6**).
17. After protein recovery from concentrators, centrifuge samples at 20,000 × *g* for 15 min to remove aggregates. Discard pellet.
18. Purified protein is stable at 4 °C for at least 6 months or at −80 °C for years.

3.4 Analysis of Purified VLPs

1. AP205 VLPs can be evaluated for particle assembly by native electrophoresis on a 0.6% TAE-agarose gel (*see* **Note 7**). Fully assembled particles are observed as distinct bands which stain for both nucleic acids and protein (Fig. 2a).
2. Particle purity and glycosylation status can be qualitatively evaluated by SDS-PAGE with Coomassie staining (Fig. 2b) (*see* **Notes 8** and **9**).
3. Intact protein mass spectrometry can be used to semiquantitatively evaluate glycosylation of the coat proteins (Fig. 3).
4. Transmission electron microscopy can be used to visualize the particle structure (Fig. 4) (*see* **Note 10**).

4 Notes

1. In the *A. pleuropneumoniae* genome, ORFs encoding the NGT and α6GlcT are arranged sequentially as part of a polycistronic operon. Previous studies have demonstrated that it is possible to amplify these two ORFs and express them in a bicistronic arrangement under the control of a single promoter [4].

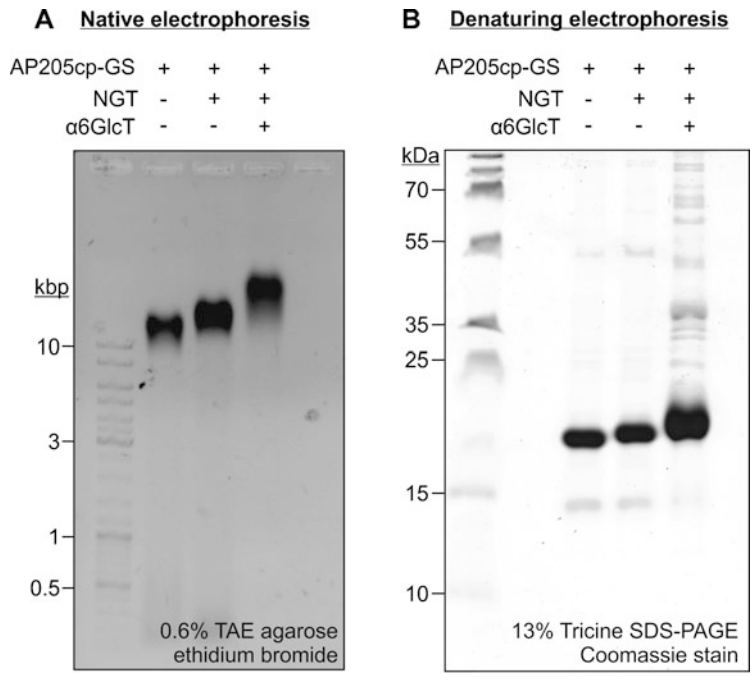

Fig. 2 Native and denaturing electrophoresis of purified VLPs. The AP205cp-GS construct was expressed alone, or in combination with the NGT and/or α6GlcT (as indicated above the gel images), then purified by affinity chromatography. (**a**) 10 μg of each purified protein was loaded on a 0.6% TAE agarose gel, containing 0.5 μg/mL ethidium bromide. Samples were electrophoresed for 40 min at 120 V in a TAE bath. The gel was imaged under UV illumination. Ethidium bromide binds to nucleic acids encapsulated in the VLPs. (**b**) Samples of each purified protein were denatured at 95 °C in reducing Laemmli buffer, then 3 μg of each was separated on 13% tricine SDS-PAGE and proteins were visualized by Coomassie staining

2. We found C-terminal tags with less than a nine-amino acid spacer to be less efficiently glycosylated, because the VLP surface sterically hinders NGT access to the glycosite.
3. The affinity tag, Strep-tag II, is not necessary for glycosylation and may be removed if purification via precipitation or size-exclusion chromatography is desired.
4. Settings apply to Qsonica Sonicators. Alternatively, cells can also be lysed using a French pressure cell press. We observed efficient lysis with three passages through the cell press at 800–1200 PSIG without lysozyme treatment.
5. Allow proteins to unbind from the Strep-Tactin beads with a 10 min incubation in elution buffer before eluting. Yields may be improved by eluting with 1 CV and repeating it four times. Pool the eluate fractions.

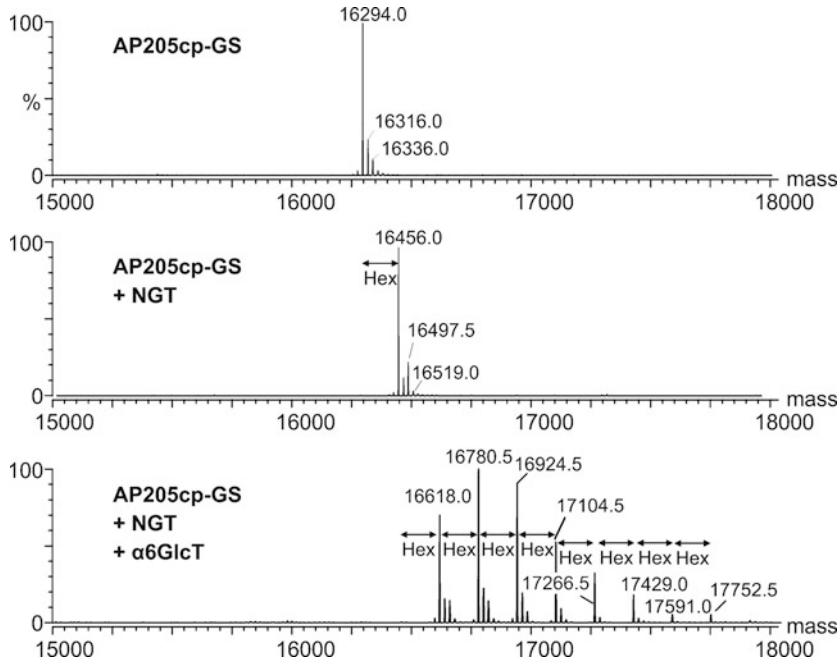

Fig. 3 AP205cp-GS is glycosylated by the NGT and α6GlcT. Purified VLPs were reduced with DTT, desalted using a C4 ZipTip, and analyzed by electrospray-TOF mass spectrometry. Spectra were acquired in positive-ion mode. The *m*/*z* data were deconvoluted into mass spectra with a resolution of 0.5 Da/channel

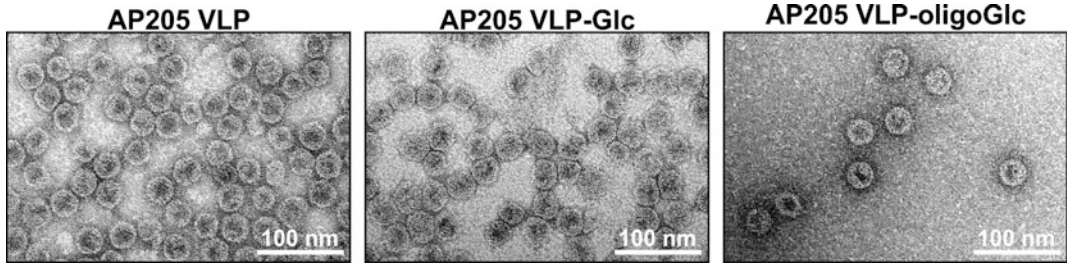

Fig. 4 Glycosylated AP205cp-GS assembles into 30 nm VLPs, as shown after expression without glycosyltransferases (left panel), coexpression with the NGT (middle panel) and with the NGT and α6GlcT (right panel). Purified VLP samples were adsorbed onto carbon-coated copper grids and stained with 2% (w/v) uranyl acetate (pH 4). Grids were examined with a transmission electron microscope fitted with a 100 kV tungsten emitter and 1376 × 1032 pixel CCD detector

6. As a step-wise alternative, one could first buffer exchange by dialysis overnight and concentrate the proteins using 100 kDa MWCO centrifugal filters the next day.
7. AP205 VLPs can be electrophoresed on agarose gels with the same buffers, reagents, and gel documentation system used for nucleic acid analysis. In our experience, separation of 5–10 μg of purified VLP, on a 0.6% agarose gel, with TAE buffer,

supplemented with 0.25 μg/mL ethidium bromide, is ideal for separation and visualization of VLPs. Ethidium bromide labels nucleic acids encapsulated within the VLPs. The protein component of VLPs can subsequently be stained by placing the agarose gel in Colloidal Coomassie Staining solution (*see* **Note 8**).

8. Increased sensitivity and reduced background staining of agarose and polyacrylamide gels, is achieved with the Colloidal Coomassie Staining and Destaining Solutions developed by Kang and colleagues [13].
9. To separate different glycoforms of AP205cp-GS, we recommend 13% tricine SDS-PAGE gels as described by Schägger [14].
10. For best results, 400-mesh carbon-coated copper grids are negatively charged in an oxygen plasma, then placed face-down on a 5 μL droplet of 20 nM VLP for 60 s. Adsorbed particles are stained with 2% (w/v) uranyl acetate (pH 4) for 30 s, then imaged.

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References

1. Ramjeet M, Deslandes V, Goure J, Jacques M (2008) Actinobacillus pleuropneumoniae vaccines: from bacterins to new insights into vaccination strategies. Anim Health Res Rev 9 (1):25–45
2. Choi KJ, Grass S, Paek S, St Geme JW 3rd, Yeo HJ (2010) The Actinobacillus pleuropneumoniae HMW1C-like glycosyltransferase mediates N-linked glycosylation of the Haemophilus influenzae HMW1 adhesin. PLoS One 5(12): e15888
3. Schwarz F, Fan YY, Schubert M, Aebi M (2011) Cytoplasmic N-glycosyltransferase of Actinobacillus pleuropneumoniae is an inverting enzyme and recognizes the NX(S/T) consensus sequence. J Biol Chem 286 (40):35267–35274
4. Cuccui J, Terra VS, Bosse JT, Naegeli A, Abouelhadid S, Li Y, Lin CW, Vohra P, Tucker AW, Rycroft AN, Maskell DJ, Aebi M, Langford PR, Wren BW, Consortium BRT (2017) The N-linking glycosylation system from Actinobacillus pleuropneumoniae is required for adhesion and has potential use in glycoengineering. Open Biol 7(1)
5. Naegeli A, Neupert C, Fan YY, Lin CW, Poljak K, Papini AM, Schwarz F, Aebi M (2014) Molecular analysis of an alternative N-glycosylation machinery by functional transfer from Actinobacillus pleuropneumoniae to Escherichia coli. J Biol Chem 289 (4):2170–2179
6. Keys TG, Wetter M, Hang I, Rutschmann C, Russo S, Mally M, Steffen M, Zuppiger M, Muller F, Schneider J, Faridmoayer A, Lin CW, Aebi M (2017) A biosynthetic route for polysialylating proteins in Escherichia coli. Metab Eng 44:293–301

7. Tissot AC, Renhofa R, Schmitz N, Cielens I, Meijerink E, Ose V, Jennings GT, Saudan P, Pumpens P, Bachmann MF (2010) Versatile virus-like particle carrier for epitope based vaccines. PLoS One 5(3):e9809
8. Shishovs M, Rumnieks J, Diebolder C, Jaudzems K, Andreas LB, Stanek J, Kazaks A, Kotelovica S, Akopjana I, Pintacuda G, Koning RI, Tars K (2016) Structure of AP205 coat protein reveals circular permutation in ssRNA bacteriophages. J Mol Biol 428 (21):4267–4279
9. Brune KD, Leneghan DB, Brian IJ, Ishizuka AS, Bachmann MF, Draper SJ, Biswas S, Howarth M (2016) Plug-and-Display: decoration of virus-like particles via isopeptide bonds for modular immunization. Sci Rep 6:19234
10. Deuschle U, Kammerer W, Gentz R, Bujard H (1986) Promoters of Escherichia coli: a hierarchy of in vivo strength indicates alternate structures. EMBO J 5(11):2987–2994
11. Schmidt TG, Skerra A (2007) The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. Nat Protoc 2(6):1528–1535
12. Porterfield JZ, Zlotnick A (2010) A simple and general method for determining the protein and nucleic acid content of viruses by UV absorbance. Virology 407(2):281–288
13. Kang D, Gho YS, Suh M, Kang C (2002) Highly sensitive and fast protein detection with coomassie brilliant blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bull Kor Chem Soc 23(11):1511–1512
14. Schägger H (2006) Tricine-SDS-PAGE. Nat Protoc 1(1):16–22

Chapter 12

Upstream and Downstream Processes for Viral Nanoplexes as Vaccines

Keven Lothert, Gregor Dekevic, Daniel Loewe, Denise Salzig, Peter Czermak, and Michael W. Wolff

Abstract

The increasing medical interest in viral nanoplexes, such as viruses or virus-like particles used for vaccines, gene therapy products, or oncolytic agents, raises the need for fast and efficient production processes. In general, these processes comprise upstream and downstream processing. For the upstream process, efficiency is mainly characterized by robustly achieving high titer yields, while reducing process times and costs with regard to the cell culture medium, the host cell selection, and the applied process conditions. The downstream part, on the other hand, should effectively remove process-related contaminants, such as host cells/cell debris as well as host cell DNA and proteins, while maintaining product stability and reducing product losses. This chapter outlines a combination of process steps to successfully produce virus particles in the controlled environment of a stirred tank bioreactor, combined with a platform-based purification approach using filtration-based clarification and steric exclusion chromatography. Additionally, suggestions for off-line analytics in terms of virus characterization and quantification as well as for contaminant estimation are provided.

Key words Upstream processing, Downstream processing, Viral nanoplexes, Virus, Virus-like particles, Mammalian cells, Stirred tank bioreactor, Polyethylene glycol, Membrane chromatography, Steric exclusion

1 Introduction

Viruses, membrane fractions of viruses, viral surface proteins, or virus-like particles are frequently used in modern medicine as ingredient in prophylactic vaccines [1–3]. Most recently, viruses have also been used as gene therapy vectors to treat patients with genetic deficiencies or cancer [4, 5]. For the latter, oncolytic viruses which selectively infect and eliminate cancer cells are also used [6–9]. Viruses and virus-like particles are commonly produced in eukaryotic host cells (mammalian, avian- and insect cells)

Keven Lothert and Gregor Dekevic shared first authorship.

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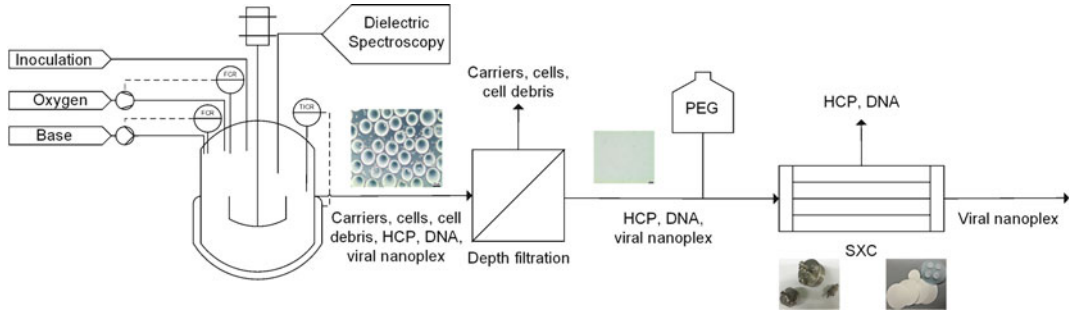

Fig. 1 Overview of the individual steps for the upstream and downstream process. Cultivation and infection of the host cells in a stirred-tank bioreactor, including the monitoring of critical process parameters, such as pH, temperature, dissolved oxygen, and the impedance via the dielectric spectroscopy. The downstream process comprises a primary clarification, to remove larger particles, such as cells and cell debris, and a chromatographic purification step, to remove host cell protein and DNA

[10]. Typical examples for these cells are primary cells, diploid cells, or continuous (transformed) cell lines like: chicken embryo fibroblast cells (CEF), epithelial kidney cells from an African green monkey (Vero cells), Madin-Darby canine kidney cells (MDCK), retina cells from a Muscovy duck (AGE1.CR® cells), transformed cells lines from a human fetus (PER.C6® cells), duck embryonic stem cells (EB66), and human embryonic kidney cells (HEK 293). Each cell line, each virus, and every pharmaceutical preparation requires the specific design of an integrated upstream and downstream process [10]. The target for these processes is to achieve a maximum volumetric viral yield, taking into account the upstream requirements and the resources to be used as well as the efficiency of the subsequent downstream process. For this, the cell culture medium with the required supplements, the production vessels, the cultivation conditions, the infection conditions (time and multiplicity of infection), the time of harvest, as well as the modes of operation have to be selected.

Here, we describe a commonly applicable initial method for the development of a process to produce enveloped virus particles, using eukaryotic host cells cultured under controlled conditions in a stirred tank bioreactor. In addition, we depict a generally employable chromatographic method for nanoplex purification and the critical process parameters for the optimization of the procedures in terms of its economics, safety, and robustness are defined (Fig. 1).

1.1 Upstream Processing of Viral Nanoplexes

In the past, viral nanoplexes have been produced in different host cells and cultured in specific medium adapted to the process requirements as listed in Table 1.

The general trend in biotechnology for cellular production systems moves to the application of suspension cell lines cultivated in fully defined media at high cell densities and to a continuous

Table 1
Culture conditions for selected viral nanoplexes and achieved yields

Virus	Cells	Medium	Cultivation type	Virus titer	References
MVA—Modified vaccinia Ankara virus	AGE1.CR.pIX, suspension	CD-U3 (chem. Def.)	Suspension, 1 L bioreactor,	1.0×10^{10} IU/mL	[11]
Lentivirus	HEK 293 T (transfection)	DMEM	Adherent, 24-well plate	2.0×10^{8} TU/mL	[12]
Influenza virus	AGE1.CR.pIX, suspension	CD-U3 (chem. Def.)	Suspension, 1 L bioreactor	3.8×10^{10} virions/mL	[11, 13]
	MDCK (adherent), MDCK. SUS2 (suspension)	EpiSerf, Smif8	Hollow fiber bioreactor	1.8×10^{10} virions/mL	[13]
MeV—Measles virus	Vero	DMEM-HG	Microcarrier in 0.5 L stirred-tank reactor	$>10^{10}$ $TCID_{50}$/mL	[9, 14]

IU infective units, *TU* transduction units, $TCID_{50}$ tissue culture infective dose

virus production. However, many viruses only replicate to high titers in anchorage-dependent cells [10]. Also, the infection of cells commonly leads to cell lysis during the production process, releasing intracellular components to the supernatant. As these can affect the product enzymatically (proteases, glycosidase, lipases, etc.), the infectivity of viral products might be compromised. Additionally, it has to be considered that the released host cell components increase the load of contaminants, which have to be removed in the subsequent downstream process. This can affect the necessary filtration area, column volumes, buffer consumptions, and the required equipment utilization, impacting the process economics. Furthermore, viral nanoplexes are frequently sensitive to chemical, physical and environmental stress [15]. Particularly, typical cultivation temperatures for the host cells can affect their infectivity. Therefore, the parameters in USP must be well chosen to avoid a damage of the virus. Critical parameters that need to be considered during a viral nanoplex production are, for example, cell concentration at infection (CCI), the time of infection (TOI), the time of harvest (TOH), the multiplicity of infection (MOI), the process temperature, the pH value, as well as the aeration and the shear stress (Table 2).

1.2 Downstream Processing of Viral Nanoplexes

The downstream process includes the clarification for the removal of remaining cells and cell debris, concentration, inactivation if noninfectious virus particles are required, nuclease treatment,

Table 2
Parameters for the optimization of the USP of viral nanoplexes

Parameter	Value	References
Physical-chemical		
T (32 °C to 37 °C)	1 h to 16 h several days	[16–19]
pH	7–9	[20, 21]
Additives (e.g., salt)	Example: $MgSO_4$ Example: $CaCl_2$	[21] [21, 22]
System-related		
Aeration strategy	Continuous aeration (0.02 vvm), head-space aeration	[14, 23],
Shear stress	Peristaltic pump, roller pump, agitator in STR ($\geq$0.25 N/m^2)	[13, 14, 24, 25]
Specific for virus production		
TOI (time of infection)	After completion of cell attachment and spreading to the microcarrier	[9]
TOH (time of harvest)	~40 h after the global permittivity maximum of dielectric spectroscopy	[9]
MOI (multiplicity of infection)	Typically 0.001–10	[9, 26, 27]

purification, polishing, and sterile filtration (depending on the virus size) [3, 28].

The cell culture-derived supernatant is a highly complex suspension comprising the target nanoplexes as well as the contaminants, such as cells, cell debris, host-cell DNA and proteins, and medium components. In consequence, the downstream process must ensure, according to the regulatory guidelines, a sufficient nanoplex purity and concentration, for which, generally, process trains of different unit operations have to be established. For this purpose a variety of methods and techniques are currently applied along the lines of the general purification schemes for biotechnological products, that is, clarification, concentration, intermediate purification, and polishing [3, 28]. These methods include, among others, centrifugation, micro- and ultrafiltration for clarification, and concentration, as a first process step. For final purification and polishing, chromatography is usually the method of choice [3, 29–31]. It is worth to note, that for the chromatography of viral nanoplexes, such as vaccines and viral vectors, membrane-based systems offer certain advantages in contrast to resins, due to their convective flow properties [5, 32]. This eliminates the limitations of pore diffusion and pore exclusion resulting in a reduced pressure

Table 3
Critical process parameters influencing the SXC performance

Critical process parameter	Recommended	Notes
Polymer	Polyethylene glycol	Other crowding agents, for example, dextranes are feasible.
Polymer concentration	6–12%	For virus particles, higher concentrations could induce precipitation [58].
PEG molecular weight	4000–12,000 g/mol	Too large polymers might cause solubility problems and increase viscosity, thus limiting the flow
pH during loading and washing	pH 5–9	SXC works best close to the isoelectric point of the nanoplex [44], however, virus stability must be maintained.
Salt amount during elution	0–1 M	Salts reduce the retention, but should not affect virus stability or infectivity [44].
Membrane composition	Regenerated cellulose	Other hydrophilic membranes with varying pore sizes have been applied successfully [58].
Number of membrane layers	10–15	To increase membrane's capacity, the diameter should be adjusted.

drop, possible higher flow rates, and improved binding capacities. For the membrane chromatography-based purification of viruses and virus particles, mainly ion exchange membranes [33–35] but also affinity [36] and pseudoaffinity [30, 31, 37–40] matrices have been applied [41]. For a broad applicability and a robust process performance, platform technologies largely independent on specific surface properties of the product should be selected. Virtually all viral nanoplexes can easily be distinguished from contaminating process components by their size. In literature, two chromatographic methods are described, that build upon this principle: the size exclusion chromatography (SEC) [42, 43] and the steric exclusion chromatography (SXC) [44]. The drawback of the SEC is the high dilution of the product and the low column capacities. The SXC enables a circumvention of these drawbacks. Therefore, and due to its general applicability for nanoplexes, this method was chosen to be described in this chapter (Table 3). However, depending on the intended product application and purity requirements, additional unit operations might be necessary. The SXC employs the mutual spatial exclusion of molecules in a solution of polyethylene glycol (PEG). This mechanism is shown in Fig. 2. In brief, a crude cell culture supernatant is mixed with a polymer-rich solution and, afterward, applied to a hydrophilic stationary phase. Depending on the size and concentration of the PEG, polymer-deficient zones develop on the surface of the nanoplexes in solution and on the stationary phase. These areas are not accessible for the PEG,

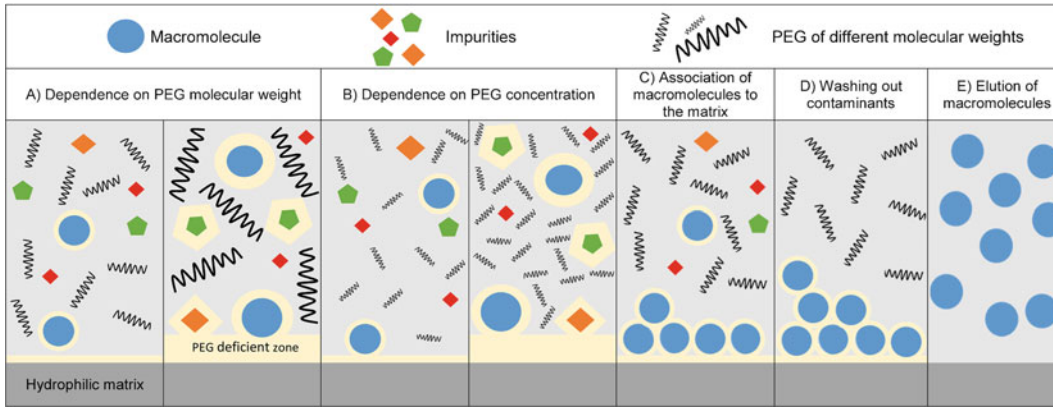

Fig. 2 Principle of steric exclusion chromatography using a hydrophilic stationary phase and polyethylene glycol (PEG) as a crowding agent. After PEG addition, zones with lower (PEG deficient zones) and higher (bulk solution) PEG concentration are formed around macromolecules in solution and on the stationary phase. Depending on the molecular weight and concentration of the PEG, these zones may also include smaller impurities. (**a**, **b**) By adjusting and maintaining the desired PEG concentration, macromolecules associate to each other and to the stationary phase, thus reducing the surface between areas of different PEG concentrations and the system's free energy. (**c**) Unaffected molecules, such as smaller impurities, are washed out. (**d**) Elution is achieved by removing the PEG from the system and subsequently dissociating the retained particles (**e**)

due to its hydrodynamic radius, resulting in a thermodynamically instable system. Due to the association of the nanoplexes with one another and to the stationary phase, the surface between PEG-rich and PEG-deficient zones as well as the systems' free energy is reduced. Under these conditions, a retention of excluded nanoplexes is possible, whereas smaller process contaminants, such as DNA and proteins, can be removed. The reduction of the PEG concentration in the mobile phase releases the associated nanoplexes and allows an elution. The whole method is based on molecular crowding mechanisms, which have already been described in the last century [45–48]. Later on, the principle was used in many applications, for example for the precipitation of proteins [49–51], viruses [52], and extracellular vesicles [53], only to name a few. During SXC, precipitation is an unwanted effect, thus, in general, lower polymer concentrations are used. The application of the method has already been described for large proteins and bacteriophages using cryogel OH-monoliths and starch-coated magnetic nanoparticles [44, 54–56]. The method's specificity is mainly dependent on the size of the target molecule, making it a convenient alternative to purify nanoplexes, such as virus particles and extracellular vesicles. The applicability for viruses has already been shown for Influenza A virus particles employing regenerated cellulose membranes with recoveries above 99% [57], and for baculovirus purifications with an average yield of 91% [58].

Table 4
Analytical techniques for the evaluation of product properties and purity

Determination of	Method (-s)	References	Notes
Particle size distribution	Resistive pulse sensing (Dynamic) light scattering Nanoparticle tracking analysis Transmission electron microscopy	[61, 62] [63–65] [66–68] [69–71]	+ Information on physical characteristics and particle concentration – No information on infectivity or antigens
Antigens	Enzyme-linked immunosorbents assay (ELISA)	[72–74]	+ Antigen concentration – No information on infectivity
Genotype	Quantitative (real-time) polymerase chain reaction (qPCR)	[75, 76]	– No information on infectivity – Knowledge of RNA/DNA sequence is required for primer selection
Infectivity	Plaque assay Tissue culture infection dose assay Flow cytometric titration	[77–79] [14, 80] [81, 82]	+ Knowledge of infective particle concentration – Long incubation times (depending on the assay)
Host cell DNA	Quant-iT™ PicoGreen™ DNA assay Threshold assay qPCR	[37, 38, 40] [37, 38] [83]	– Distinction between viral and host cell DNA only possible using qPCR
Host cell proteins	Bradford assay Bicinchonic acid (BCA) assay	[84, 85] [85]	– No distinction between viral and host cell protein

1.2.1 Evaluation of the Purification Process Performance

In order to ensure the required product quality, appropriate analytical methods have to be established. Depending on the target nanoplex, the process-related contaminants and the intended application, there is a broad range of possible assays available. Parameters to be evaluated include nanoplex concentration, morphological appearance and aggregation as well as antigen presence and infectivity (Table 4). Furthermore, contaminants, such as host-cell DNA and protein levels, need to be monitored, according to the regulatory demands for the product. For instance, cell culture-derived vaccines should contain final levels of less than 100 μg total protein per strain and dose, and less than 10 ng DNA per dose [3, 59, 60]. Additionally, DNA fragments need to be smaller than 200 base pairs, often resulting in the need for an additional nuclease treatment. Table 4 lists possible approaches to evaluate product properties and impurity concentrations. Due to the vast amount of available applications, this overview is not exhaustive.

2 Materials

All materials should be understood as examples. Equipment of similar function, and reagents with an appropriate purity of a different manufacturer might be used as well.

2.1 Upstream Processing Based on an Example of Vero Cells

2.1.1 Thawing Cells

1. Cells (Vero cells in defined low passage number, # CCL-81, ATCC, Manassas, VA, USA) in cryovial.
2. Water bath (37 °C) (WNB 22, Memmert GmbH + Co. KG, Schwabach, Germany).
3. Growth medium DMEM-HG (Biochrom, Berlin, Germany) (*see* **Note 1**).
4. 10 mM HEPES buffer (Biochrom, Berlin, Germany).
5. 4 mM L-glutamine (Biochrom, Berlin, Germany).
6. 70% (v/v) ethanol (AppliChem GmbH, Darmstadt, Germany).
7. Sterile serological 2 mL pipette (Sarstedt, Nümbrecht, Germany).
8. 15-mL centrifuge tube (Sarstedt, Nümbrecht, Germany).
9. Centrifuge (Heraeus Megafuge X1R, Thermo Fisher Scientific, Darmstadt, Germany).
10. T-25 flask (Sarstedt, Nümbrecht, Germany).
11. 37 °C incubator (HeraCell 240, Thermo Fisher Scientific, Waltham, MA, USA).
12. Sterile workbench (NuAire CellGard ES NU-480, MN, USA).

2.1.2 Cell Passaging

1. Deionized water (Milli-Q Academic, Q-Gard® 1, Merck KGaA, Darmstadt, Germany).
2. DMEM-HG (Biochrom, Berlin, Germany).
3. FBS (Biochrom, Berlin, Germany).
4. 10 mM HEPES buffer (Biochrom, Berlin, Germany).
5. 4 mM L-glutamine (Biochrom, Berlin, Germany).
6. 75–175 cm^2 T-flasks (Sarstedt, Nümbrecht, Germany).
7. Phosphate-buffered saline (PBS), w/o Ca^{2+} and Mg^{2+} (#L1825, Biochrom, Berlin, Germany) (PBS composition: NaCl—8000, KCl—200, Na_2HPO_4—1150, KH_2PO_4—200, $MgCl_2$-$6H_2O$—100, $CaCl_2$—100, all values in mg/L).
8. Trypsin (Biochrom, Berlin, Germany).
9. 70% (v/v) ethanol (AppliChem GmbH, Darmstadt, Germany)
10. Sterile serological and aspiration pipettes (Sarstedt, Nümbrecht, Germany).
11. 1.5 mL tubes (Eppendorf, Hamburg, Germany).

12. 15-mL and/or 50-mL centrifuge tube (Sarstedt, Nümbrecht, Germany).
13. Aspiration system (Vacusafe, Integra, Biebertal, Germany).
14. Centrifuge (Heraeus Megafuge X1R, Thermo Fisher Scientific, Darmstadt, Germany).
15. Incubator at 37 °C (HERAcell® 240i CO_2 Incubator, Thermo Fisher Scientific, Waltham, USA).
16. Incident light microscope (DM1i, Leica, Wetzlar, Germany).

2.1.3 Cell Counting and Cell Viability Determination via a Counting Chamber

1. Hemocytometer/counting chamber, Neubauer improved (Marienfeld, Königshofen, Germany).
2. PBS (#L1825, Biochrom, Berlin, Germany).
3. Incident light microscope (DM1i, Leica, Wetzlar, Germany).
4. Pipette and pipette tips (Sarstedt, Nümbrecht, Germany).
5. 0.4% trypan blue solution (Sigma-Aldrich, Hamburg, Germany).

2.1.4 Cell Counting and Cell Viability Determination via Crystal Violet

1. Centrifuge (Heraeus Megafuge X1R, Thermo Fisher Scientific, Darmstadt, Germany).
2. 1.5 mL tubes (Eppendorf, Hamburg, Germany).
3. 0.1% (w/v) crystal violet staining solution (Carl Roth, Karlsruhe, Germany).
4. 0.1 M citric acid (Carl Roth, Karlsruhe, Germany).
5. Hemocytometer/counting chamber, Neubauer improved (Marienfeld, Königshofen, Germany).
6. Incident light microscope (DM1i, Leica, Wetzlar, Germany).

2.1.5 Bioreactor Preparation

1. 1-L glass STR, working volume: 0.5 L (Z611000110; Applikon, Biotechnology, Delft, Netherlands).
2. Software: Process control (BioPAT® MFCS SCADA, Sartorius AG, Göttingen, Germany).
3. Reactor control unit (BIOSTAT® B-DCU Twin, Sartorius Stedim Biotech GmbH, Göttingen, Germany).
4. 3 × 45° pitched-blade impeller, diameter: 4.5 cm (Sartorius Stedim Biotech GmbH, Göttingen, Germany).
5. Sparger for aeration (Sartorius Stedim Biotech GmbH, Göttingen, Germany).
6. Sensors for monitoring:
   (a) pH sensor (Z001023551, Applikon Biotechnology, JG Delft, Netherlands).
   (b) PT100 temperature sensor (Sartorius Stedim Biotech GmbH, Göttingen, Germany).

(c) Oxygen probe (VisiFerm DO, Hamilton, Bonaduz, Switzerland).

(d) Biomass monitoring: online permittivity (ArcView, Hamilton, Hoechst im Odenwald, Germany & Futura, Aber Instruments, Aberystwyth, UK).

7. Water bath at 37 °C (WNB 22, Memmert GmbH + Co. KG, Schwabach, Germany).
8. Centrifuge tubes, 15- and 50-mL (Sarstedt, Nümbrecht, Germany).
9. Silicon tubes 3–5 mm inner diameter (Carl Roth GmbH, Karlsruhe, Germany).
10. Air filters, 0.2 μm poresize (Midisart 2000® PTFE, Sartorius AG, Goettingen, Germany).
11. Glass bottle with connector caps for base, harvest and bottom drain (medium and inoculation).
12. Sterile syringe 10 mL, 20 mL (B. Braun, Melsungen, Germany).
13. 15- and 50-mL centrifugation tubes (Sarstedt, Nümbrecht, Germany).
14. 1.5 and 2 mL reaction tubes (Sarstedt, Nümbrecht, Germany).
15. Male and female luer lock adapter and lids (Sarstedt, Nümbrecht, Germany).
16. Y-tube connector (Bürkle GmbH, Bad Bellingen, Germany).
17. 1 M NaOH (Carl Roth GmbH, Karlsruhe, Germany)
18. Pressurized air, O_2 and CO_2 for aeration and pH control.

2.1.6 Cell Expansion and Nanoplex Production in the STR

1. Preculture of, for example, Vero cells (# CCL-81, ATCC, Manassas, VA, USA).
2. Prewarmed (37 °C) DMEM-HG growth medium, supplemented with 10% (v/v) FBS, 10 mM HEPES, and 4 mM $_L$-glutamine (Biochrom, Berlin, Germany).
3. Sterile bottles (2 × 0.5 L) with silicon tubes and Luer lock adapters.
4. Microcarrier (Cytodex 1, GE Healthcare, Uppsala, Sweden).
5. Infectious recombinant Measles virus strain (e.g., MVvac2 GFP (P): Paul-Ehrlich-Institute, Langen, Germany).
6. 10-mL single-use syringes (B. Braun, Melsungen, Germany).

2.2 Downstream Processing

2.2.1 Harvest of the Viral Nanoplex-Containing Supernatant, and Primary Clarification

1. 1-L glass STR, working volume: 0.5 L (model number Z611000110; Applikon, Biotechnology, Delft, Netherlands)—filled with microcarriers, cell culture medium, Vero cells (grow on microcarrier) and viral nanoplexes in supernatant.
2. SciPress Sensors (Parker Hannifin Manufacturing Ltd., Durham, England).
3. Balance (TE4101, Sartorius Stedim Biotech GmbH, Göttingen, Germany).
4. WinWedge software (Tal Technologies Inc., Philadelphia, PA, USA).
5. Peristaltic pump (Sartorius Stedim Biotech GmbH, Göttingen, Germany) with Tandem 1082 pump head, with a Masterflex Pharmed #15 (Cole Parmer GmbH, Wertheim, Germany) tube and transparent silicon tubes (VWR International, Radnor, PA, USA) with sterile Luer lock connections (Sarstedt, Nümbrecht, Germany).
6. Opticap XL 1 Capsule with Polygard®-CR 5.0 μm (Nominal)—sterile (Merck KGaA, Darmstadt, Germany).
7. 1 L PBS—Sterile (#L1825, Biochrom, Berlin, Germany).
8. Sodium hydroxide (Carl Roth GmbH, Karlsruhe, Germany).
9. Sterile bottles (0.5 L, Schott AG, Mainz, Germany).
10. Sterile workbench (ESCO Infinity Class II, Esco Lifesciences GmbH, Friedberg, Germany).

2.2.2 Preparation of Buffers and Membranes

1. PBS (#L1825, Biochrom, Berlin, Germany).
2. Deionized water (Milli-Q Academic, Q-Gard® 1, Merck KGaA, Darmstadt, Germany).
3. PEG 8000 (biotechnology grade, VWR International, Radnor, PA, USA).
4. Sodium chloride (Sigma-Aldrich, Hamburg, Germany).
5. Sodium hydroxide 1 M NaOH (Carl Roth GmbH, Karlsruhe, Germany).
6. 100-mL, 250-mL, and 1-L glass beakers (Carl Roth GmbH, Karlsruhe, Germany).
7. 100-mL, 250-mL, and 500-mL measuring cylinders (Carl Roth GmbH, Karlsruhe, Germany).
8. Analytical scale (Kern & Sohn GmbH, Balingen-Frommern, Germany).
9. Magnetic stirring bar (Carl Roth GmbH, Karlsruhe, Germany).

10. Thermomixer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany).
11. 0.22 µm bottle-top filters (Corning, Corning, New York, USA).
12. Vacuum pump (#181-0067P, VWR International, Radnor, PA, USA).
13. 500-mL, 250-mL, and 100-mL glass bottles (Carl Roth GmbH, Karlsruhe, Germany).
14. Ultrasonic bath (Ultrasonic cleaner (VWR International, Radnor, PA, USA).
15. Regenerated cellulose membranes, 1 µm pore size (Whatman RC60, 50 mm circles, GE Healthcare Life Sciences, Uppsala, Sweden).
16. 13 mm puncher (hardware store).
17. Rubber hammer (hardware store).
18. Petri dish (between 5 and 10 cm diameter, Sarstedt Nümbrecht, Germany).

2.2.3 Preparation of the Sample

1. Clarified virus harvest (from Subheading 2.2.1).
2. PBS supplemented with 16% (w/v) PEG 8000 (from Subheading 2.2.2).
3. 50-mL centrifuge tubes (Sarstedt, Nümbrecht, Germany).
4. 2-mL reaction tubes (Sarstedt, Nümbrecht, Germany).
5. Sterile single-use pipette tips (Serological pipettes, Sarstedt, Nümbrecht, Germany).
6. Pipette holder/rubber bulb (Pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
7. 10-mL single-use syringes (B. Braun, Melsungen, Germany).
8. Single-use cannulas, blunt with Luer lock attachment (B. Braun, Melsungen, Germany).
9. Luer lock syringe cap male/female (B. Braun, Melsungen, Germany).

2.2.4 Performing the SXC

1. 10-mL superloop (GE Healthcare Life Sciences, Uppsala, Sweden)
2. FPLC system (e.g., Äkta Pure 25; GE Healthcare Life Sciences, Uppsala, Sweden).
3. Buffers prepared in Subheading 2.2.2.
4. 15-mL centrifuge tubes (Sarstedt, Nümbrecht, Germany).
5. Inline filter holder device 13 mm (Pall Life Sciences, Port Washington, NY, USA).

2.2.5 Quantification and Characterization of the Purified Virus Particles by Flow Cytometry

1. 24-well adherent cell culture plate (Nunc™ Thermo Fisher Scientific, Waltham, MA, USA).
2. Host cells, infectable by the virus (Vero cells, ATCC, Manassas, VA, USA).
3. Cell culture medium (DMEN, Sigma-Aldrich, Hamburg, Germany).
4. PBS (Biochrom, Berlin, Germany).
5. Pipette tips 100, 1000 µL (Sarstedt, Nümbrecht, Germany).
6. Piston-operated pipettes 100 and 1000 µL (Eppendorf, Hamburg, Germany).
7. Sterile single-use pipette tips (serological pipettes, Sarstedt, Nümbrecht, Germany).
8. Pipette holder/rubber bulb (Pipetus, Hirschmann Laborgeräte, Eberstadt, Germany).
9. 50-mL centrifuge tubes (Sarstedt, Nümbrecht, Germany).
10. 2-mL reaction tubes (Sarstedt, Nümbrecht, Germany).
11. Virus standard at 1E+8 infective particles (in-house standard).
12. CO_2-incubator (HeraCell 240, Thermo Fisher Scientific, Waltham, MA, USA).
13. Trypsin–EDTA solution (0.25%/0.02%, Merck, Darmstadt, Germany).
14. Fetal calf serum (FCS) (Merck, Darmstadt, Germany).
15. 96-well U-bottom plate (Nunc™ Thermo Fisher Scientific, Waltham, MA, USA).
16. Centrifuge with plate rotor (Multifuge 3L-R, Heraeus, Hanau, Germany).
17. Flow cytometer (Guava® easyCyte, Millipore Merck, Darmstadt, Germany).
18. Deionized water (Milli-Q Academic, Q-Gard® 1, Merck KGaA, Darmstadt, Germany).
19. Guava instrument cleaning fluid (ICF, Merck, Darmstadt, Germany).
20. 0.5-mL and 1.5-mL tubes for waste, water and ICF (Sarstedt, Nümbrecht, Germany).
21. Zetasizer Nano ZS90 (Malvern Panalytical, Malvern, United Kingdom).
22. Semi-micro cuvettes, polystyrol (Sarstedt, Nümbrecht, Germany).
23. PBS (Biochrom, Berlin, Germany).

2.2.6 Quantification of the Total Protein and DNA (Process Contaminant Estimation)

1. 50-mL centrifuge tubes (Sarstedt, Nümbrecht, Germany).
2. 2-mL reaction tubes (Sarstedt, Nümbrecht, Germany).
3. Pipette tips 10, 100, 1000 µL (Sarstedt, Nümbrecht, Germany).
4. Piston-operated pipettes 10, 100, and 1000 µL (Eppendorf, Hamburg, Germany).
5. Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).
6. PBS (Biochrom, Berlin, Germany).
7. 96-well flat bottom microplate without surface modifications (Nunc™ Thermo Fisher Scientific, Waltham, MA, USA).
8. Incubator (Binder, Tuttlingen, Germany).
9. Plate reader (BioTek™ Cytation™ 3, BioTek, Winooski, VT, USA).
10. Deionized water (Milli-Q Academic, Q-Gard® 1, Merck KGaA, Darmstadt, Germany).
11. Quant-iT™ PicoGreen® dsDNA kit (Thermo Fisher Scientific, Waltham, MA, USA).
12. Black 96-well, flat bottom microplate (Corning, Corning, New York, USA).

3 Methods

All methods described using the equipment and reagents stated above. Application of comparable devices and chemicals from alternative different manufacturers is possible.

3.1 Upstream Processing Based on an Example of Vero Cells

3.1.1 Thawing Cells

1. Prepare prewarmed (37 °C) DMEM-HG growth medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mM HEPES, and 4 mM L-glutamine.
2. Thaw cells in the cryovial rapidly in a 37 °C water bath (*see* **Note 2**).
3. Sterilize the cryovial with 70% (v/v) ethanol and work under aseptic conditions from this point on.
4. Using a 2 mL pipette, transfer 1 mL prewarmed complete growth medium into the cryovial and gently mix the cells thoroughly by pipetting up and down.
5. Transfer the 2 mL cell suspension into 9 mL prewarmed complete growth medium in a 15 mL centrifuge tube.
6. Centrifuge the cells at 300 × *g*, 5 min, RT.
7. Remove and discard the supernatant (residual DMSO).

8. Resuspend the cell pellet in the prewarmed complete growth medium.
9. Transfer the cell suspension into a 75 cm^2 T-flask. The preferred cell density is $5 \times 10^3/cm^2$.
10. Place the T-flask into an 37 °C incubator and grow the cells to 80–90% confluency before passaging.

3.1.2 Cell Passaging

Subculture cells when confluency reaches 80–90% respectively at the log phase, or 2–3 per week. Work under aseptic conditions.

1. Prepare prewarmed (37 °C) DMEM-HG growth medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mM HEPES and 4 mM $_L$-glutamine.
2. Cool PBS (w/o Ca^{2+} and Mg^{2+}).
3. Remove and discard the used medium.
4. Wash cells 2× with 0.3 mL/cm^2 PBS.
5. Detach cells with 0.012 mL/cm^2 0.25% (w/v) trypsin at 37 °C for 8 min. Verify the detachment of the cells with an incident light microscope (*see* **Note 3**).
6. Resuspend the detached cells in 0.12 mL/cm^2 complete growth medium (*see* **Note 4**).
7. Take a 100 µL cell-sample in a 1.5 mL tube and determine the cell concentration (*see* Subheading 3.1.3).
8. Centrifuge cell suspension at 300 × *g*, 5 min, RT in a 15 or 50 mL centrifugation tube.
9. Remove and discard the supernatant (residual trypsin).
10. Resuspend the pellet in the appropriate volume of fresh prewarmed complete growth medium.
11. Seed out cells in an initial cell concentration of 5×10^3 cell/cm^2 into 75–175 cm^2 T-flask.
12. Incubate cells in an incubator at 37 °C.

3.1.3 Cell Counting and Cell Viability Determination via a Counting Chamber

1. Use manufacturer's instruction to prepare the Neubauer counting chamber.
2. Take a 200 µL sample from your culture in a 1.5 mL tube.
3. Prepare an appropriate dilution of the cell suspension with PBS to obtain a final cell count of approx. 150 cells.
4. Mix 50 µL of cell suspension with 50 µL trypan blue 0.5% solution. Wear protective gloves, clothing and eye and face protection when working with trypan blue.
5. Rapidly transfer the stained cells to the prepared Neubauer counting chamber and count the cells using the inverse light microscope.

6. Calculate the cell concentration and viability according the manufacturer's instructions. Dispose trypan blue-contaminated equipment according to local, state and federal regulations.

3.1.4 Cell Counting and Cell Viability Determination via Crystal Violet

The cell numbers attached on the microcarrier are determined by the counting of nuclei [86].

1. Centrifuge 1 mL of microcarrier cell suspension sample at 300 × *g* for 5 min, RT.
2. Discard 0.9 mL of the supernatant.
3. Incubate the pellet with 0.9 mL 0.1% crystal violet in 0.1 M citric acid at RT for at least 24 h (alternatively: 30 min, 37 °C, 500 rpm in a thermomixer).
4. Determine the dyed nuclei by means of the Neubauer counting chamber under the incident light microscope.
5. Calculate the cell density according to the manufacturer's instruction of the Neubauer counting chamber.

3.1.5 Bioreactor Preparation

1. Install and connect the following tubes and probes to the STR: (*see* **Note 5**).
   (a) pH probe: calibrate (before autoclaving) according to the manufacturer's instructions.
   (b) pO_2 sensor: calibrate (after medium fill-up) according to the manufacturer's instructions.
   (c) Dielectric spectroscopy: connect to amplifier and signal transformation box.
   (d) Temperature sensor.
   (e) Exhaust condenser including sterile filter.
   (f) Connect the sterile air filter to the gas inlets.
   (g) Acid and base tubes.
   (h) Tubes for sampling.
   (i) $_{L}$-sparger for oxygen supply.
2. Calibrate the pumps for acid and base, if necessary, according to the manufacturer's instructions.
3. Cover all probes and plastic parts (e.g., sterile air filter) with aluminum foil.
4. Autoclave the STR (*see* **Note 6**) and prepared bottles (1 × medium, 1 × inoculation, 1 × base).
5. Let the STR cool down to RT after autoclaving (*see* **Note 7**).
6. Fill the medium into the reactor under the sterile working bench:
   (a) Prepare a sterile medium bottle with a bottom drain tubing with Luer lock lids.

(b) Use a peristaltic pump to fill the medium into the STR (*see* **Note 8**).

7. Fill up the thermal jacket with VE water and start heating the medium to 37 °C via the external loop (*see* **Note 9**).
8. Start stirring at 110 rpm.
9. Fill up the base tube (dead volume) manually with 1 M NaOH (*see* **Note 10**) and start pH regulation.

3.1.6 Cell Expansion in the STR

Microcarrier (e.g., Cytodex 1) may provide the growth surface required for the adherently growing cells in the STR.

1. Prepare and sterilize the microcarrier Cytodex 1 (3 g/L) (GE Healthcare, Uppsala, Sweden) according to the manufacturer's recommendations.
2. Split the DMEM medium:
   (a) for conditioning in the bioreactor vessel (350 mL),
   (b) for cell inoculation (150 mL).
3. Harvest cells from T-175 flask as described in Subheading 3.1.2.
4. Inoculate at the following cell density: 5×10^3 cell/cm^2.
5. Allow cells to adhere to the microcarrier (4–7 h) at 70 rpm constant agitation rate.

3.1.7 Nanoplex Production in the STR

1. A stable conductivity signal from the dielectric spectroscopy indicates the time point at which the cells (e.g., Vero) completed their attachment and spreading on the microcarrier. This represents the initial time point of infection.
2. To ensure a single infection cycle, infect the cells with a high MOI: ~30 $TCID_{50}$/cell. Lower MOIs may lead to several infection cycles (*see* **Note 11**).
3. Optimal TOH is reached ~40 h after a global maximum of the permittivity signal, which represents the detachment of the cells from the microcarrier, rather than a maximum of syncytial abundance. Stop the reactor run and start harvesting.

3.2 Downstream Processing

3.2.1 Harvest of the Viral Nanoplex-Containing Supernatant and Primary Clarification

Before starting to harvest, make sure you have prepared the following **steps 1–10** to have the clarification equipment ready at the point of harvest (**step 11**).

1. Connect the tubes to the Schott flasks (with outlet for the feed; min. Volume 1 L) and place the depth filter capsule with the correct orientation (flow direction is indicated on the capsule) into the closed system.
2. Steam-sterilize the capsule, tubes, flasks and liquids at 121 °C for 20 min (autoclaving).

3. Place pressure sensors under the sterile workbench and rinse the flow channel of the sensors with 1 M NaOH (e.g., 3 × 10 mL with a serological pipette). Rinse the flow channel with 20 mM Tris buffer (pH 7.4) afterward.
4. Set up equipment under a sterile workbench.
   (a) Connect pump and balance to the power outlet of the bench.
   (b) Place pump tubing into the pump head.
   (c) Clamp the depth filter capsule to a tripod rod with flow direction from top to bottom.
5. Clamp one pressure sensor in front of the entrance and one behind the outlet of the capsule.
6. Connect balance and pressure sensors to the ports of the peristaltic pump. Additionally, connect the pump to a PC with the appropriate software (WinWedge and Microsoft Excel).
7. Place the permeate flask on the balance and set the pressure sensors equal to zero.
8. Equilibrate the capsule with at least 1 L of 20 mM TRIS buffer (pH 7.4) and fill the buffer into the feed vessel.
9. Start the pump (filtration rate of 150 mL/min), monitor the pressure, and record the permeate weight.
10. Discard the permeated buffer.
11. Connect a flask (e.g., 1 L) sterilely to the sampling tube of the bioreactor and transfer the content of the bioreactor to the flask, by closing the exhaust air connection with a clamp and the activation of the aeration of the bioreactor (pneumatic conveyance). Important: The pressure equalization must be guaranteed in the flask (*see* **Note 12**).
12. Disconnect the flask (sterilely) from the bioreactor and connect it to the filtration system inside the clean bench.
13. Switch on the pump with a flow rate of 150 mL/min to pump the cell culture supernatant through the capsule.
14. Keep the filtrate in sterile bottles/tubes and store it intermediately at −80 °C, or proceed directly with the chromatographic purification (*see* **Note 13**).

3.2.2 Preparation of Buffers and Membranes

1. Prepare ca. 500 mL PBS solution with 8% (w/v) PEG 8000 to be used as the running buffer A. Dissolve the PEG using the magnetic stirring thermomix device at 30–40 °C and gently mix (250 U/min) (*see* **Notes 14** and **15**).
2. Prepare ca. 50 mL PBS solution with 16% (w/v) PEG 8000 to be used as the sample dilution buffer.

3. Prepare ca. 200 mL of a PBS solution supplemented with 0.5 M NaCl for use as the running buffer B.
4. Prepare ca. 500 mL of deionized water.
5. Prepare 500 mL of 0.5 M NaOH and 1 M NaCl in water for use as the cleaning buffer.
6. Filter all buffers using the 0.22 μm bottletop filter connected to a vacuum pump into fresh glass bottles.
7. Degas all buffers by placing the bottles in an ultrasonic bath for at least 10 min with the cap loosely placed on top (*see* **Note 16**).
8. Prepare membranes of 13 mm diameter to be used as stationary phase as follows:
   (a) Use 13 mm puncher and rubber hammer to punch discs of appropriate size from the 50 mm cellulose sheets.
   (b) Prepare about 12 discs per chromatographic run.
   (c) Place the punched discs into a petri dish filled with PBS until use (*see* **Notes 17** and **18**).

3.2.3 Preparation of the Chromatographic Feed

Depending on the volume to be purified, slightly different parameters have to be applied. In the following, the method is described for an initial method testing. Further information on a potential scale-up are found in the respective notes. Always prepare the sample directly before performing the SXC. Longer storage times of the sample–PEG mixture might change the sample composition due to an enhanced virus aggregation.

1. Add 6 mL of the clarified virus harvest to a fresh tube.
2. Let 6 mL of the 16% PEG solution slowly run in PBS while gently shaking the tube (*see* **Note 19**).
3. Soak up 10 mL of the sample directly into a syringe using a blunt cannula (*see* **Note 20**).
4. Remove and discard the cannula and close the syringe using a Luer-lock syringe cap (*see* **Notes 21** and **22**).

3.2.4 Steric Exclusion Chromatography

1. Assemble the filter device according to the respective manufacturer's instructions. Usually, the set-up is as follows from bottom to top: lower part of the filter holder, sealing ring, support screen, sealing ring, upper part of the filter holder (*see* **Notes 23** and **24**).
2. Place ten layers of the cellulose membranes on top of the support screen and top them with the sealing ring (*see* **Note 25**).
3. Close the filter holder device and insert it into the flow path at the usual column position (*see* **Note 26**).

4. Flush the system and the membrane stack with the running Buffer A for at least 60 column volumes (*see* **Note 27**).
5. Load the sample from the syringe to the 10 mL sample loop (*see* **Note 28**).
6. Perform the actual SXC runs comprising the following steps:
   (a) Equilibration with at least 5 mL of buffer A (*see* **Note 29**).
   (b) Sample application using the sample loop (*see* **Note 30**).
   (c) Washing with at least 5 mL buffer A (*see* **Note 31**).
   (d) Elution with 5–10 mL elution buffer B (*see* **Notes 32–34**).
7. Fractionate the entire volumes for **step 6b–d** for off-line analytics.
8. Clean the system using cleaning buffer and flush system afterward with water.
9. (a) Start next SXC purification *or*
   (b) Fill system with 20% ethanol in water for storage.
10. Discard the used membranes and store the virus samples at −80 °C until performing the offline analytics (*see* **Notes 35–39**).

3.2.5 Quantification of the Purified Virus Particles by Flow Cytometry

1. Prepare standard calibration range by diluting the virus stock 7 times with subsequent 1:2 dilutions in cell culture medium (e.g., use 350 µL medium +350 µL preceding virus standard) (*see* **Notes 40** and **41**).
2. Seed host cells at 1E+07 cells mL with 1 mL per well into the 24-well plate. For each standard and sample prepare three wells of cells (*see* **Note 42**).
3. Infect the seeded cells directly with 100 µL of the virus suspension (100 µL/well for standards and samples).
4. Incubate at 37 °C and 8% CO_2 for 16 h.
5. Discard the supernatant and wash all wells twice with 1 mL PBS.
6. Add 200 µL of trypsin–EDTA solution and allow incubation for 3–5 min at 37 °C.
7. Add 50 µL FCS.
8. Carefully resuspend the cells and transfer the content of each well into a new well on the 96-well U-bottom plate.
9. Centrifuge the plate for 5 min at 500 × *g*.
10. Discard the supernatant and resuspend cells in 100 µL PBS (*see* **Notes 43** and **44**).
11. Measure the samples in the flow cytometer as follows.

(a) Adjust settings and gates by using blank and highest standards to optimally differentiate between infected (fluorescing) and noninfected cells.

(b) Plot the amount of fluorescing cells against the virus concentration and calculate sample concentration from the linear range of the curve.

(c) Determine the total infective virus content in each fraction using the sample volume of the SXC.

(d) Calculate the recovery in % by comparing the total infective virus amount in the individual fractions with the feed solution (*see* **Note 45**).

3.2.6 Size Determination Using Dynamic Light Scattering

1. Transfer 500 μL of the samples into individual semi-micro cuvettes (*see* **Note 46**).
2. Start a new manual measurement in the Zetasizer Nano ZS90 software with the following conditions:
   (a) Dispersant refractive index: 1.45.
   (b) Viscosity of the dispersant: 0.954 cP.
   (c) 90° angle
   (d) Number of measurements: 3.
   (e) Data processing: multiple narrow modes.
3. Afterward, check the detected size populations and record the mean values and standard deviations.
4. If necessary, prepare different sample dilutions in PBS to exclude buffer effects, and repeat the measurement (*see* **Note 47**).

3.2.7 Quantification of Total Protein Amounts

1. Prepare the standard calibration samples according to the manufacturer's instructions (*see* **Note 48**).
2. Mix working reagent A and B in the ratio 50:1 (A:B) in a fresh 50-ml centrifuge tube. Use that solution within 1 h.
3. Transfer 25 μL of each sample and standard into a clear 96-well flat bottom microplate using two wells per sample (duplicate measurements).
4. Add 200 mL of the reaction mix to each well, gently shake plate, and cover it with a lid.
5. Incubate the plate at 37 °C for 30 min (*see* **Notes 49** and **50**).
6. Use a plate reader to detect the absorbance at 562 nm.
7. Calculate the mean for each duplicate measurement after blank subtraction.
8. Prepare the standard calibration curve by plotting the blanked absorbance versus the protein concentration, and use it to calculate the concentration of your samples (*see* **Notes 51**–**53**).

9. Determine the total protein content in each fraction using the sample volume, and calculate the recovery in % by comparing the total protein amount in the individual fractions with the total protein amount in the feed solution.

3.2.8 Determination of the Total dsDNA Amount

1. Prepare 1× TE-buffer in a 50-mL reaction tube, by diluting the kit-contained 20× buffer with deionized water. For the above performed chromatographic run with four samples, about 12 mL should be prepared.
2. Prepare the standard calibration samples using the lambda-DNA stock solution contained in the kit, as described in the instructions.
3. Dilute the Quant-iT™ PicoGreen® dsDNA reagent 1:200 with 1× TE-buffer in a fresh 50-mL reaction tube. Prepare enough reagent to apply 100 µL of the mix per well, and consume within 1 h.
4. Pipet 100 µL of each standard and blank (duplicates) into a black 96-well microplate.
5. Transfer 20 µL of each chromatographic sample in duplicates (*see* **Note 54**).
6. Add 80 µL 1× TE buffer to each 20 µL sample.
7. Add 100 µL working reagent to all wells (standards, blanks and samples).
8. Shake the plate gently and let it incubate in the dark for 5 min.
9. Measure the emission at 520 nm after fluorescence excitation at 480 nm in a plate reader.
10. Prepare the standard calibration curve and calculate DNA concentrations and recoveries as described in Subheading 3.2.7 for the total protein amount (*see* **Note 55**).

4 Notes

Upstream processing

Thawing cells

1. Use up to 10% FBS (Biochrom, Berlin, Germany).
2. Ensure the thread of the cryovial is above the warm water when thawing the cells to avoid a contamination risk.

Cell passaging

3. Apply the shake-off technique, if cells don't detach. Use the heel of your hand to gently knock via the side of the T-flask, until the cells detach. Check the detachment under the light microscope.

4. FBS in the medium neutralizes/inhibits the trypsin's activity. It is crucial to remove the remaining trypsin from the cells.

Bioreactor preparation

5. The tips of electrodes (e.g., oxygen electrode) may have a concave form which can lead to the entrapment of air/oxygen bubbles. Make sure, that the tip of the electrode is installed below the stirred bubbles in the bioreactor.

   All sampling tubes are to be connected with Luer lock adapters, including all bottles (acid, base, medium, inoculum, etc.).

6. (a) Do not autoclave the amplifier of the dielectric spectroscopy.
   (b) Open tubing clamps during autoclave so that the hot steam reaches all surfaces.
   (c) Add 10–20 mL PBS into the 1 L bioreactor to prevent a drying-out of the pH electrode.
7. Do not autoclave the medium, or any of the medium supplements, as essential components will denature irreversibly due to heat and pressure exposure.
8. Fill up the medium under the clean bench to avoid the risk of contamination.
9. Set the maximum heating temperature in the thermal loop to 39–40 °C to avoid a denaturation of medium components (e.g., proteins) by overheating when warming up the medium to a temperature of 37 °C.
10. Prepare a 1 M NaOH solution, and sterilize it with a 0.2 μm filter. Use a sterile filter in the lid, and attach silicon tubes. Set up the silicon tube end with a Luer lock adapter to connect these to the bioreactor.

Nanoplex production in the STR

11. Start the cell infection after the cells have completed their attachment to the microcarrier.

Downstream processing
Initial sample clarification

12. An immediate clarification of the complete harvest is highly recommended. A storage of the harvest can lead to an additional cell disruption, releasing enzymes that modify the target an increase the product contact time to these enzymes. This can potentially affect virus infectivity and stability.
13. Membrane-based filtration (depth and tangential flow filtration) is the method of choice for the clarification of larger volumes. However, for small scale samples, centrifugation might be considered.

Preparation of buffers and membranes

14. Instead of using a PBS buffer, theoretically any buffer of choice may be used depending on the requirements of the virus to be produced. Be aware of the buffer osmolarity and ionic strength to avoid virus aggregation.
15. PEG can be dissolved by slightly heating up the solution. Overheating must be avoided.
16. All solutions and buffers need to be filtered and degassed, in order to increase the shelf life of the buffers and to prevent the gassing-out of the liquids during the chromatographic run.
17. Cellulose membranes can be autoclaved in the respective buffer without PEG, shortening the swelling process of the cellulose membranes and, thus, improving the process robustness.
18. Store unused membranes in 20% ethanol and at 4 °C.

Preparation of the sample

19. In order to prevent virus dilution during sample preparation, buffer stocks containing elevated PEG concentrations of up to 32% PEG 8000 are recommended.
20. When preparing the sample to be loaded onto the chromatography column, the sample volume for the off-line analytics must be considered.
21. Sample preparation should be done under slight constant stirring in an appropriate beaker, and for larger scale processes an inline preparation is recommended.
22. The PEG concentration and the PEG molecular weight must be adjusted individually for each virus. As an initial condition, 8% PEG 8000 are recommended to test the principal applicability of the method. However, for an optimal chromatographic process, the critical process parameters must be optimized, preferably by a Design-of-Experiment (DoE) approach. In general, it can be said, that the smaller the nanoplexes to be purified are, the higher is the required PEG concentration, or PEG molecular weight. PEG concentrations above 14% should not be used, in order to prevent the risk of virus precipitation.

Performing the SXC

23. The minimum requirements for the chromatographic system are two independent pumps and a UV_{280}-detector. It is even possible, to load the sample and sequentially push the appropriate buffers through the filter holder by a syringe, and to collect the product in the final elution step. However, for a better process control, the system pressure and the conductivity should be monitored. An additional light scattering detection increases the detection possibilities of nanoplexes significantly

and allows a differentiation of the contaminating proteins and virus particles. Furthermore, the system should contain a fraction collector, to allow a robust and reproducible collection of the chromatographic fractions.

24. Depending on the batch size of the virus to be purified, larger membrane holder modules can be used to scale up the membrane surface area (or volume) in relation to the viral load. For an initial method development and optimization, a 13 mm device will be sufficient.
25. Setting up the membrane stack within the membrane holder, use forceps with rounded (not sharp!) edges and avoid air bubbles between the individual layers.
26. If necessary, a layer of PTFE-band can be used to properly seal the filter holder.
27. The chromatography system is commonly stored in 20% ethanol solution. Before flushing the system with buffer, the system needs to be rinsed with water to remove the ethanol.
28. Loading the sample loop by a syringe can be challenging for larger loop sample volumes. Thus, it might be easier to directly prefill the loop under the bench for sample volumes between 50 and 150 mL. For sample volumes above 150 mL, an external sample pump should be used.
29. Prior to the loading of the virus particles, the system needs to be equilibrated with at least ten column volumes, until all relevant signals are constant.
30. Directly before the sample is loaded onto the column, the UV_{280}-detector and the light scattering detector must be set to zero.
31. After sample loading, the subsequent washing step should be completed after five to ten column volumes, or once the detector signals reach the baseline.
32. Priming the system tubing with the elution buffer, allows for a faster elution and higher concentrated product fraction.
33. Elution is usually completed after ten column volumes. However, for small column volumes, in relation to the system volume, a small fraction of the virus particles may still be in tubing of the system. Here, a decision must be made between a complete virus elution and a higher virus concentration.
34. The buffer system for the virus elution does not have to correspond with the sample buffer, allowing for a buffer exchange within the chromatography run according to the requirements of the subsequent unit operation.

35. Membranes are intended for a single-use application. However, it is possible to reuse membrane stacks several times after washing them with 0.5 M NaOH in buffer.
36. SXC works best near the isoelectric point of the virus particle. Thus, the buffer's pH may need an adjustment for an optimal product recovery and purity. However, it is crucial to test the virus stability of the respective virus for the applied pH, particularly in the case of a pH below 7.
37. The virus recovery can be improved by increasing the ionic strength (e.g., addition of NaCl) in the elution buffer. NaCl concentrations of up to 1 M show a positive effect. However, as for the pH above, the stability of the respective virus needs to be confirmed for these conditions.
38. It is strongly recommended to evaluate the capacity of the membrane stack for the respective nanoplex. This can be done by using an online light scattering monitor, or by performing offline virus quantifications from fractions during sample loading.
39. A storage of the chromatographic samples should be done at −80 °C. However, virus infectivity can be impaired by the freezing step; hence, protective agents (e.g., sucrose) may be added, or infectivity assays must be done directly after the chromatographic experiment.

    Quantification and characterization of the purified virus particles

40. If the virus infectivity is not relevant for the anticipated product, other quantification methods, such as qPCR, ELISA, or particle counting techniques, are suitable alternatives.
41. For virus titration via the FACS assay, it is essential to have a calibration standard based on an alternative quantification method. The assay must be completely validated for each individual type of virus, cells, and culture conditions.
42. The host cells for the assay must be chosen with regard to the infectivity of the targeted virus. Furthermore, the virus must carry either the genetic information of a fluorescent protein to be subsequently expressed in the infected cells for a possible detection, or the viral proteins on the surface of infected cells must be labeled by fluorescently tagged antibodies.
43. Before removing the supernatant from the centrifuged plate, visually check for cell pellets. The supernatant may then be removed by pipetting or by flipping the plate. This requires practice, and the presence of pellets should be checked before and afterward.

44. The volume of PBS for the cell resuspension can be adapted on the applied FACS equipment, to provide a sufficient cell concentration.
45. If the sample virus concentration is beyond the linear range of the calibration curve, a further dilution of the virus sample must be analyzed with the assay.
46. For the size determination using light scattering detection, a sample concentration above 1E+07 particles/mL is recommended. Whether lower virus concentrations can be analyzed by this method, depends on the sample composition, its purity and most important the actual size of the nanoplex.
47. There are multiple ways to determine the size and shape of the virus particles. Light scattering is only one possibility. Alternatives include transmission electron microscopy, tunable resistive pulse sensing, disc centrifugation and others. Each method has advantages and drawbacks, and should be applied according to the product's requirements.

Quantification of process contaminants

48. As for most types of assays, the protein standards must be dissolved in a buffer resembling your sample buffer the most, or the buffer differences must be considered during assay validation.
49. Incubation can be done at room temperature, if a heating device is not available. However, in this case, the incubation time needs to be adjusted.
50. Directly after mixing in the sample with the protein assay reagent, a color change is observed. However, the reaction does not have a true endpoint. After 30 min of incubation at 37 °C, the reaction is nearly completed, and the further color development will be too slow to affect the measurement significantly. However, corresponding samples must be measured at the same time point.
51. A new calibration curve should be prepared for each plate measured. Although the assay is robust, deviations over time, or differences in individual measurements, can be reduced by a corresponding calibration curve on every plate.
52. Nonused standards can be stored at 4 °C for one week.
53. If sample concentrations are beyond of the validated calibration range (absorbance higher than the highest calibration), a dilution must be prepared and remeasured. An extrapolation of values is not valid.
54. If samples contain varying amounts of salts or assay-interfering components, the samples must be dialyzed to ensure a valid analysis. However, the molecular weight cut off (MWCO) of

the dialysis tubing must be small enough so as to not alter the concentration of the analytes. A MWCO of 5,000–14,000 (excluding molecules with molecular masses of 5 kDa to 14 kDa) can generally be recommended.

55. The DNA assay may cause problems when purifying DNA-viruses. Damaged or ruptured virus particles can increase the measured total DNA concentration.

References

1. Nachbagauer R, Krammer F (2017) Universal influenza virus vaccines and therapeutic antibodies. Clin Microbiol Infect 23(4):222–228. https://doi.org/10.1016/j.cmi.2017.02.009
2. Patel A, Dunlop J, Owsianka A et al (2015) Current and future prophylactic vaccines for hepatitis C virus. Vaccines Dev Ther 31. https://doi.org/10.2147/VDT.S48437
3. Wolff MW, Reichl U (2011) Downstream processing of cell culture-derived virus particles. Expert Rev Vaccines 10(10):1451–1475. https://doi.org/10.1586/erv.11.111
4. Santiago-Ortiz JL, Schaffer DV (2016) Adeno-associated virus (AAV) vectors in cancer gene therapy. J Control Release 240:287–301. https://doi.org/10.1016/j.jconrel.2016.01.001
5. Nestola P, Peixoto C, Silva RRJS et al (2015) Improved virus purification processes for vaccines and gene therapy. Biotechnol Bioeng 112 (5):843–857. https://doi.org/10.1002/bit.25545
6. Kaufman HL, Kohlhapp FJ, Zloza A (2015) Oncolytic viruses: a new class of immunotherapy drugs. Nat Rev Drug Discov 14 (9):642–662. https://doi.org/10.1038/nrd4663
7. Lawler SE, Speranza M-C, Cho C-F et al (2017) Oncolytic viruses in cancer treatment: a review. JAMA Oncol 3(6):841–849. https://doi.org/10.1001/jamaoncol.2016.2064
8. Weiss K, Gerstenberger J, Salzig D et al (2015) Oncolytic measles viruses produced at different scales under serum-free conditions. Eng Life Sci 15(4):425–436. https://doi.org/10.1002/elsc.201400165
9. Grein TA, Loewe D, Dieken H et al (2018) High titer oncolytic measles virus production process by integration of dielectric spectroscopy as online monitoring system. Biotechnol Bioeng 115(5):1186–1194. https://doi.org/10.1002/bit.26538
10. Genzel Y, Rödig J, Rapp E et al (2014) Vaccine production: upstream processing with adherent or suspension cell lines. Methods Mol Biol 1104:371–393. https://doi.org/10.1007/978-1-62703-733-4_23
11. Vázquez-Ramírez D, Jordan I, Sandig V et al (2019) High titer MVA and influenza A virus production using a hybrid fed-batch/perfusion strategy with an ATF system. Appl Microbiol Biotechnol 103(7):3025–3035. https://doi.org/10.1007/s00253-019-09694-2
12. Jiang W, Hua R, Wei M et al (2015) An optimized method for high-titer lentivirus preparations without ultracentrifugation. Sci Rep 5 (5):13875. https://doi.org/10.1038/srep13875
13. Tapia F, Vogel T, Genzel Y et al (2014) Production of high-titer human influenza A virus with adherent and suspension MDCK cells cultured in a single-use hollow fiber bioreactor. Vaccine 32(8):1003–1011. https://doi.org/10.1016/j.vaccine.2013.11.044
14. Grein TA, Loewe D, Dieken H et al (2019) Aeration and shear stress are critical process parameters for the production of oncolytic measles virus. Front Bioeng Biotechnol 7:78. https://doi.org/10.3389/fbioe.2019.00078
15. Firquet S, Beaujard S, Lobert P-E et al (2015) Survival of enveloped and non-enveloped viruses on inanimate surfaces. Microbes Environ 30(2):140–144. https://doi.org/10.1264/jsme2.ME14145
16. Black FL (1959) Growth and stability of measles virus. Virology 7:184–192. https://doi.org/10.1016/0042-6822(59)90186-2
17. Kohn A, Yassky D (1962) Growth of measles virus in kb cells. Virology 17:157–163. https://doi.org/10.1016/0042-6822(62)90092-2
18. Weiss K, Salzig D, Mühlebach MD et al (2012) Key parameters of measles virus production for oncolytic virotherapy. Am J Biochem Biotechnol 8(2):81–98. https://doi.org/10.3844/ajbbsp.2012.81.98
19. Trabelsi K, Majoul S, Rourou S et al (2012) Development of a measles vaccine production process in MRC-5 cells grown on Cytodex1 microcarriers and in a stirred bioreactor. Appl

Microbiol Biotechnol 93:1031–1040. https://doi.org/10.1007/s00253-011-3574-y
20. Weiss K, Salzig D, Röder Y et al (2013) Influence of process conditions on measles virus stability. Am J Biochem Biotechnol 9 (3):243–254. https://doi.org/10.3844/ajbbsp.2013.243.254
21. Loewe D, Häussler J, Grein TA et al (2019) Forced degradation studies to identify critical process parameters for the purification of infectious measles virus. Viruses 11(8). https://doi.org/10.3390/v11080725
22. Rapp F, Butel JS, Wallis C (1965) Protection of measles virus by sulfate ions against thermal inactivation. J Bacteriol 90:132–135
23. Chisti Y (2000) Animal-cell damage in sparged bioreactors. Trends Biotechnol 18:420–432. https://doi.org/10.1016/S0167-7799(00)01474-8
24. Crouch CF, Fowler HW, Spier RE (1985) The adhesion of animal cells to surfaces: the measurement of critical surface shear stress permitting attachment or causing detachment. J Chem Tech Biotechnol 35:273–281
25. Cherry RS, Papoutsakis ET (1989) Growth and death rates of bovine embryonic kidney cells in turbulent microcarrier bioreactors. Bioprocess Eng 4:81–89. https://doi.org/10.1007/BF00373735
26. Baczko K, Lazzarini RA (1979) Efficient propagation of measles virus in suspension cultures. J Virol 31:854–855
27. Sviben D, Forčić D, Kurtović T et al (2016) Stability, biophysical properties and effect of ultracentrifugation and diafiltration on measles virus and mumps virus. Arch Virol 161 (6):1455–1467. https://doi.org/10.1007/s00705-016-2801-3
28. Wolff MW, Reichl U (2008) Downstream processing: from egg to cell culture-derived influenza virus particles. Chem Eng Technol 31 (6):846–857. https://doi.org/10.1002/ceat.200800118
29. Weigel T, Soliman R, Wolff MW et al (2019) Hydrophobic-interaction chromatography for purification of influenza A and B virus. J Chromatogr B Analyt Technol Biomed Life Sci 1117:103–117. https://doi.org/10.1016/j.jchromb.2019.03.037
30. Wolff MW, Siewert C, Lehmann S et al (2010) Capturing of cell culture-derived modified Vaccinia Ankara virus by ion exchange and pseudo-affinity membrane adsorbers. Biotechnol Bioeng 105(4):761–769. https://doi.org/10.1002/bit.22595
31. Wolff MW, Siewert C, Hansen SP et al (2010) Purification of cell culture-derived modified vaccinia ankara virus by pseudo-affinity membrane adsorbers and hydrophobic interaction chromatography. Biotechnol Bioeng 107 (2):312–320. https://doi.org/10.1002/bit.22797
32. Orr V, Zhong L, Moo-Young M et al (2013) Recent advances in bioprocessing application of membrane chromatography. Biotechnol Adv 31(4):450–465. https://doi.org/10.1016/j.biotechadv.2013.01.007
33. Grein TA, Michalsky R, Vega López M et al (2012) Purification of a recombinant baculovirus of Autographa californica M nucleopolyhedrovirus by ion exchange membrane chromatography. J Virol Methods 183 (2):117–124. https://doi.org/10.1016/j.jviromet.2012.03.031
34. Peixoto C, Ferreira TB, Sousa MFQ et al (2008) Towards purification of adenoviral vectors based on membrane technology. Biotechnol Prog 24(6):1290–1296. https://doi.org/10.1002/btpr.25
35. Nestola P, Peixoto C, Villain L et al (2015) Rational development of two flowthrough purification strategies for adenovirus type 5 and retro virus-like particles. J Chromatogr A 1426:91–101. https://doi.org/10.1016/j.chroma.2015.11.037
36. Opitz L, Salaklang J, Büttner H et al (2007) Lectin-affinity chromatography for downstream processing of MDCK cell culture derived human influenza A viruses. Vaccine 25 (5):939–947. https://doi.org/10.1016/j.vaccine.2006.08.043
37. Weigel T, Solomaier T, Wehmeyer S et al (2016) A membrane-based purification process for cell culture-derived influenza A virus. J Biotechnol 220:12–20. https://doi.org/10.1016/j.jbiotec.2015.12.022
38. Fortuna AR, van Teeffelen S, Ley A et al (2019) Use of sulfated cellulose membrane adsorbers for chromatographic purification of cell cultured-derived influenza A and B viruses. Sep Purif Technol 226:350–358. https://doi.org/10.1016/j.seppur.2019.05.101
39. Opitz L, Lehmann S, Reichl U et al (2009) Sulfated membrane adsorbers for economic pseudo-affinity capture of influenza virus particles. Biotechnol Bioeng 103(6):1144–1154. https://doi.org/10.1002/bit.22345
40. Carvalho SB, Fortuna AR, Wolff MW et al (2018) Purification of influenza virus-like particles using sulfated cellulose membrane adsorbers. J Chem Technol Biotechnol 93 (7):1988–1996. https://doi.org/10.1002/jctb.5474

41. Hoffmann D, Leber J, Loewe D et al (2019) Purification of new biologicals using membrane-based processes. In: Basile A, Charcosset C (eds) Current trends and future developments on (bio-) membranes. Elsevier, Amsterdam, pp 123–150
42. Transfiguracion J, Jorio H, Meghrous J et al (2007) High yield purification of functional baculovirus vectors by size exclusion chromatography. J Virol Methods 142(1–2):21–28. https://doi.org/10.1016/j.jviromet.2007.01.002
43. Kalbfuss B, Wolff M, Morenweiser R et al (2007) Purification of cell culture-derived human influenza A virus by size-exclusion and anion-exchange chromatography. Biotechnol Bioeng 96(5):932–944. https://doi.org/10.1002/bit.21109
44. Lee J, Gan HT, Latiff SMA et al (2012) Principles and applications of steric exclusion chromatography. J Chromatogr A 1270:162–170. https://doi.org/10.1016/j.chroma.2012.10.062
45. Ingham KC (1978) Precipitation of proteins with polyethylene glycol: characterization of albumin. Arch Biochem Biophys 186 (1):106–1013
46. Atha DH, Ingham KC (1981) Mechanism of precipitation of proteins by polyethylene glycols. J Biol Chem 256(23):12108–12117
47. Polson A (1977) A theory for the displacement of proteins and viruses with polyethylene glycol. Prep Biochem 7:129–154
48. Arakawa T, Timasheff SN (1985) Mechanism of poly(ethylene glycol) interaction with proteins. Biochemistry 24:6756–6762
49. Timasheff SN, Arakawa T (1988) Mechanism of protein precipitation and stabilization by co-solvents. J Cryst Growth 90:39–46
50. Sim S-L, He T, Tscheliessnig A et al (2012) Branched polyethylene glycol for protein precipitation. Biotechnol Bioeng 109 (3):736–746. https://doi.org/10.1002/bit.24343
51. Wang Y, Sarkar M, Smith AE et al (2012) Macromolecular crowding and protein stability. J Am Chem Soc 134(40):16614–16618. https://doi.org/10.1021/ja305300m
52. Lewis GD, Metcalf TG (1988) Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl Environ Microbiol 54 (8):1983–1988
53. Ludwig A-K, de Miroschedji K, Doeppner TR et al (2018) Precipitation with polyethylene glycol followed by washing and pelleting by ultracentrifugation enriches extracellular vesicles from tissue culture supernatants in small and large scales. J Extracell Vesicles 7 (1):1528109. https://doi.org/10.1080/20013078.2018.1528109
54. Gagnon P, Toh P, Lee J (2014) High productivity purification of immunoglobulin G monoclonal antibodies on starch-coated magnetic nanoparticles by steric exclusion of polyethylene glycol. J Chromatogr A 1324:171–180. https://doi.org/10.1016/j.chroma.2013.11.039
55. Tao S-P, Zheng J, Sun Y (2015) Grafting zwitterionic polymer onto cryogel surface enhances protein retention in steric exclusion chromatography on cryogel monolith. J Chromatogr A 1389:104–111. https://doi.org/10.1016/j.chroma.2015.02.051
56. Wang C, Bai S, Tao S-P et al (2014) Evaluation of steric exclusion chromatography on cryogel column for the separation of serum proteins. J Chromatogr A 1333:54–59. https://doi.org/10.1016/j.chroma.2014.01.059
57. Marichal-Gallardo P, Pieler MM, Wolff MW et al (2017) Steric exclusion chromatography for purification of cell culture-derived influenza A virus using regenerated cellulose membranes and polyethylene glycol. J Chromatogr A 1483:110–119. https://doi.org/10.1016/j.chroma.2016.12.076
58. Lothert K, Sprick G, Beyer F et al (2019) Membrane-based steric exclusion chromatography for the purification of a recombinant baculovirus and its application for cell therapy. J Virol Methods 275:113756. https://doi.org/10.1016/j.jviromet.2019.113756
59. (1998) WHO Expert Committee on biological standardization. World Health Organ Tech Rep Ser 878: i–vi, 1–101
60. Sheng-Fowler L, Lewis AM, Peden K (2009) Issues associated with residual cell-substrate DNA in viral vaccines. Biologicals 37 (3):190–195. https://doi.org/10.1016/j.biologicals.2009.02.015
61. Akpinar F, Yin J (2015) Characterization of vesicular stomatitis virus populations by tunable resistive pulse sensing. J Virol Methods 218:71–76. https://doi.org/10.1016/j.jviromet.2015.02.006
62. Yang L, Yamamoto T (2016) Quantification of virus particles using nanopore-based resistive-pulse sensing techniques. Front Microbiol 7:1500. https://doi.org/10.3389/fmicb.2016.01500
63. Murphy RM (1997) Static and dynamic light scattering of biological macromolecules: what can we learn? Curr Opin Biotechnol 8

(1):25–30. https://doi.org/10.1016/S0958-1669(97)80153-X

64. Citkowicz A, Petry H, Harkins RN et al (2008) Characterization of virus-like particle assembly for DNA delivery using asymmetrical flow field-flow fractionation and light scattering. Anal Biochem 376(2):163–172. https://doi.org/10.1016/j.ab.2008.02.011
65. Driskell JD, Jones CA, Tompkins SM et al (2011) One-step assay for detecting influenza virus using dynamic light scattering and gold nanoparticles. Analyst 136(15):3083–3090. https://doi.org/10.1039/C1AN15303J
66. Vestad B, Llorente A, Neurauter A et al (2017) Size and concentration analyses of extracellular vesicles by nanoparticle tracking analysis: a variation study. J Extracell Vesicles 6(1):1344087. https://doi.org/10.1080/20013078.2017.1344087
67. Soo CY, Song Y, Zheng Y et al (2012) Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. Immunology 136(2):192–197. https://doi.org/10.1111/j.1365-2567.2012.03569.x
68. Kramberger P, Ciringer M, Štrancar A et al (2012) Evaluation of nanoparticle tracking analysis for total virus particle determination. Virol J 9:265. https://doi.org/10.1186/1743-422X-9-265
69. Harris A, Cardone G, Winkler DC et al (2006) Influenza virus pleiomorphy characterized by cryoelectron tomography. Proc Natl Acad Sci U S A 103(50):19123–19127. https://doi.org/10.1073/pnas.0607614103
70. Ruigrok RW, Andree PJ, van Hooft Huysduynen RA et al (1984) Characterization of three highly purified influenza virus strains by electron microscopy. J Gen Virol 65 (Pt 4):799–802. https://doi.org/10.1099/0022-1317-65-4-799
71. Pease LF, Lipin DI, Tsai D-H et al (2009) Quantitative characterization of virus-like particles by asymmetrical flow field flow fractionation, electrospray differential mobility analysis, and transmission electron microscopy. Biotechnol Bioeng 102(3):845–855. https://doi.org/10.1002/bit.22085
72. Li Y, Wang Q, Bergmann SM et al (2019) Preparation of monoclonal antibodies against KHV and establishment of an antigen sandwich ELISA for KHV detection. Microb Pathog 128:36–40. https://doi.org/10.1016/j.micpath.2018.12.034
73. Pfefferkorn M, Böhm S, Schott T et al (2018) Quantification of large and middle proteins of hepatitis B virus surface antigen (HBsAg) as a novel tool for the identification of inactive HBV carriers. Gut 67(11):2045–2053. https://doi.org/10.1136/gutjnl-2017-313811
74. Rajendran M, Sun W, Comella P et al (2018) An immuno-assay to quantify influenza virus hemagglutinin with correctly folded stalk domains in vaccine preparations. PLoS One 13(4):e0194830. https://doi.org/10.1371/journal.pone.0194830
75. Jensen KS, Adams R, Bennett RS et al (2018) Development of a novel real-time polymerase chain reaction assay for the quantitative detection of Nipah virus replicative viral RNA. PLoS One 13(6):e0199534. https://doi.org/10.1371/journal.pone.0199534
76. Abachin E, Convers S, Falque S et al (2018) Comparison of reverse-transcriptase qPCR and droplet digital PCR for the quantification of dengue virus nucleic acid. Biologicals 52:49–54. https://doi.org/10.1016/j.biologicals.2018.01.001
77. Rush BS, Coughlin ML, Sanyal G (2018) In vitro infectivity of oncolytic Newcastle Disease Virus: correlation between plaque and fluorescent focus assays. J Virol Methods 251:69–74. https://doi.org/10.1016/j.jviromet.2017.09.029
78. Tobita K, Sugiura A, Enomoto C et al (1975) Plaque assay and primary isolation of influenza a viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. Med Microbiol Immunol 162(1):9–14. https://doi.org/10.1007/BF02123572
79. Tatsumoto N, Miyauchi T, Arditi M et al (2018) Quantification of infectious sendai virus using plaque assay. Bio Protoc 8(21). https://doi.org/10.21769/BioProtoc.3068
80. Karlsson EA, Meliopoulos VA, Tran V et al (2018) Measuring influenza virus infection using bioluminescent reporter viruses for in vivo imaging and in vitro replication assays. Methods Mol Biol 1836:431–459. https://doi.org/10.1007/978-1-4939-8678-1_21
81. Lambeth CR, White LJ, Johnston RE et al (2005) Flow cytometry-based assay for titrating dengue virus. J Clin Microbiol 43 (7):3267–3272. https://doi.org/10.1128/JCM.43.7.3267-3272.2005
82. Vlasak J, van Hoang M, Christanti S et al (2016) Use of flow cytometry for characterization of human cytomegalovirus vaccine particles. Vaccine 34(20):2321–2328. https://doi.org/10.1016/j.vaccine.2016.03.067
83. Konz JO, Lee AL, Lewis JA et al (2005) Development of a purification process for adenovirus: controlling virus aggregation to improve the clearance of host cell DNA. Biotechnol

Prog 21(2):466–472. https://doi.org/10.1021/bp049644r

84. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72 (1–2):248–254. https://doi.org/10.1016/0003-2697(76)90527-3
85. Smith PK, Krohn RI, Hermanson GT et al (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150 (1):76–85. https://doi.org/10.1016/0003-2697(85)90442-7
86. Weber C, Gokorsch S, Czermak P (2007) Expansion and chondrogenic differentiation of human mesenchymal stem cells. Int J Artif Organs 30:611–618. https://doi.org/10.1177/039139880703000709

Chapter 13

Whole-Cell Vaccine Preparation: Options and Perspectives

Punit Kumar, Sunita, Kashyap Kumar Dubey, and Pratyoosh Shukla

Abstract

Vaccines are biological preparations to elicit a specific immune response in individuals against the targetted microorganisms. The use of vaccines has caused the near eradication of many critical diseases and has had an everlasting impact on public health at a relatively low cost. Most of the vaccines developed today are based on techniques which were developed a long time ago. In the beginning, vaccines were prepared from tissue fluids obtained from infected animals or people, but at present, the scenario has changed with the development of vaccines from live or killed whole microorganisms and toxins or using genetic engineering approaches. Considerable efforts have been made in vaccine development, but there are still many diseases that need attention, and new technologies are being developed in vaccinology to combat them. In this chapter, we discuss different approaches for vaccine development, including the properties and preparation of whole-cell vaccines.

Key words Vaccines, Diseases, Inactivation, Attenuation, Conjugated vaccines, Whole-cell vaccines

1 Introduction

As per the World Health Organization (WHO), a vaccine may be described as a biological preparation that is used to improve immunity against a particular disease. Vaccines contain live, weakened, or killed forms of the microorganisms such as bacteria and viruses, its toxins or one of its surface proteins which cause diseases (Fig. 1). Vaccines are prepared for human and animal uses. The agents present in vaccines provoke the immune system of the individual to generate antibodies or required cellular responses and destroy the foreign agent and remembers it so that the immune system can recognize and eliminate the microorganism which may be encountered later [1]. Alternatively, there is one more category of vaccines called subunit vaccines, and such vaccines contain specific compo-

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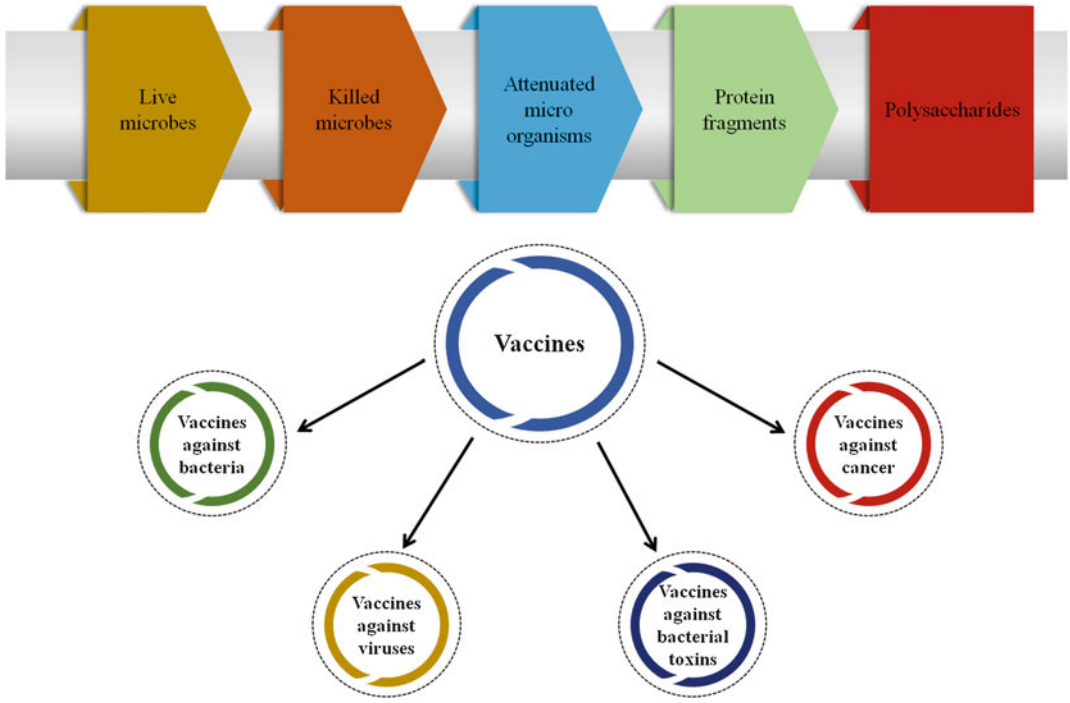

Fig. 1 Demonstration of different types of vaccines (vaccine against bacteria, virus, toxins, and cancer) and constituents used to design a vaccine (microorganisms in living, killed, and attenuated forms, proteins, and polysaccharides)

nents of pathogens instead of the complete microorganism to trigger an immune response in the host.

Vaccination is a well-recognized cost-effective contribution to quality and prolongation of life expectancy, mortality, child survival, morbidity, and disability [2–4]. Most of the countries in the world have adopted an effective immunization program to deliver particular vaccines to selected beneficiaries, mainly infants, children, and pregnant women. It is supposed that vaccines are available against at least 27 causative agents, and more agents are being identified for the development of vaccines. The number of targeted antigens through immunization programs varies among different countries; however, vaccines against diarrhea due to rotavirus, diphtheria, hepatitis B, measles, pertussis, poliomyelitis, *Streptococcus pneumoniae*, and tetanus are involved in immunization programs of most of the countries in the world [3, 5].

The success of a vaccine depends on many factors, and of them some important factors are mentioned below [6]:

- Type and dose of vaccine.
- The use of adjuvant or excipients in the preparation of the vaccine.
- The schedule of immunization.

- The route of administration of the vaccine.
- The immune status of the individuals being vaccinated, age, and disease.

2 History of Vaccine Development

The history of vaccines and vaccination belongs to human efforts in the prevention against disease in society (Table 1). It is assumed that smallpox and other infectious diseases were known to ancient people. The initial efforts for vaccine development were made for smallpox as it was found that people affected with smallpox were further protected from future infection of disease [3]. Today, initial developments in vaccinology are attributed to the result of Edward Jenner's efforts of prevention of smallpox through inoculation of people with cowpox virus [33]. The period between 1930 and 1950 was considered the transitional era in vaccine development, and during this period chick embryos and minced tissues were used for in vitro growth of viruses and rickettsiae for vaccine preparation. Past studies have contributed to the development of effective vaccines as the availability of more information through the study of natural infections, animal models, and seroepidemiology has led to the development of vaccines [34]. Modern vaccinology started in about 1950 following the considerable advancements made prior to and during World War II. Its quest has been dependent on research and development in microbiology, molecular biology, cell culture, and immunology, which have contributed to develop many live, killed, and the recombinant-expressed vaccines [33].

Though the vaccines have been successfully created for infectious diseases which are controlled by preexisting antibodies and for other transmissible diseases, further immunological mechanisms need to be developed for full protection. Nowadays, "new vaccines" are a particularly urgent requirement of society, where economic development, globalization, and immigration of people are causing the appearance or reappearance of old and new infectious agents [2]. Modern techniques of growth and maintenance of cell culture, development of efficient fermentation processes, isolation of proteins, and formulation of vaccines have enabled manufacturers to maintain purity, safety, and specificity.

Researchers have used novel technologies for the development of vaccines. It is clear that development of vaccines has been led by advancements in immunology, microbiology, and cell biology to identify the protection mediated by antibodies and lymphocytes, and selection of attenuated mutants during cell culture. Included in this are outcomes of the experiments conducted in animals where immune responses exhibited against natural infection were analyzed, which successfully led the way to develop vaccines [35],

Table 1
Development of vaccines though different approaches against some common diseases

Disease	Causative microorganism	Development of vaccine	References
Bacterial meningitis	*Neisseria meningitidis*	Polysaccharide–protein conjugate, capsular polysaccharide-based vaccines, noncapsular antigens, membrane proteins	[7]
Botulism	*Clostridium botulinum*	Pentavalent vaccine against BoNT serotypes A-E, monovalent vaccine against BoNT serotype F, toxoid vaccines, next-generation vaccines using synthetic peptide, neurotoxic carboxy-terminal fragments (rBoNT(HC))	[8–10]
Cholera	*Vibrio cholerae*	Subunit toxoid, live oral cholera vaccine, cholera toxin B subunit vaccine, cholera toxin recombinant B subunit vaccine	[11, 12]
Diphtheria	*Corynebacterium diphtheriae*	Diphtheria toxoid vaccine, recombinant fragments of diphtheria toxin (DT)	[13, 14]
Plague	*Yersinia pestis*	Live attenuated and killed whole-cell vaccines, attenuated mutants, subunit vaccines, recombinant vaccine	[15, 16]
Pertussis	*Bordetella pertussis*	Haemagglutinins vaccine, pertussis toxin, acellular and whole-cell vaccine,	[17, 18]
Polio	Poliovirus	Inactivated Salk polio vaccine, Sabin oral polio vaccine, inactivated poliovirus vaccine,	[19–21]
Rabies	Rabies virus	Recombinant human adenovirus, edible vaccines in plants, oral vaccine, live modified rabies virus vaccine strain SAG-2, live attenuated rabies vaccine, a genetically modified rabies vaccine	[22–24]
Smallpox	Variola virus	Live animal poxvirus, subunit vaccine, second- and third-generation smallpox vaccines	[25–27]
Tetanus	*Clostridium tetani*	Tetanus toxoid vaccine	[28, 29]
Typhoid	*Salmonella typhi*	Live attenuated bacteria, recombinant conjugate,	[30–32]

and passive administration of antibodies towards particular antigen to demonstrate that specific antigen must be present in vaccines [36]. Different mechanisms of vaccine development have been employed by researchers such as attenuation, cell culture, reassortment, inactivation, subunit vaccines, and genetic engineering [37–41].

Attenuation is the very basic mechanism of vaccine development. Live attenuated vaccines are one of the most successful and cost-effective vaccines. Such vaccines protect against acute diseases caused by human and veterinary viral and bacterial infections, but

chronic infections like HIV are more challenging due to safety and efficacy concerns [42]. This process had been used by Pasteur and his colleagues to develop vaccines against the diarrheal disease-causing agent in chickens, that is, *Pasteurella multocida* [36], animal poxvirus (probably horsepox) against smallpox [43], bovine tuberculosis bacteria against human tuberculosis [44, 45]. The development of vaccines for measles, mumps, oral polio, rubella, tetravalent dengue, and varicella was made by an in vitro selection of clones by cell-culture passage [42]. The principle behind the development of live, attenuated viral vaccines is that its virulence may be sufficiently attenuated by successively subculturing while retaining the required antigens to induce immune responses. It is also suggested that passage in cell culture causes cells to grow in the culture medium, and the mutants will grow comprising modified harmful genes that control the infection of the organism in the host [36]. This method enabled the selection and isolation of mutants for the oral polio vaccine, which was not able to cause paralysis. These mutations were at least moderately lost during the growth of attenuated cells in the human intestine, and this causes infrequent incidences of postvaccination paralysis [46].

Inactivation is an important method for vaccine development. These are also recognized as killed whole-cell vaccines. Inactivation of the agent is done by heat treatment, chemical treatment, formalin treatment, β-propiolactone treatment, and so on so that infectivity of the agent is eliminated and it loses its ability to replicate, but its immunogenicity is retained [36, 47]. These vaccines generally offer a shorter duration of protection and thus require booster doses for long-term immunity [41]. This method has been used to develop a large number of vaccines such as vaccines for cholera, plague, typhoid, pertussis, polio, hepatitis A, influenza, and yellow fever vaccine [47–49].

Reassortment is recognized as a key evolutionary mechanism present in segmented RNA viruses [50]. This mechanism enables the development of virus having RNA segments of two viruses by cocultivation of two viruses. This method has enabled the development of live and inactivated influenza and rotavirus vaccines [51].

Encapsulated bacteria cause diseases among infants, the elderly, and immunocompromised persons. It was observed that antibodies against the polysaccharide capsule increased phagocytosis, and this property led to the development of capsular polysaccharide vaccines. The immune response triggered by polysaccharide antigens protects against encapsulated bacteria. Polysaccharide vaccines have been reported against *Neisseria meningitidis*, *Salmonella typhi*, and *Streptococcus pneumoniae*. Capsular polysaccharides for vaccine preparations have been reported for the development of the meningococcal polysaccharide vaccine [30, 52, 53], pneumococcal polysaccharides vaccines [54], and *Haemophilus influenzae* type b capsular vaccine [55]. It was also found that such vaccines

contributed little protective immunity to infants and young children [56]. Moreover, investigators have used conjugates of capsular polysaccharides and proteins to enhance immunogenicity. Conjugate polysaccharide vaccines have been developed against the diseases caused by encapsulated bacteria such as *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae*. These vaccines have reduced global childhood morbidity and mortality, with their efficacy to induce long-lasting immunity in a range of age groups [52, 57–60].

Adjuvants are used in conjunction with vaccines to induce a fast, effective, and prolonged humoral or cellular immune response against the antigen. The significance of adjuvants is increasing because highly purified subunit and synthetic vaccines are more specific, costly, but less immunogenic. So a potent adjuvant should cause the use of fewer antigens to induce the desired immune response. The some examples of adjuvants are biodegradable polymeric microspheres, liposomes, oil adjuvants, lipopolysaccharide, and aluminum salts [61–63]. Some of the vaccine components are mentioned in Fig. 1 that can be significantly used in the process of developing vaccines.

Subunits are another category of vaccines developed by using part of the antigen of the causative microorganism. Subunit vaccines use only part of antigen from a target pathogen to induce an immune response. Thus, a specific antigen is isolated from a pathogen and presented as an antigen of its own [41]. Purified antigens may be surface molecules, subcellular fragments, and toxoids which require different carriers to be transported. Immune responses raised by subunit vaccine are based on the type of antigen [38]. The high levels of purity of subunit vaccines contribute to enhancement in the specificity of a vaccine and a significant reduction in adverse effects. Such vaccines may be a capsular polysaccharide (*Streptococcus pneumoniae*, *Haemophilus influenzae*), inactivated protein toxins (diphtheria, pertussis, and tetanus), and viral coat proteins (hepatitis B vaccines). Sometimes capsular polysaccharide vaccines are chemically conjugating with protein components and used as an antigen for the development of vaccines [64–66]. Conjugated vaccines are also considered as a subclass of a subunit vaccine, which contains a polysaccharide-based antigen [67]. Such types of vaccines have been reported for influenza virus using viral hemagglutinin (HA) protein, rabies vaccine using rabies glycoprotein, a vaccine against *Salmonella*, and meningitis and acellular pertussis vaccines using one to five proteins from the pertussis bacillus [17, 18, 68–72].

Researchers have used the genetic engineering approach to enhance the efficacy and increased the production of vaccines in different hosts [73]. Investigators also have discussed the role of gene editing tools for cost-effective production of antibodies and therapeutic proteins [74, 75]. In this approach, the genes of

pathogens which encode antigens to stimulate immune response are expressed in microbial cells. Varieties of expression systems are available with a variety of advantages and produce the required antigens in large quantities [76]. Using this approach many types of vaccines have been developed such as Ty21a vaccine for typhoid, CVD103-HgR cholera vaccine, cholera toxin B, human papilloma virus (HPV) vaccine using L1 protein, hepatitis B virus, bivalent oral vaccine for typhoid fever and cholera-related diarrhea, and *Salmonella enterica* serovar Enteritidis antigens [77–79].

3 Whole-Cell Vaccines and Their Uses

The concept of whole-cell vaccines arrived more than 60 years ago. According to a WHO report, the whole-cell vaccine design is based on the strain selection, and it should be performed by knowing the complete information about the characterization of the strain [80]. The whole-cell vaccine can be cultured in various seed lot systems; different media are also available, but synthetic media are preferentially used for production purposes [81] as it is found that synthetic media have defined components suitable for specific microbial growth. In addition, there is a very low chance of contamination of other types of microbes in synthetic media. In designing whole-cell vaccines, the inactivation of the toxin is an important factor, and it can be done by different methods such as chemical treatment, acid treatment, and radiation [82]. The final product must be analyzed to check that no heat-labile toxins are present in it. WHO has developed the guidelines for quality control, standardization, and production of whole-cell vaccines. For example, strains of *B. pertussis* used in vaccine production should be well characterized and as more components than just the strain are used in vaccines preparation, the bacterial content before detoxification and killing must be determined by comparison with 10 IU of WHO opacity standard. The concentration of bacteria in a single human vaccine dose should not be more than 20 IU and not less than 4.0 IU with a lower limit of the estimated potency not less than 2.0 IU. The storage of whole-cell vaccines should be done at 2–8 °C [83, 84]. The intracerebral mouse protection test (Kendrick test) is also considered an effective assay to analyze the potency of whole-cell pertussis vaccines and it is also assumed that this is the only test showing a correlation with protection in children [85, 86]. It has been reported that whole-cell vaccines can functionally be used in infants and toddlers in several countries for a long duration of periods depending on dose and the immune reaction of individuals. Bacterial cell components must also be analyzed for virulence and immunogenicity while designing the whole-cell vaccines.

There are different types of whole-cell vaccines available such as pertussis, cholera, and meningitis [87]. Pertussis or whooping cough is caused by *Bordetella pertussis*, which is a contagious disease. Whole-cell pertussis vaccines are one of the widely used vaccines for immunization and researchers are also analyzing the potency of acellular pertussis vaccines. Whole-cell pertussis vaccine is available, and it is considered as a part of the trivalent vaccine consisting of diphtheria–tetanus–pertussis (DTP) [88].

The reoccurrence of multidrug-resistant (MDR) bacteria like *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and methicillin-resistant-*Staphylococcus aureus* (MRSA), and the absence of any licensed vaccines against these pathogens are responsible for the increasing rate of mortality. Cabral et al. [89] reported that an effective whole-cell vaccine could be developed for D-glutamate of the bacterial cell wall. Along with, they also tried this strategy to develop vaccines against these MDR associated pathogens. It is very promising as it does not need the identification of any specific virulence factor, and it can be potentially applied for the designing of live attenuated vaccines for other bacterial pathogens [90]. The examples of different types of whole-cell vaccines are mentioned in Table 2, and the mechanism of the whole vaccine to induce immune cells is explained in the Fig. 2. Whole-cell vaccines, along with antigens, can be sold at a lower price as compared to acellular vaccines [100].

There is a lot of practice in the field of cancer therapeutics, yet it is a deadly disease responsible for the death of many people. In clinical trials, modified whole-cell cancer vaccines emerged as an important form of immunotherapy [101]. It is more advantageous as compared to a specific protein and peptide antigen as it exposes all sources of antigens. Moreover, it removes the requirement for the identification of the most optimal antigen that targets a specific type of cancer [102]. Essentially, multiple tumor antigens can be targeted at a time and generate broader immune responses more than one tumor antigen. Additionally, the novel tumor antigen can be identified by exploring the serologic response and immunized lymphocytes. This will also help in categorizing the significance of a response to a specific tumor antigen by comparing immune responses of pre- and post vaccination [103]. The modification of the whole-cell vaccine can enhance the expression of cytokines, chemokines, and costimulatory molecules that is responsible for an immune response [104]. The approach was found to be safe in the case of phase I and phase II trials in different cancer patients. The assessment of vaccine induced immunity can be measured by delayed-type hypersensitivity (DTH) to autologous tumor cells [105].

In many developing countries, cholera remains an important public health concern. Cholera is still a fatal disease in poor countries and transmitted by contaminated food and water with

Table 2
Development of whole-cell vaccines against various pathogens in pipeline

Pathogen	Diseases	Symptoms	References
Chlamydia abortus	Ovine enzootic abortion	Abortion in sheep and goats Birth of weak offspring Utero infections	[91]
Chlamydia felis	Conjunctivitis	Inflammation of feline conjunctiva, rhinitis, and respiratory problems	[92]
Chlamydia pneumoniae	Pneumonia	Runny or stuffy nose, Fatigue, Low-grade fever, hoarseness or loss of voice, sore throat, slowly worsening cough	[93]
Salmonella typhi	Typhoid	High fever, diarrhea, and vomiting	[32]
Vibrio cholerae	Cholera	Loss of fluid electrolytes and severe dehydration	[94]
Yersinia pestis	Plague	Abdominal pain, diarrhea, vomiting. Bleeding from mouth, nose or rectum, or under the skin. Blackening and death of tissue (gangrene) in extremities.	[95]
Bordetella pertussis	Whole-cell pertussis	Mild coughing, sneezing, runny nose, low fever	[96]
Influenza virus	Influenza	High fever, runny nose, sore throat, muscle pains, headache, coughing, sneezing, and tiredness	[97]
Polio virus	IPV	Muscle weakness, loss of muscle,	[98]
Hepatitis A virus	Hepatitis A	Yellow eyes and skin, dark urine, Pain in the belly, loss of appetite, nausea, fever, diarrhea, fatigue	[98]
Rabies virus	Rabies	Neurological problems and a fear of light and water	[99]

feces. The risk of death can rise to 70% if there is no proper treatment. There is an estimation of 1.3–4 million cases and 21,000–143,000 annual deaths globally [106]. There has been good progress in the development of cholera vaccines. The killed whole-cell cholera vaccine is now becoming an effective tool against cholera. Currently two common vaccines for cholera which are WHO prequalified are Dukoral and Shanchol. Dukoral comprises inactivated whole-cell *V. cholera* with a recombinant B subunit of cholera toxin. SANCHOL is a bivalent (*Vibrio cholerae* O1 and O139) Indian whole-cell cholera vaccine [107]. Cholera becomes more deadly during pregnancy and adversely affects the health of pregnant women, and infection in pregnancy has been associated with abortions and stillbirths. Cholera can be one of the reasons for pregnancy loss, increasing the possibility 2–36% [108, 109]. Therefore it is important to take the necessary steps to treat this disease. The treatment and prevention of it is possible with the help of a

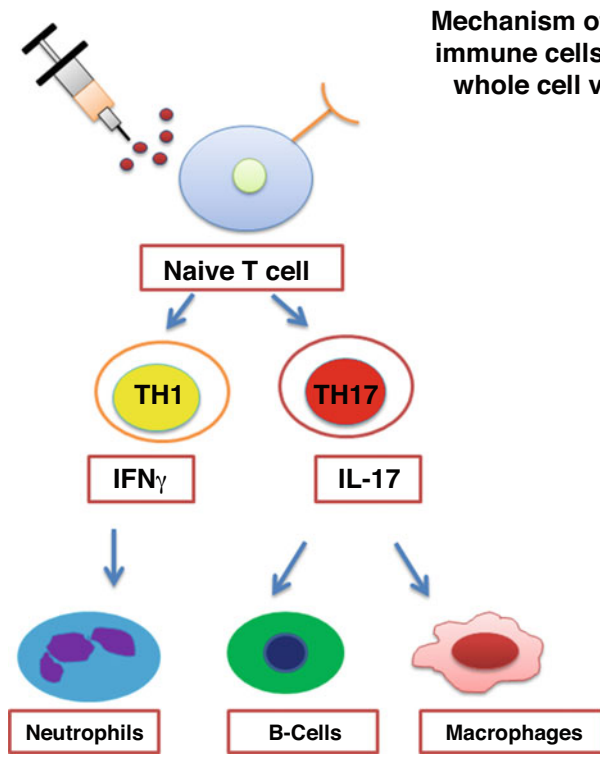

Fig. 2 Demonstration of mechanisms eliciting immune cells through the whole-cell vaccine

whole-cell killed oral cholera vaccine (OCV). Additionally, the vaccine does not produce any adverse effects on the pregnancy outcome as it cannot replicate in the gut and oral route intake. It was also observed that no harmful effects on pregnancy were seen during the mass vaccination in Tanzania. However, a clinical study showed that OCV has no harmful effect on pregnant women thus indicating that OCV is safe with no loss of fetal life in pregnant women while they are suffering from cholera [110, 111]. Therefore, it should be recommended that pregnant women take OCV when they are at high risk of losing a fetus while suffering from cholera.

Malaria is also one of the common causes of morbidity and mortality in tropical and subtropical regions. It can be controlled by developing an efficient vaccine that should be cost-effective also [112]. This infection is transmitted by the bite of female Anopheles mosquito, which injects sporozoites in the skin. Researchers are trying to develop a vaccine based on antigens of blood stage parasites [113]. Richards et al. reported that a whole killed blood lysate vaccine could be significantly applied to protect against the liver-stage as well as blood-stage malaria infections [114].

4 Preparation of Whole-Cell Vaccines

There are different strategies to make bacterial and viral vaccines as a whole-cell vaccine. Generally, the virus multiplies to cause infection; however, the vaccine virus multiplies fewer than 20 times to induce memory B cells [115]. Different methods such as physical, chemical, and radiation technology are mentioned for the attenuation of the pathogens in Fig. 3. A vaccine virus does not multiply rapidly, so it will not cause any diseases. Weakened or live vaccines are advantageous as they are responsible for providing lifelong immunity. The virus can be inactivated by killing via treatment with chemicals [116]. Generally, formalin, glutaraldehyde, phenol, and propiolactone are used to inactivate pathogens. The killing can inactivate the virus so that it is incapable of multiplying and causing disease in the human body. Different vaccines such as hepatitis A, polio, influenza, and rabies are prepared by applying this technology. Hypochlorous acid is produced in vivo by activated phagocytes and is a potent oxidant. It is antibacterial in nature and targets proteins [117]. It has a very high redox potential and destroys bacteria by stealing electrons from it. Hakim et al. [118] mentioned that slightly acidic hypochlorous acid water (SAHW) inactivates bacterial cells below 2.6 $\log_{10}$ CFU/ml in the exposure of 5 s. A high concentration of hypochlorous acid is present in SAHW, which is a chlorine-based solution. Hypochlorous acid has a low molecular weight and is uncharged so it can easily enter into the cell wall. It reacts highly in both oxidative reactions as well as substitutive reactions [119]. It has the potential to denature the essential components of the cells like DNA, RNA, mitochondria, and enzymes. Chiang et al. [120] reported that hypochlorous acid could be used to enhance the immunogenicity of dendritic cells by inducing primary necrosis in tumor cells.

Radiation sterilization is one of the techniques that are used to develop different types of vaccines [121]. It can significantly remove chemical contaminants and destroy nucleic acids through penetration without damaging the antigens on the surface of the pathogen. The development of a pneumococcal vaccine that is cost-effective and serotype independent is a global challenge. Gamma irradiations can be used to sterilize many biological products [122] and thus probably it can be used as an inactivation technique to generate a whole-cell vaccine. Chen et al. [84] reported that un-encapsulated *Streptococcus pneumoniae* strain Rx1 could be inactivated with the help of γ irradiations for the development of a pneumococcal vaccine that is serotype independent and generate B cells and IL 17 responses. Intranasal vaccination with γ-irradiated Streptococcus pneumoniae whole-cell vaccine provides serotype-independent protection mediated by B-cells and innate IL-17 responses [123].

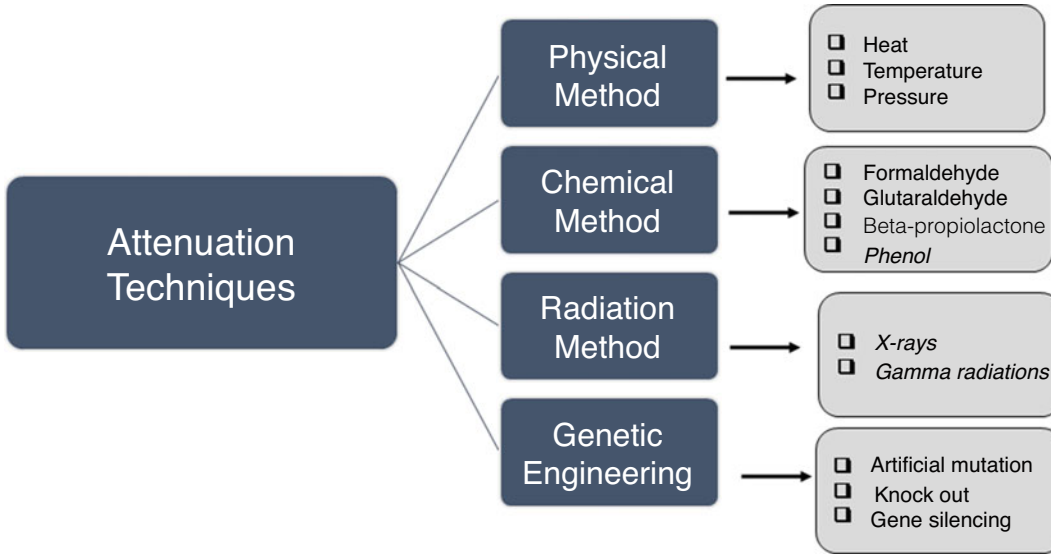

Fig. 3 Demonstration of different techniques for the attenuation of pathogens in whole-cell vaccine preparation

In consideration with safety and stability of the whole cell, the vaccine should never cause any disease as it does not constitute any living components and is more stable than live attenuated vaccines [81]. However, attenuated vaccines can stimulate cytotoxic T cells which are applied for the treatment of intracellular pathogens and cancer. In inactivate vaccines, the pathogens cannot divide, but some of the components of pathogens are left (polysaccharide capsules of pneumococcus) which are then recognized by the immune system and provoke an adaptive immune response [124]. These vaccines are given in multiple doses as they are not active, and the immune system does not require a response in the first dose but usually responds after the second or third dose [125].

5 Conclusion and Prospects

Whole-cell vaccines have been used for 60 years, and these vaccines are standardized to Opacity Units. It has been suggested that the potency of whole-cell vaccines should be as per the standards set by regulating agencies. In the preparation of whole-cell vaccines, many factors influence the processing of vaccines such as strain selection, type of culture medium for growth of strains, and inactivation of the toxin. It is found that whole-cell vaccines are being administrated to infants and toddlers in several countries for long durations. Researchers have reported the development of whole-cell vaccines against many infectious agents with examples that include whooping cough caused by *Bordetella pertussis* as part of the

trivalent vaccine comprising diphtheria–tetanus–pertussis (DTP), D-glutamate of the bacterial cell wall to control MDR, cholera, *Streptococcus pneumoniae*, malaria, Hepatitis A, polio, influenza, and rabies. The development of tools and techniques in molecular biology, bioinformatics, cell biology, and immunology has significantly contributed to the development of more potent vaccines. Thus, by using these techniques (such as epitope prediction, development of antimicrobial peptides, and protein engineering), novel therapeutic proteins may be produced, and these proteins may have the potential for future use in immunization.

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References

1. WHO, Vaccines. https://www.who.int/topics/vaccines/en/. Accessed 31 May 2019
2. Bragazzi NL, Gianfredi V, Villarini M, Rosselli R, Nasr A, Hussein A, Martini M, Behzadifar M (2018) Vaccines meet big data: state-of-the-art and future prospects. From the Classical 3Is ("Isolate-Inactivate-Inject") Vaccinology 1.0 to Vaccinology 3.0, vaccinomics, and beyond: a historical overview. Front Public Health 6:62. https://doi.org/10.3389/fpubh.2018.00062
3. Lahariya C (2014) A brief history of vaccines & vaccination in India. Indian J Med Res 139 (4):491–511
4. McCullers JA, Dunn JD (2008) Advances in vaccine technology and their impact on managed care. P T 33(1):35–41
5. WHO, Children: reducing mortality, 19 September 2018. https://www.who.int/en/news-room/fact-sheets/detail/children-reducing-mortality. Accessed 07 June 2019
6. Gizurarson S (1996) Optimal delivery of vaccines: clinical pharmacokinetic considerations. Clin Pharmacokinet 30(1):1–15
7. Jódar L, Feavers IM, Salisbury D, Granoff DM (2002) Development of vaccines against meningococcal disease. Lancet 359 (9316):1499–1508
8. Byrne MP, Smith LA (2000) Development of vaccines for prevention of botulism. Biochimie 82(9–10):955–966
9. Smith LA (1998) Development of recombinant vaccines for botulinum neurotoxin. Toxicon 36(11):1539–1548
10. Baldwin MR, Tepp WH, Przedpelski A, Pier CL, Bradshaw M, Johnson EA, Barbieri JT (2008) Subunit vaccine against the seven serotypes of botulism. Infect Immun 76 (3):1314–1318
11. Levine MM (2010) Immunogenicity and efficacy of oral vaccines in developing countries: lessons from a live cholera vaccine. BMC Biol 8(1):129
12. Ryan ET, Calderwood SB (2000) Cholera vaccines. Clin Infect Dis 31(2):561–565
13. Lobeck K, Drevet P, Léonetti M, Fromen-Romano C, Ducancel F, Lajeunesse E, Lemaire C, Ménez A (1998) Towards a recombinant vaccine against diphtheria toxin. Infect Immun 66(2):418–423
14. Rappuoli R, Malito E (2014) History of Diphtheria vaccine development. In: Burkovski A (ed) Corynebacterium diphtheriae and related toxigenic species. Springer, Dordrecht

15. Titball RW, Williamson ED (2004) Yersinia pestis (plague) vaccines. Expert Opin Biol Ther 4(6):965–973
16. Morris SR (2007) Development of a recombinant vaccine against aerosolized plague. Vaccine 25(16):3115–3117
17. Sato Y, Sato H (1999) Development of acellular pertussis vaccines. Biologicals 27 (2):61–69
18. Granström M (2011) The history of pertussis vaccination: from whole-cell to subunit vaccines. In: Plotkin S (ed) History of vaccine development. Springer, New York
19. Sutter RW, Prevots DR, Cochi SL (2000) Poliovirus vaccines. Progress toward global poliomyelitis eradication and changing routine immunization recommendations in the United States. Pediatr Clin N Am 47 (2):287–308
20. Baicus A (2012) History of polio vaccination. World J Virol 1(4):108–114. https://doi.org/10.5501/wjv.v1.i4.108
21. Liu Y, Wang J, Liu S, Du J, Wang L, Gu W, Xu Y, Zuo S, Xu E, An Z (2017) Introduction of inactivated poliovirus vaccine leading into the polio eradication endgame strategic plan; Hangzhou, China, 2010-2014. Vaccine 35 (9):1281–1286. https://doi.org/10.1016/j.vaccine.2017.01.034
22. Yusibov V, Hooper DC, Spitsin SV, Fleysh N, Kean RB, Mikheeva T, Deka D, Karasev A, Cox S, Randall J, Koprowski H (2002) Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. Vaccine 20(25–26):3155–3164
23. Dubey KK, Luke GA, Knox C, Kumar P, Pletschke BI, Singh PK, Shukla P (2018) Vaccine and antibody production in plants: developments and computational tools. Brief Funct Genomics 17(5):295–307. https://doi.org/10.1093/bfgp/ely020
24. Yang DK, Kim HH, Lee SH, Jeong WH, Tark D, Cho IS (2017) A genetically modified rabies vaccine (ERAGS) induces protective immunity in dogs and cattle. Clin Exp Vaccine Res 6(2):128–134. https://doi.org/10.7774/cevr.2017.6.2.128
25. Rosenthal SR, Merchlinsky M, Kleppinger C, Goldenthal KL (2001) Developing new smallpox vaccines. Emerg Infect Dis 7(6):920
26. Parrino J, Graham BS (2006) Smallpox vaccines: past, present, and future. J Allergy Clin Immunol 118(6):1320–1326
27. Moss B (2011) Smallpox vaccines: targets of protective immunity. Immunol Rev 239 (1):8–26. https://doi.org/10.1111/j.1600-065X.2010.00975.x
28. Figueiredo D, Turcotte C, Frankel G, Li Y, Dolly O, Wilkin G, Marriott D, Fairweather N, Dougan G (1995) Characterization of recombinant tetanus toxin derivatives suitable for vaccine development. Infect Immun 63(8):3218–3221
29. Verma R, Khanna P (2012) Tetanus toxoid vaccine: elimination of neonatal tetanus in selected states of India. Hum Vaccin Immunother 8(10):1439–1442. https://doi.org/10.4161/hv.21145
30. Guzman CA, Borsutzky S, Griot-Wenk M, Metcalfe IC, Pearman J, Collioud A, Favre D, Dietrich G (2006) Vaccines against typhoid fever. Vaccine 24(18):3804–3811
31. Szu SC (2013) Development of Vi conjugate–a new generation of typhoid vaccine. Expert Rev Vaccines 12(11):1273–1286
32. Jackson BR, Iqbal S, Mahon B, Centers for Disease Control and Prevention (CDC) (2015) Updated recommendations for the use of typhoid vaccine—advisory committee on immunization practices. MMWR Morb Mortal Wkly Rep 64(11):305–308
33. Hilleman MR (2000) Vaccines in historic evolution and perspective: a narrative of vaccine discoveries. J Hum Virol 3(2):63–76
34. Plotkin SA, Plotkin SL (2011) The development of vaccines: how the past led to the future. Nat Rev Microbiol 9(12):889–893. https://doi.org/10.1038/nrmicro2668
35. Plotkin SA, Gilbert PB (2012) Nomenclature for immune correlates of protection after vaccination. Clin Infect Dis 54(11):1615–1617
36. Plotkin S (2014) History of vaccination. Proc Natl Acad Sci U S A 111(34):12283–12287. https://doi.org/10.1073/pnas.1400472111
37. Stauffer F, El-Bacha T, Da Poian AT (2006) Advances in the development of inactivated virus vaccines. Recent Pat Antiinfect Drug Discov 1(3):291–296
38. Scott C (2004) Classifying vaccines: from cowpox to the cutting edge. BioProcess Int 2:14–23
39. Plotkin SA (2009) Vaccines: the fourth century. Clin Vaccine Immunol 16 (12):1709–1719. https://doi.org/10.1128/CVI.00290-09
40. Vartak A, Sucheck SJ (2016) Recent advances in subunit vaccine carriers. Vaccines (Basel) 4 (2):12. https://doi.org/10.3390/vaccines4020012
41. The history of vaccines; Different types of vaccines. https://www.historyofvaccines.org/content/articles/different-types-vaccines. Accessed 09 June 2019

42. Minor PD (2015) Live attenuated vaccines: historical successes and current challenges. Virology 479–480:379–392
43. Baxby D (2003) Smallpox vaccination techniques. 2. Accessories and aftercare. Vaccine 21 (13–14):1382–1390
44. Luca S, Mihaescu T (2013) History of BCG vaccine. Maedica (Buchar) 8(1):53–58
45. Collins ND, Barrett AD (2017) Live attenuated yellow fever 17D vaccine: a legacy vaccine still controlling outbreaks in modern day. Curr Infect Dis Rep 19(3):14. https://doi.org/10.1007/s11908-017-0566-9
46. Pliaka V, Kyriakopoulou Z, Markoulatos P (2012) Risks associated with the use of live-attenuated vaccine poliovirus strains and the strategies for control and eradication of paralytic poliomyelitis. Expert Rev Vaccines 11 (5):609–628
47. Werzberger A, Mensch B, Kuter B, Brown L, Lewis J, Sitrin R, Miller W, Shouval D, Wiens B, Calandra G, Ryan J (1992) A controlled trial of a formalin-inactivated hepatitis A vaccine in healthy children. N Engl J Med 327(7):453–457
48. Wood JM, Robertson JS (2004) From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. Nat Rev Microbiol 2(10):842
49. Monath TP, Lee CK, Julander JG, Brown A, Beasley DW, Watts DM, Hayman E, Guertin P, Makowiecki J, Crowell J, Levesque P (2010) Inactivated yellow fever 17D vaccine: development and nonclinical safety, immunogenicity and protective activity. Vaccine 28(22):3827–3840
50. Vijaykrishna D, Mukerji R, Smith GJ (2015) RNA virus reassortment: an evolutionary mechanism for host jumps and immune evasion. PLoS Pathog 11(7):e1004902. https://doi.org/10.1371/journal.ppat.1004902
51. Clark HF, Offit PA, Plotkin SA, Heaton PM (2006) The new pentavalent rotavirus vaccine composed of bovine (strain WC3)-human rotavirus reassortants. Pediatr Infect Dis J 25 (7):577–583
52. Tan LK, Carlone GM, Borrow R (2010) Advances in the development of vaccines against Neisseria meningitidis. N Engl J Med 362(16):1511–1520
53. Ni Y, Springer MJ, Guo J, Finger-Baker I, Wilson JP, Cobb RR, Turner D, Tizard I (2017) Development of a synthetic Vi polysaccharide vaccine for typhoid fever. Vaccine 35(51):7121–7126. https://doi.org/10.1016/j.vaccine.2017.10.081
54. Butler JC, Breiman RF, Campbell JF, Lipman HB, Broome CV, Facklam RR (1993) Pneumococcal polysaccharide vaccine efficacy: an evaluation of current recommendations. JAMA 270(15):1826–1831
55. Peltola H (2000) Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. Clin Microbiol Rev 13 (2):302–317
56. Lee CJ, Lee LH, Koizumi K (2002) Polysaccharide vaccines for prevention of encapsulated bacterial infections: part 1. Inf Med 19(3):127–133
57. AlonsoDeVelasco E, Verheul AF, Verhoef J, Snippe H (1995) Streptococcus pneumoniae: virulence factors, pathogenesis, and vaccines. Microbiol Mol Biol Rev 59(4):591–603
58. Dekleva ML (2002) Vaccine technology. In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology. Wiley, New York. https://doi.org/10.1002/0471250589.ebt215
59. Kelly DF, Moxon ER, Pollard AJ (2004) Haemophilus influenzae type b conjugate vaccines. Immunology 113(2):163–174. https://doi.org/10.1111/j.1365-2567.2004.01971.x
60. Maiden MC (2013) The impact of protein-conjugate polysaccharide vaccines: an endgame for meningitis? Philos Trans R Soc Lond Ser B Biol Sci 368(1623):20120147. https://doi.org/10.1098/rstb.2012.0147
61. Gupta RK (1998) Aluminum compounds as vaccine adjuvants. Adv Drug Deliv Rev 32 (3):155–172
62. Marciani DJ (2003) Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. Drug Discov Today 8(20):934–943
63. Alving CR, Peachman KK, Rao M, Reed SG (2012) Adjuvants for human vaccines. Curr Opin Immunol 24(3):310–315
64. Mulard L (2007) Carbohydrates and vaccines: from purified polysaccharides to semi-synthetic glycoconjugate vaccines. Ann Pharm Fr 65(1):14–32
65. Colombo C, Pitirollo O, Lay L (2018) Recent advances in the synthesis of glycoconjugates for vaccine development. Molecules 23 (7):1712. https://doi.org/10.3390/molecules23071712
66. Gupta SK, Shukla P (2018) Glycosylation control technologies for recombinant therapeutic proteins. Appl Microbiol Biotechnol 102(24):10457–10468

67. Vartak A, Sucheck S (2016) Recent advances in subunit vaccine carriers. Vaccine 4(2):12
68. Zhang N, Zheng BJ, Lu L, Zhou Y, Jiang S, Du L (2015) Advancements in the development of subunit influenza vaccines. Microbes Infect 17(2):123–134. https://doi.org/10.1016/j.micinf.2014.12.006
69. Astray RM, Jorge SA, Pereira CA (2017) Rabies vaccine development by expression of recombinant viral glycoprotein. Arch Virol 162(2):323–332. https://doi.org/10.1007/s00705-016-3128-9
70. MacLennan CA, Martin LB, Micoli F (2014) Vaccines against invasive Salmonella disease: current status and future directions. Hum Vaccin Immunother 10(6):1478–1493. https://doi.org/10.4161/hv.29054
71. Baliban SM, Yang M, Ramachandran G, Curtis B, Shridhar S, Laufer RS et al (2017) Development of a glycoconjugate vaccine to prevent invasive Salmonella Typhimurium infections in sub-Saharan Africa. PLoS Negl Trop Dis 11(4):e0005493. https://doi.org/10.1371/journal.pntd.0005493
72. Micoli F, Romano MR, Tontini M, Cappelletti E, Gavini M, Proietti D, Rondini S, Swennen E, Santini L, Filippini S, Balocchi C (2013) Development of a glycoconjugate vaccine to prevent meningitis in Africa caused by meningococcal serogroup X. PNAS 110(47):19077–19082
73. Dertzbaugh MT (1998) Genetically engineered vaccines: an overview. Plasmid 39 (2):100–113
74. Gupta SK, Shukla P (2017) Gene editing for cell engineering: trends and applications. Crit Rev Biotechnol 37(5):672–684. https://doi.org/10.1080/07388551.2016.1214557
75. Dangi AK, Sinha R, Dwivedi S, Gupta SK, Shukla P (2018) Cell line techniques and gene editing tools for antibody production: a review. Front Pharmacol 9:630. https://doi.org/10.3389/fphar.2018.00630
76. Nascimento IP, Leite LC (2012) Recombinant vaccines and the development of new vaccine strategies. Braz J Med Biol Res 45 (12):1102–1111
77. Buck CB, Day PM, Trus BL (2013) The papillomavirus major capsid protein L1. Virology 445(1–2):169–174
78. Roland KL, Cloninger C, Kochi SK, Thomas LJ, Tinge SA, Rouskey C, Killeen KP (2007) Construction and preclinical evaluation of recombinant Peru-15 expressing high levels of the cholera toxin B subunit as a vaccine against enterotoxigenic Escherichia coli. Vaccine 25(51):8574–8584
79. Gustavsson M, Do TH, Lüthje P, Tran NT, Brauner A, Samuelson P, Truong NH, Larsson G (2015) Improved cell surface display of Salmonella enterica serovar Enteritidis antigens in Escherichia coli. Microb Cell Factories 14:47. https://doi.org/10.1186/s12934-015-0227-3
80. Peng W, Liu S, Meng J, Huang J, Huang J, Tang D, Dai Y (2019) Profiling the TRB and IGH repertoire of patients with H5N6 Avian Influenza Virus Infection by high-throughput sequencing. Sci Rep 9(1):7429
81. Auniņš JG (2009) Viral vaccine production in cell culture. In: Flickinger MC (ed) Encyclopedia of industrial biotechnology: bioprocess, bioseparation, and cell technology. Wiley, New York, pp 1–35
82. Karch CP, Burkhard P (2016) Vaccine technologies: from whole organisms to rationally designed protein assemblies. Biochem Pharmacol 120:1–14
83. WHO Expert Committee on Biological Standardization. WHO technical report series, no. 941—Fifty-sixth report, Annex 6: recommendations for whole-cell pertussis vaccine, 301–33. WHO HQ, Geneva, 2007. http://apps.who.int/medicinedocs/en/cl/CL7.13/clmd,50.html
84. Chen Z, He Q (2017) Immune persistence after pertussis vaccination. Hum Vaccin Immunother 13(4):744–756
85. Xing D, Markey K, Das RG, Feavers I (2014) Whole-cell pertussis vaccine potency assays: the Kendrick test and alternative assays. Expert Rev Vaccines 13(10):1175–1182. https://doi.org/10.1586/14760584.2014.939636
86. Corbel MJ, Xing DK (2004) Toxicity and potency evaluation of pertussis vaccines. Expert Rev Vaccines 3(1):89–101
87. Holmgren J, Levine MM (2015) Vaccines against bacterial enteric infections. In: Mestecky J, Strober W, Russel MW, Kelsall BL, Cheroutre H, Lambrecht BN (eds) Mucosal immunology, vol 1, 4th edn. Academic, New York, pp 1047–1082. https://doi.org/10.1016/C2010-1-65194-2
88. Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J (1995) A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. N Engl J Med 334 (6):349–356
89. Cabral MP, García P, Beceiro A, Rumbo C, Pérez A, Moscoso M, Bou G (2017) Design of live attenuated bacterial vaccines based on

D-glutamate auxotrophy. Nat Commun 8:15480

90. Serruto D, Serino L, Masignani V, Pizza M (2009) Genome-based approaches to develop vaccines against bacterial pathogens. Vaccine 27(25–26):3245–3250
91. Essig A, Longbottom D (2015) Chlamydia abortus: new aspects of infectious abortion in sheep and potential risk for pregnant women. Curr Clin Microbiol Rep 2(1):22–34
92. Phillips S, Quigley BL, Timms P (2019) Seventy years of Chlamydia vaccine research–limitations of the past and directions for the future. Front Microbiol 10:70. https://doi.org/10.3389/fmicb.2019.00070
93. Sharma A, Rajan G, Kharb R, Biswas S (2016) Genome wide analysis of Chlamydia pneumoniae for candidate vaccine development. Curr Comput Aided Drug Des 12(3):206–215
94. Bi Q, Ferreras E, Pezzoli L et al (2017) Protection against cholera from killed whole-cell oral cholera vaccines: a systematic review and meta-analysis. Lancet Infect Dis 17 (10):1080–1088
95. Chowdhury SR, Sarkar A, Kumar RP, Kumar V, Saxena D, Barman BD (2018) Periodontal vaccine–a review. J Adv Med Dent Scie Res 6(3):4–9
96. de Castro JT, Miyaji EN, Soares-Schanoski A, Debrie AS, Bezerra MF, Akamatsu MA, de Oliveira MS (2018) Whole cell pertussis vaccine expressing the Pneumococcal Surface protein A as an approach for a double vaccine. Int J Infect Dis 73:356
97. Soema PC, Kompier R, Amorij JP, Kersten GF (2015) Current and next generation influenza vaccines: formulation and production strategies. Eur J Pharm Biopharm 94:251–263
98. Kraan H, ten Have R, van der Maas L, Kersten G, Amorij JP (2016) Incompatibility of lyophilized inactivated polio vaccine with liquid pentavalent whole-cell-pertussis-containing vaccine. Vaccine 34(38):4572–4578
99. Zhang W, Cheng N, Wang Y, Zheng X, Zhao Y, Wang H, Yang S (2019) Adjuvant activity of PCP-II, a polysaccharide from Poria cocos, on a whole killed rabies vaccine. Virus Res 270:197638. https://doi.org/10.1016/j.virusres.2019.06.001
100. Domenech de Cellès M, Magpantay FM, King AA, Rohani P (2016) The pertussis enigma: reconciling epidemiology, immunology and evolution. Proc R Soc Lond B Biol Sci 283(1822):20152309
101. Mattia G, Puglisi R, Ascione B, Malorni W, Carè A, Matarrese P (2018) Cell death-based treatments of melanoma: conventional treatments and new therapeutic strategies. Cell Death Dis 9(2):112. https://doi.org/10.1038/s41419-017-0059-7
102. Yarchoan M, Johnson BA III, Lutz ER, Laheru DA, Jaffee EM (2017) Targeting neoantigens to augment antitumour immunity. Nat Rev Cancer 17(4):209
103. Keenan BP, Jaffee EM (2012) Whole cell vaccines—past progress and future strategies. Semin Oncol 39(3):276–286. https://doi.org/10.1053/j.seminoncol.2012.02.007
104. Barouch DH, Letvin NL, Seder RA (2004) The role of cytokine DNAs as vaccine adjuvants for optimizing cellular immune responses. Immunol Rev 202(1):266–274
105. Kongsted P, Borch TH, Ellebaek E, Iversen TZ, Andersen R, Met Ö, Svane IM (2017) Dendritic cell vaccination in combination with docetaxel for patients with metastatic castration-resistant prostate cancer: a randomized phase II study. Cytotherapy 19 (4):500–513
106. World Health Organization fact sheet Cholera. https://www.who.int/news-room/fact-sheets/detail/cholera. Accessed 28 July 2019
107. Gupta SS, Bharati K, Sur D, Khera A, Ganguly NK, Nair GB (2016) Why is the oral cholera vaccine not considered an option for prevention of cholera in India? Analysis of possible reasons. Indian J Med Res 143 (5):545–551. https://doi.org/10.4103/0971-5916.187102
108. Ciglenecki I, Bichet M, Tena J, Mondesir E (2013) Cholera in pregnancy: outcomes from a specialized cholera treatment unit for pregnant women in Leogane. Haiti PLoS Negl Trop Dis 7:e2368
109. Grout L, Martinez-Pino I, Ciglenecki I, Keita S, Traore B, Delamou D, Serafini M (2015) Pregnancy outcomes after a mass vaccination campaign with an oral cholera vaccine in Guinea: a retrospective cohort study. PLoS Negl Trop Dis 9(12):e0004274
110. Khan AI, Ali M, Lynch J, Kabir A, Excler JL, Khan MA, Islam MT, Akter A, Chowdhury F, Saha A, Khan IA, Desai SN, Kim DR, Saha NC, Singh AP, Clemens JD, Qadri F (2019) Safety of a bivalent, killed, whole-cell oral cholera vaccine in pregnant women in Bangladesh: evidence from a randomized placebo-controlled trial. BMC Infect Dis 19 (1):422. https://doi.org/10.1186/s12879-019-4006-3
111. Khan AI, Chowdhury F, Leung DT, Larocque RC, Harris JB, Ryan ET, Calderwood SB, Qadri F (2015) Cholera in pregnancy: clinical

and immunological aspects. Int J Infect Dis 39:20–24. https://doi.org/10.1016/j.ijid.2015.08.006

112. Greenwood BM, Fidock DA, Kyle DE, Kappe SH, Alonso PL, Collins FH, Duffy PE (2008) Malaria: progress, perils, and prospects for eradication. J Clin Invest 118(4):1266–1276
113. Ellis RD, Sagara I, Doumbo O, Wu Y (2010) Blood stage vaccines for Plasmodium falciparum: current status and the way forward. Hum Vaccin 6(8):627–634
114. Richards JS, Beeson JG (2009) The future for blood-stage vaccines against malaria. Immunol Cell Biol 87(5):377–390
115. Depelsenaire ACI, Kendall MAF, Young PR, Muller DA (2017) Introduction to vaccines and vaccination. In: Skwarczynski M, Toth I (eds) Micro and nanotechnology in vaccine development. Elsevier, Amsterdam
116. Barrett AD (2015) 9 licensed vaccines for humans. Vaccinology: an essential guide. 152–180
117. Pattison DI, Davies MJ (2001) Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds. Chem Res Toxicol 14:1453–1464. https://doi.org/10.1021/tx0155451
118. Hakim H, Alam MS, Sangsriratanakul N, Nakajima K, Kitazawa M, Ota M, Toyofuku C, Yamada M, Thammakarn C, Shoham D, Takehara K (2016) Inactivation of bacteria on surfaces by sprayed slightly acidic hypochlorous acid water: in vitro experiments. J Vet Med Sci 78(7):1123–1128. https://doi.org/10.1292/jvms.16-0075
119. Pattison DI, Hawkins CL, Davies MJ (2003) Hypochlorous acid-mediated oxidation of lipid components and antioxidants present in low-density lipoproteins: absolute rate constants, product analysis, and computational modeling. Chem Res Toxicol 16(4):439–449
120. Chiang CL, Kandalaft LE, Tanyi J, Hagemann AR, Motz GT, Svoronos N, Montone K, Mantia-Smaldone GM, Smith L, Nisenbaum HL, Levine BL, Kalos M, Czerniecki BJ, Torigian DA, Powell DJ Jr, Mick R, Coukos G (2013) A dendritic cell vaccine pulsed with autologous hypochlorous acid-oxidized ovarian cancer lysate primes effective broad antitumor immunity: from bench to bedside. Clin Cancer Res 19(17):4801–4815
121. Seo HS (2015) Application of radiation technology in vaccines development. Clin Exp Vaccine Res 4(2):145–158
122. da Silva Aquino KA (2012) Sterilization by gamma irradiation. In Gamma radiation. IntechOpen. https://doi.org/10.5772/34901
123. Babb R, Chen A, Hirst TR et al (2016) Intranasal vaccination with γ-irradiated Streptococcus pneumoniae whole-cell vaccine provides serotype-independent protection mediated by B-cells and innate IL-17 responses. Clin Sci 130(9):697–710
124. Parham P (2014) The immune system. Garland Science, New York
125. Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11(4s):S45

Chapter 14

O-Antigen Extraction, Purification, and Chemical Conjugation to a Carrier Protein

Francesca Micoli, Carlo Giannelli, and Roberta Di Benedetto

Abstract

A variety of bacterial infections have been tackled by glycoconjugates over the recent years, and more vaccines are either under development at preclinical level or in clinical trials. So far, licensed glycoconjugate vaccines have made use of capsular polysaccharides or derived fragments. Today, many glycoconjugates are making use of other classes of sugars, in particular, the O-antigen portion of lipopolysaccharide molecules. Here, we report a simplified method for O-antigen extraction and purification that avoids the step of lipopolysaccharide isolation. Also, a selective chemistry for terminal linkage of O-antigen chains to a carrier protein is described, together with analytical methods for intermediates and final conjugate characterization.

Key words O-antigen, Polysaccharide, Glycoconjugation, Vaccine, Purification, *Salmonella*

1 Introduction

Surface polysaccharides from bacteria have been used for many years in vaccine applications, being both essential virulence factors and targets for protective antibodies. Covalent conjugation to an appropriate carrier protein was discovered to be an important means to increase the immunogenicity of polysaccharides [1, 2]. Glycoconjugates provide T cell-dependent immunogenicity against the saccharide hapten. With the involvement of T cells, immunological memory is invoked, and affinity maturation and isotype switching occur. Differently from polysaccharides, glycoconjugate vaccines are effective in young infants [3, 4].

Licensed glycoconjugate vaccines are produced by chemical conjugation of capsular polysaccharides to prevent meningitis caused by meningococcus, pneumococcus and *Haemophilus influenzae* type b. However, other classes of carbohydrates, including the O-antigen portion of lipopolysaccharide (LPS) molecules in gram-negative bacteria, represent attractive targets for glycoconjugates. O-antigen-based conjugate vaccines have been proposed for

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many human pathogens including *Salmonella* [5], *Shigella* species [6], *Vibrio cholerae* [7], and *Escherichia coli* [8].

Lipopolysaccharide (LPS) is the major component of the surface of most gram-negative bacteria. It is constituted by a polysaccharide chain of repeating units, the O-antigen, linked to a core region containing 10–12 sugar units, that, in turn, is covalently bound to lipid A through the 2-keto-3-deoxyoctonate (KDO). Lipid A is highly conserved and exerts endotoxic activity, while the O-antigen chain differs between serovars and is a major contributor to the serological specificity of bacteria.

In most published protocols for producing O-antigen-based vaccines, O-antigen purification is carried out after LPS extraction and hydrolysis. Traditional methods for O-antigen extraction involve sedimentation of the bacteria, inactivation of the culture by formalin fixation, hot phenol extraction of the LPS [9], and treatment of the extracted LPS with acetic acid or anhydrous hydrazine [10] for LPS hydrolysis prior to O-antigen purification, usually performed by treatments with enzymes and size-exclusion chromatography to remove contaminants such as proteins and nucleic acids. The traditional process for O-antigen isolation and purification overall is complex and time consuming and involves manipulation of large volumes of hazardous phenol and toxic intermediate LPS.

Here, we describe a simplified method for O-antigen isolation, which employs acetic acid hydrolysis taking place directly in the bacterial culture, avoiding operator exposure to pathogenic bacteria. This treatment cleaves the labile linkage between KDO, at the proximal end of the core oligosaccharide, and lipid A, releasing the O-antigen chain attached to the core sugars (Fig. 1). The hydrolyzed O-antigen chain and core sugars are indicated here as OAg for simplicity. We also describe the steps for OAg purification. The process described has been built based on the structural characteristics of *Salmonella* OAg [11] and can be generally applied to OAg with neutral sugar chains. The extraction procedure is general and applicable to any LPS.

We also describe a conjugation protocol for linkage of the OAg to an appropriate carrier protein, based on the sequential insertion of adipic acid dihydrazide (ADH) and adipic acid bis(N-hydroxy succinimide) ester (SIDEA) as linkers, using the terminus KDO, thus leaving the repeating units of the O-antigen chain unmodified [12–14]. This protocol is generic and independent from O-antigen chain structural features. The complete panel of analytical methods for purified OAg, conjugate and intermediates characterization is also described.

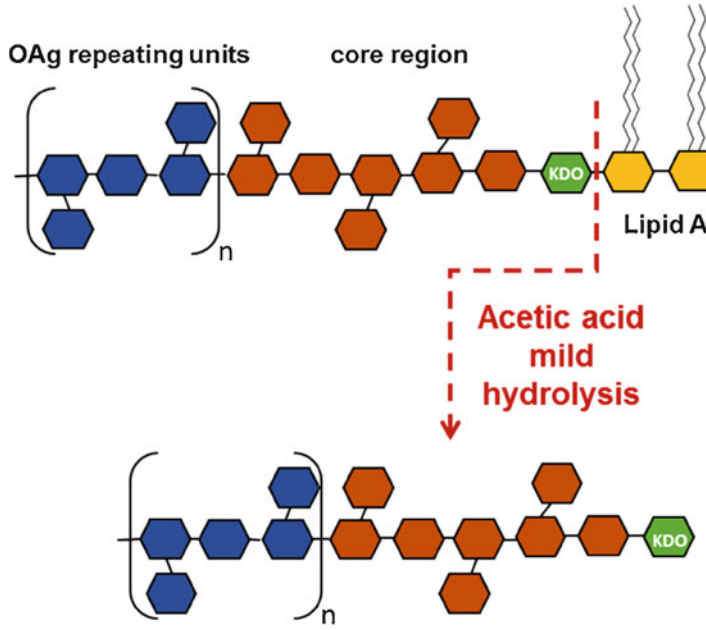


Fig. 1 O-antigen isolation through mild acetic acid hydrolysis: the labile linkage between KDO, at the proximal end of the core, and lipid A is cleaved releasing the O-antigen chain attached to the core sugars

2 Materials

Prepare all solutions using ultrapure water (grade 1, >18 MΩ-cm at 25 °C; prepared by purifying deionized water) and analytical grade reagents. Prepare and store all reagents at room temperature (RT), filter all the solutions using a 0.22 μm filtration system (e.g., Merck Stericup), unless otherwise indicated.

2.1 OAg purification

1. NaCl 1 M: Add about 900 mL water in a 1 L graduated cylinder equipped with a magnetic stir bar. Weigh 58.44 g NaCl and transfer into the cylinder. Mix on a magnetic stirrer (*see* **Note 1**) until all the powder is dissolved. Add water up to 1 L total volume and mix again.
2. Citrate buffer 200 mM pH 2.7 (*see* **Note 2**): Weigh 28.9 g citric acid monohydrate and 13.3 g sodium citrate monobasic and transfer into a cylinder filled with about 900 mL water. Mix until the powder is completely dissolved; adjust the pH if needed (*see* **Note 3**) and add water up to 1 L total volume.
3. Citrate buffer 20 mM pH 3: Weigh 2.3 g citric acid monohydrate and 1.9 g sodium citrate monobasic and prepare a 1 L buffer solution, as described in **item 2**.

4. Citrate buffer 20 mM pH 3, NaCl 1 M: Weigh 2.3 g citric acid monohydrate, 1.9 g sodium citrate monobasic and 58.44 g NaCl and prepare a 1 L buffer solution, as described in **item 2**.
5. Sodium phosphate monobasic (NaH_2PO_4) 500 mM: Weigh 60 g NaH_2PO_4 and prepare a 1 L solution.
6. Calcium chloride ($CaCl_2$) 5 M: Weigh 554.9 g $CaCl_2$ and prepare a 1 L solution.
7. Tangential flow filtration (TFF) 30 KDa Sartorius Hydrosart 200 cm^2 membrane (stabilized cellulose-based membrane).
8. Sartobind S MA75 filter (housing polysulfone membrane matrix stabilized cellulose, nominal pore size >3 μm strong cation exchanger, membrane area 75 cm^2).

2.2 OAg Conjugation to Carrier Protein and Conjugate Purification

1. Acetate buffer 100 mM pH 4.5: Withdraw 3.4 mL acetic acid and 3.3 g sodium acetate (AcONa) and prepare a 1 L buffer solution, as described in Subheading 2.1, **item 2**.
2. Citrate buffer 100 mM pH 3: Weigh 11.1 g citric acid monohydrate and 10 g sodium citrate monobasic and prepare a 1 L buffer solution, as described in Subheading 2.1, **item 2**.
3. Phosphate buffer 100 mM pH 7.2: Weigh 3.2 g NaH_2PO_4 and 10.4 g sodium phosphate dibasic (Na_2HPO_4) and prepare a 1 L buffer solution, as described in Subheading 2.1, **item 2**.
4. Phosphate buffer 50 mM pH 7.2, NaCl 150 mM: Weigh 1.8 g NaH_2PO_4, 4.9 g Na_2HPO_4, and 8.8 g NaCl and prepare a 1 L buffer solution, as described in Subheading 2.1, **item 2**.
5. 16/90 cm Sephacryl S-300 HR column.

2.3 Analytical Methods

1. HiTrap desalting column, 5 mL, prepacked with Sephadex G-25 Superfine (GE Healthcare).
2. Phenol sulfuric colorimetric method.
   (a) 5% Phenol solution.
   - Weigh on technical balance a 100 mL glass bottle with its screw cap and set it as tare.
   - Under chemical hood transfer some phenol in the bottle and close it.
   - Weigh on the technical balance (tared with the empty bottle and cap) the amount of phenol transferred in the bottle.
   - Repeat operations described in the previous sections in order to reach a quantity close to 5 g.
   - Calculate the quantity of water needed to obtain a 5% (w/v) phenol solution using the following formula:

     $$\text{Water to add (mL)} = \text{phenol weight (g)} \times 20.$$

- Under chemical hood add the required quantity of water using a suitable graduated cylinder and mix the bottle in order to dissolve the phenol.

(b) 0.5 mg/mL glucose (Glc) solution.

- Weight on analytical balance about 22 mg of Glc monohydrate, transfer it into a glass bottle.
- Calculate the quantity of water needed to obtain a 0.5 mg/mL Glc solution using the following formula:

$$\text{Water to add (mL)} = \text{Glc weight (mg)} \times 1.82.$$

- Add the required quantity of water using a suitable graduated cylinder and mix the bottle in order to dissolve Glc.

3. High-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD).

(a) Rhamnose (Rha), galactose (Gal), glucose (Glc), mannose (Man) sugars mix (neutral sugar) standard solution 11.25 μg/mL each.

- In different 2 mL vials, weigh accurately about 9 mg of each standard and dissolve them in a suitable amount of water added by weighting on balance, in order to obtain solutions at 4.5 mg/mL. To calculate the amount of water and sugar to be added to each vial use the following formulas:

$$\mu\text{g sugar} = \text{weight } \mu\text{g} \times \text{purity}\% \times \frac{\text{no hydrated sugar MW}}{\text{hydrated sugar MW}}$$

(*see* **Note 4**).

(If the sugar is not in the hydrated form the last ratio present in the formula is equal to 1)

$$\mu\text{L water} = \frac{\mu\text{g sugar}}{4.5}$$

- Weigh an empty 500 mL glass bottle on the technical balance and transfer 1 mL of each sugar solution prepared in previous section; then add water up to achieve a total weight of 400 g in order to obtain a solution with a concentration of 11.25 μg/mL for each standard.

(b) N-acetylglucosamine (GlcNAc) standard solution 11.25 μg/mL.

- In a 2 mL vial weigh about 9 mg of GlcNAc and dissolve it in a suitable amount of water added by weighting on balance, in order to obtain a solution at 4.5 mg/mL. To

calculate the amount of water and sugar to be added, use the following formulas:

$$\mu g\ \text{sugar} = \text{weight}\ \mu g \times \text{purity}\% \times \frac{\text{no hydrated sugar MW}}{\text{hydrated sugar MW}}$$

(*see* **Note 4**)

(If the sugar is not in the hydrated form, the last ratio present in the formula is equal to 1)

$$\mu L\ \text{water} = \frac{\mu g\ \text{sugar}}{4.5}$$

- Weigh an empty 500 mL glass bottle on the technical balance and transfer 1 mL of the sugar solution prepared in previous section; then add water up to achieve a total weight of 400 g in order to obtain a solution with a concentration of 11.25 μg/mL for GlcNAc.

(c) Thermo Dionex CarboPac PA10 column (4 × 250 mm) coupled with a PA10 guard column (4 × 50 mm).

(d) TFA 8 M solution.

- Under chemical hood, with the glass cylinder, measure 30 mL of TFA and transfer it into the glass bottle.
- With a pipette, add in the bottle 20.5 mL of water and gently mix.

(e) TFA 4 M solution.

- Dilute 10 mL of TFA 8 M adding 10 mL of water.

(f) Eluents.

- Sodium hydroxide (NaOH) 50 mM.
  - Fill the eluent bottle with 2 L of water using the volumetric flask.
  - Degas for 15 min by bubbling Helium.
  - With a pipette, add 5.2 mL of NaOH 50% (Baker, cod. 7067) to the bottle.
  - Degas for 10 min more.
- NaOH 500 mM.
  - Fill the eluent bottle with 2 L of water using the volumetric flask.
  - With a pipette, remove 26 mL of water from the bottle.
  - Degas for 15 min by bubbling Helium.
  - With a pipette, add 26 mL of NaOH 50% to the bottle.
  - Degas for 10 min more.

- Sodium acetate (AcONa) 1 M NaOH 100 mM.
  - Degas 1.5 L of water for 15 min by bubbling Helium.
  - Add, directly in the AcONa container, about 500 mL of degassed water and dissolve the whole content of AcONa (Thermo Dionex, cod. 059326). Transfer the solution in the 1 L volumetric flask.
  - Wash twice the AcONa bottle with about 100 mL more of degassed water and add the solution to the 1 L volumetric flask.
  - Fill the 1 L volumetric flask with degassed water, up to 1 L of volume for the AcONa solution.
  - Place a 0.22 μm vacuum-cup filter on an empty eluent 2 L bottle, connect it to the vacuum system and filter the prepared 1 L solution.
  - Degas the solution for 10 min by bubbling Helium.
  - With a pipette, add 5.2 mL of NaOH 50% to the bottle.
  - Degas with Helium for 10 min more.

4. Micro BCA.
   (a) Thermo micro BCA Protein Assay kit (BSA calibration curve range: 5–20 μg/mL).
5. Charles River Endosafe-PTS instrument cartridges for chromogenic kinetic LAL (Limulus amoebocyte lysate).
6. Size-exclusion high-performance liquid chromatography (HPLC-SEC).
   (a) Tosoh TSK gel G3000 PWXL column (30 cm × 7.8 mm; cod. 808021) with a Tosoh TSK gel PWXL guard column (4.0 cm × 6.0 mm; cod. 808033).
   (b) Tosoh TSK gel 6000 PW column (30 cm × 7.5 mm; cod. 805765) connected in series with a Tosoh TSK gel 5000 PW column (30 cm × 7.5 mm; cod. 805764) with Tosoh TSK gel PWH guard column (7.5 cm x 7.5 mm; cod. 806732).
   (c) 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% ACN, pH 7.2.
     - Weigh 5.85 g of NaCl and put the powder inside the 1 L glass graduated cylinder.
     - Weigh 3.22 g of NaH_2PO_4 and put the powder inside the 1 L glass graduated cylinder.
     - Weigh 10.37 g of Na_2HPO_4 and put the powder inside the 1 L glass graduated cylinder.
     - Add water up to 850 mL in the 1 L graduated cylinder. Put a magnetic stir bar inside the cylinder and solubilize the powder placing the cylinder on a magnetic stirrer.

- Measure, in a 50 mL graduated cylinder, 50 mL of acetonitrile (ACN) and add them in the 1 L cylinder with buffer solution.
- Add water to bring the total volume to 1 L.
- Leave the cylinder on the stirrer until the solution is completely homogeneous.
- Measure the pH with the electrode (7.20 ± 0.06).
- Filter by vacuum the solution with a 0.22 μm filter.

(d) λ-DNA Molecular Weight Marker III 0.12–21.2 kb (Roche; cod. 10528552001): dilute it 20× with HPLC eluent before injection.

(e) Sodium azide (NaN_3) solution (200 mg/L solution).

(f) Semicarbazide solution (100 mg semicarbazide hydrochloride + 90.5 mg of AcONa anhydrous in 10 mL of water).

(g) 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) ammonium salt 40 μg/mL solution (the concentration value is referred to ammonium salt).

7. Nuclear magnetic resonance (NMR) spectroscopy.
   (a) Sodium deuteroxide (NaOD 4 M).
   - Weigh on the balance about 160 mg of NaOH and transfer in a tube.
   - Add the quantity of deuterium hydroxide (D_2O) needed to achieve 4 M solution:

$$\mu L\ D_2O = \frac{\text{mg NaOH}}{160} \times 1000$$

   - Close the tube and mix with vortex mixer. Keep the tube closed until use in order to limit NaOH carbonation.

8. 2,4,6-trinitrobenzene sulfonate (TNBS) colorimetric method.
   (a) Sodium hydrogen carbonate 4% solution (1 g sodium hydrogen carbonate dissolved in 25 mL of water).
   (b) TNBS 0.1% solution (50 μL of TNBS 1 M solution in 14.6 mL of water).
   (c) Adipic acid dihydrazide (ADH) 600 nmol/mL in water.
   (d) ADH 100 nmol/mL in water (500 μL of ADH 600 nmol/mL diluted with 2500 μL of water).

9. Reversed-phase high-performance liquid chromatography (RP-HPLC).
   (a) Kinetex C18 column (Phenomenex, 2.6 mm 100A 15,064.6 mm, cod. 00F-4462-E0).

(b) Pyrene butyric acid (PBA) 2.5 mM.

- Weigh about 7.21 mg of PBA and transfer it in a tube.
- Calculate the volume of dimethyl sulfoxide (DMSO) required to dissolve PBA with the following equation: mL of DMSO = PBA weight (mg)/0.721.

  (Where 0.721 = 2.5 mM × 288.34 (PBA MW)/1000)

  Add the calculated volume of DMSO into the tube to dissolve PBA.
- Remark: measure DMSO volume only with pipettes equipped with polypropylene tips.

(c) Pyridine 20% solution in DMSO (mix in a tube 0.348 mL of pyridine and 1.39 mL of DMSO).

- Remark: measure DMSO and pyridine volumes only with pipettes equipped with polypropylene tips.

(d) Adipic acid dihydrazide (ADH) 10 mg/mL standard solution (150 mg of ADH dissolved in 15 mL of water).

(e) ADH 50 μg/mL standard solution (dilute 100 μL of ADH 10 mg/mL standard solution by adding 19.9 mL of water).

(f) ADH 2 μg/mL standard solution (dilute 1000 μL of ADH 50 μg/mL standard solution by adding 24.0 mL water).

(g) 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) 2 M solution in water.

- Weigh 668 mg of EDC, transfer it in a 15 mL tube, and store the tube at −20 °C. When the EDC solution will be needed to derivatize the samples add 1742 μL of water and dissolve by vortexing to obtain the solution.
- Remark: EDC powder must be dissolved immediately before its use.

(h) 65% ACN solution (add 26 mL of ACN to 14 mL of water).

10. Free saccharide analysis.

(a) Deoxycholate sodium salt 1% solution.

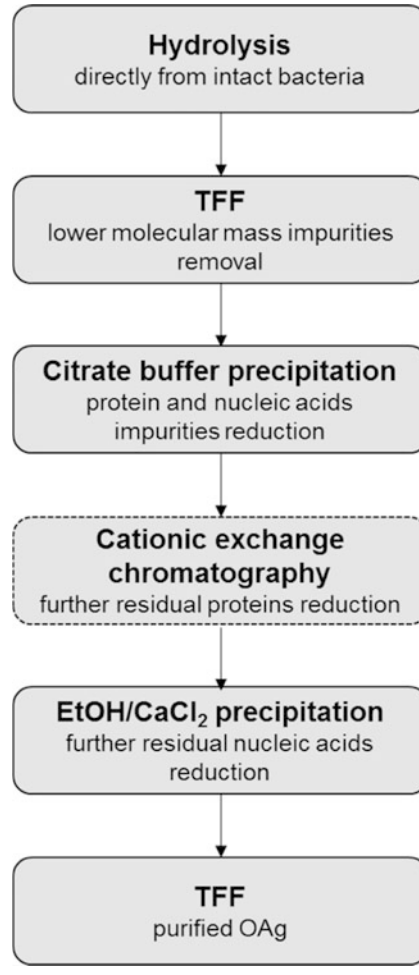
- Weigh 1000 ± 25 mg of deoxycholate sodium salt and transfer the powder in a 250 mL glass bottle.
- With the graduated cylinder add to the bottle a total amount of 100 mL of water.
- Place in the bottle the magnetic stirring bar and homogenize all the content by mixing on a magnetic stirrer.

3 Methods

3.1 OAg Extraction and Purification

OAg extraction is performed directly on intact bacteria avoiding the step of LPS extraction, hydrolyzing the OAg from the lipid A while still attached to the bacterial membrane. The O-antigen chain plus the core is released in solution, while the lipid A remains attached to the bacterial membrane and pelleted by centrifugation. Here we report the extraction, purification (Scheme 1), and analytical characterization of *S. paratyphi* A OAg, as an example (Fig. 2). The same procedure has been applied to OAg from *S. typhimurium*, *S. enteritidis*, and *Shigella flexneri* strains and can be applied with modifications, for what concerns the purification steps, to OAg from other strains.

1. Add acetic acid to the bacterial culture at a final concentration of 1–2% (v/v), check the pH is in the range of 3.5–4.7, and incubate at 100 °C for 6 h (*see* **Note 5**).
2. At the end of hydrolysis, add 28% NH_4OH, under chemical hood or directly in the bioreactor, based on the scale used, to increase the pH to around 6 and collect the supernatant by centrifugation. Indicate this solution as "post-hydrolysis supernatant."
3. Concentrate the "post hydrolysis supernatant" (*see* **Note 6**) five- to tenfold by Tangential Flow Filtration (TFF) with a 30-kDa molecular weight cut-off (MWCO), 200-cm^2 Hydrosart membrane. After concentration, perform diafiltration against 20 diavolumes of 1 M NaCl and another 10 diavolumes of water (*see* **Note 7**). Collect the retentate and indicate this solution as "TFF-1 retentate."
   - Remark: membrane with a different molecular weight cut-off could be used according to the OAg size.
4. Add citrate buffer 200 mM pH 2.7 with continuous stirring to the "TFF-1 retentate" to have a final citrate concentration of 20 mM. Check that pH of the OAg solution is around 3. Mix at RT for 30 ± 5 min. After this time, collect the supernatant after centrifugation (12,000 RCF at 15 °C for 30 min) (*see* **Note 8**). Indicate this solution as "post-pH 3" (*see* **Note 9**).
5. To "post-pH 3" (*see* **Note 10**) add, while stirring at RT, 500 mM Na_2HPO_4, absolute ethanol (EtOH), and 5 M $CaCl_2$ to give 18 mM NaH_2PO_4, 24% EtOH, (v/v), and 200 mM $CaCl_2$ concentrations in the final mixture. Perform this addition within a chemical hood. Mix at room temperature for 30 ± 5 min. After this time, collect the supernatant after centrifugation (12,000 RCF at 15 °C for 30 min) (*see* **Note 11**). Indicate this solution as "post-EtOH/$CaCl_2$."



Scheme 1 Flowchart of O-antigen purification process starting from the biomass

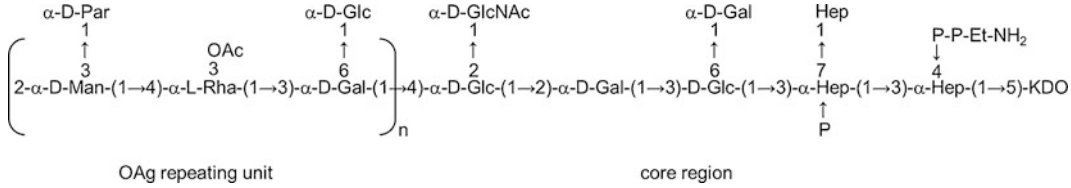


Fig. 2 Structure of *S. paratyphi* A O-antigen chain linked to the core region, after acetic acid hydrolysis

6. Concentrate the "post-EtOH/$CaCl_2$" tenfold using TFF with a 30-kDa molecular weight cut-off (MWCO), 200-cm^2 Hydrosart membrane. After concentration, perform diafiltration against 10 diavolumes of water (*see* **Note 7**). Collect the retentate and indicate this solution as "TFF-2 retentate."
7. Filter "TFF-2 retentate" through a 0.22 μm PES Nalgene filter, indicate the solution as "post 0.22 μm" and store at 4 °C.

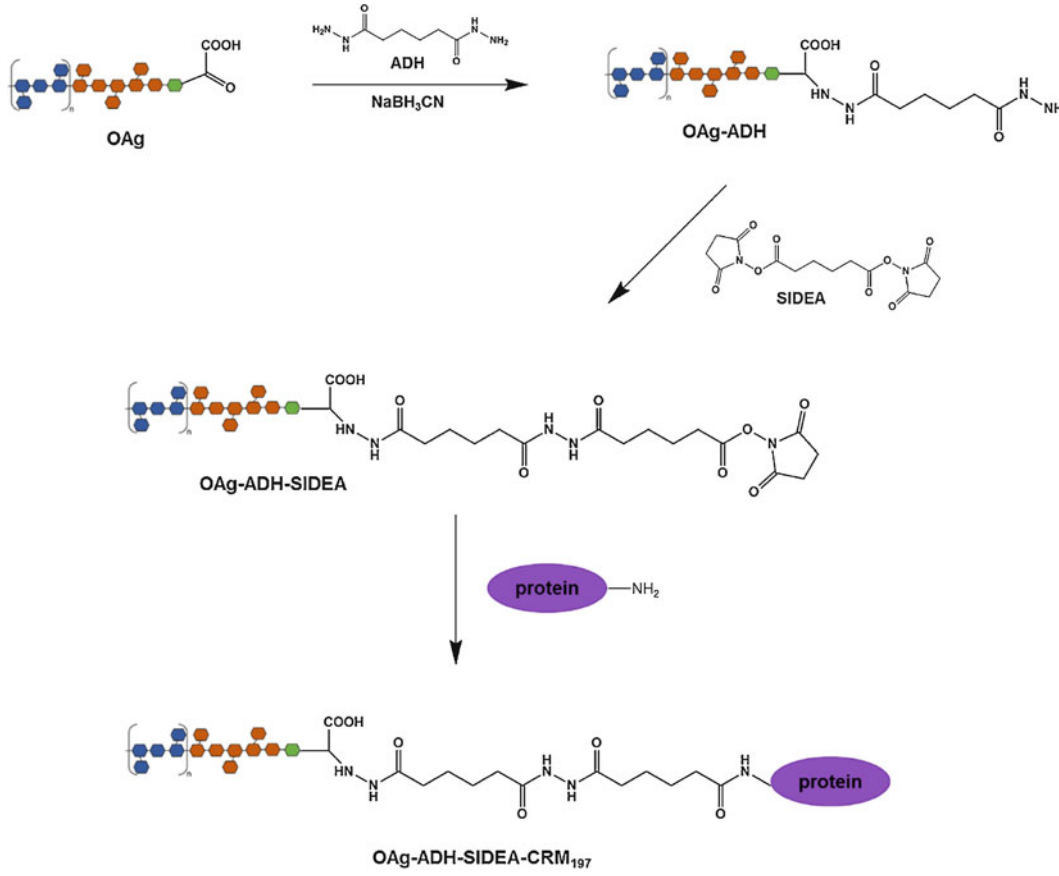
8. Characterize "post 0.22 μm" and all the intermediates by (*see* **Note 12**):
   (a) Phenol sulfuric acid assay [15] for quantifying total sugar content and HPAEC-PAD analysis for sugar composition, content and yields of purification steps.
   (b) Calculate average molecular weight distribution based on the molar ratio of Rha to GlcNAc, considering that Rha is present per each OAg chain repeating unit and that GlcNAc is a unique sugar of the core region (*see* **Note 13**).
   (c) HPLC-SEC for molecular size distribution.
   (d) micro-BCA for protein content. Express it as ratio percentage relative to sugar content (w/w).
   (e) Nucleic acids analysis by UV spectroscopy at wavelength 260 nm, assuming that a nucleic acid concentration of 50 μg/mL gives an OD260 of 1. Express it as a ratio percentage relative to sugar content (w/w).
9. Characterize "post 0.22 μm" also by:
   (a) ^{1}H NMR for OAg identity and O-acetyl content.
   (b) Semicarbazide assay for KDO quantification (*see* **Note 14**).
   (c) 2,4,6-trinitrobenzene sulfonate (TNBS) colorimetric method for NH2 groups concentration [16].
      - Express it as a molar ratio % NH_2 groups to GlcNAc. NH_2 groups can be present in OAg samples as pyrophosphoethanolamine residues in the core region [13].
   (d) Chromogenic kinetic LAL (Limulus amoebocyte lysate) to measure endotoxin level.

3.2 OAg Conjugation to Carrier Protein and Conjugate Purification

Conjugation is performed by OAg activation at the terminus KDO with ADH through its ketone group by reductive amination, followed by reaction of OAg-ADH with SIDEA and conjugation with CRM_{197}. Here the conjugation of *S. paratyphi* OAg is reported as an example (Scheme 2). The same method can be applied to OAg from other strains.

3.2.1 OAg Derivatization

1. Dry the desired amount of "post 0.22 μm" through rotating evaporator (*see* **Note 15**).
2. Solubilize dried OAg ("post 0.22 μm") in 100 mM AcONa pH 4.5 at a concentration of 20–40 mg/mL. When OAg is completely solubilized (*see* **Note 16**), add ADH and then NaBH3CN as solids, both with a ratio 1.2:1 by weight with respect to the OAg. Perform this operation within a chemical hood.
3. Mix the solution at 30 °C for 1 h.



Scheme 2 Conjugation scheme. OAg is derivatized at the terminus KDO with ADH followed by SIDEA linkage and conjugation to carrier protein

4. Purify the reaction mixture through desalting against water on a G-25 column and designate the derivatized OAg as "OAg-ADH."
5. Characterize "OAg-ADH" by:
   (a) Phenol sulfuric acid assay for quantifying total sugar content and HPAEC-PAD analysis for sugar composition and content.
   (b) HPLC-SEC for molecular size distribution.
   (c) TNBS colorimetric method and free ADH RP-HPLC analysis for % activation with ADH linker.
   - Calculate selective activation of the terminus KDO as moles of linked ADH per mole of KDO %, indicating the % of activated OAg chains.
   - Correct total NH_2 groups by subtracting the number of NH_2 groups eventually already present in underivatized OAg and the number of free ADH detected by RP-HPLC.

6. Dry the "OAg-ADH" through rotating evaporator (*see* **Note 15**).
7. Solubilize dried OAg-ADH in water/DMSO 1:9 (v/v) at a concentration of 50 mg/mL. When the polysaccharide is completely solubilized, add triethylamine (TEA) (molar ratio TEA/total NH2 groups = 5) and then SIDEA (molar ratio SIDEA/total NH2 groups = 12) (*see* **Note 17**). Perform this operation within a chemical hood.
8. Mix the solution at RT for 3 h.
9. Purify the reaction mixture through precipitation with 100 mM citrate buffer pH 3 and EtOH.
   (a) Add to the reaction mixture 2× reaction mixture volume of 100 mM citrate pH 3 and mix at 4 °C for 30 min. Under these conditions, unreacted SIDEA precipitates and is discarded after centrifugation (12,000 RCF at 4 °C for 30 min).
   (b) Add to the supernatant absolute EtOH to have 80% EtOH final concentration (v/v). The derivatized OAg precipitates and is recovered after centrifugation (12,000 RCF at 4 °C for 30 min).
   (c) Wash the pellet after centrifugation twice with 1.5 volumes of 100% EtOH with respect to the initial reaction mixture volume.
11. Lyophilize the pellet and indicate it as "OAg-ADH-SIDEA."
12. Characterize "OAg-ADH-SIDEA" (*see* **Note 18**) by:
   (a) Phenol sulfuric acid assay for quantifying total sugar content and HPAEC-PAD analysis for sugar composition and content.
   (b) HPLC-SEC for molecular size distribution.
   (c) Total active esters group quantification by A260, RP-HPLC for free SIDEA quantification.
      - Calculate % of derivatization with SIDEA as molar ratio of linked active ester groups (subtracting the moles of free active ester groups quantified by RP-HPLC) to total NH_2 groups before derivatization measured by TNBS. The ratio indicates the % in moles of NH_2 groups activated with this reaction.

3.2.2 Conjugation to CRM_{197} and Conjugate Purification

1. Solubilize "OAg-ADH-SIDEA" in phosphate buffer pH 7.2 and add CRM197 to give a protein concentration of 20 mg/mL and a molar ratio of active ester groups to CRM197 of 30 to 1.
2. Mix the reaction at RT for 2 h.

3. Analyze the conjugation mixture by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC-SEC to verify conjugate formation, comparing it with free protein and free saccharide (Fig. 3).
4. Purify the conjugate by size exclusion chromatography on 16/90 cm Sephacryl S-300 HR column eluting at 0.5 mL/min in 50 mM phosphate buffer, 150 mM NaCl, pH 7.2 (*see* **Note 19**).
5. Characterize the conjugate by:
   (a) micro-BCA for total protein content.
   (b) Phenol sulfuric acid assay for quantifying total sugar content and HPAEC-PAD analysis for sugar composition and content.
   (c) OAg to protein w/w ratio.
   (d) HPLC-SEC for molecular size distribution.
   (e) ^{1}H NMR for OAg identity and O-acetyl content.
   (f) Free saccharide analysis (*see* **Note 20**).
   - Conjugate precipitation with deoxycholate for separation from free saccharide.
   - Quantification of free saccharide (e.g., through HPAEC-PAD).

3.3 OAg and glycoconjugate characterization

3.2.3 Analytical Methods

1. Phenol sulfuric assay [15].
   (a) Calibration curve preparation.
   - Using water, prepare in labeled tubes, in duplicate, the dilutions of the Glc standard solution (0.5 mg/mL), as indicated in Table 1.
   - Label each tube with the corresponding standard concentration.

   (b) Sample preparation.
   - Ensure that sample concentration is in the range of the calibration curve. If not, dilute it with water (mix samples before dilution in order to homogenize the vial content).
   - Put 200 μL of each sample dilution in a tube. Label each tube with the corresponding sample name. Each sample is analyzed in duplicate.

   (c) Sample/Standard treatment.
   - Put the standard and sample tubes in a suitable rack which must remain within a chemical hood for all the following procedure.

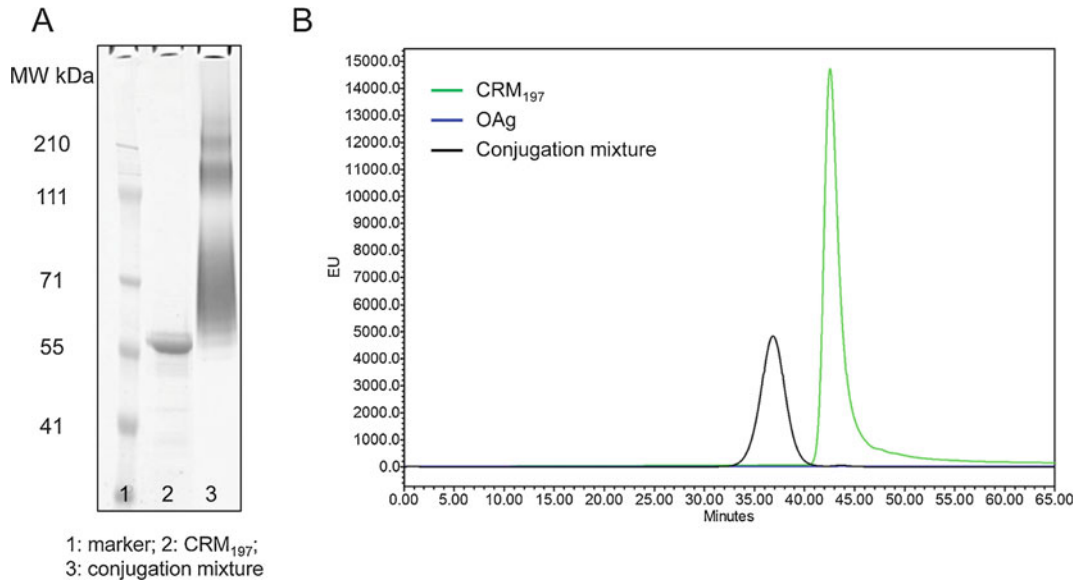


Fig. 3 (**a**) SDS-PAGE analysis of conjugation mixture in comparison to unconjugated carrier protein: lane 1: marker, lane 2: CRM_{197} (2 μg loaded), lane 3: *Shigella flexneri* 6 OAg-ADH-SIDEA-CRM_{197} (10 μg loaded). (**b**) HPLC-SEC profiles (fluorescence emission detection) of *Shigella flexneri* 6 OAg-ADH-SIDEA-CRM_{197} (100 μg/mL in protein) in comparison to free CRM_{197} (100 μg/mL) and free OAg. 80 μL of each sample was run on Tosoh TSK gel 6000 + 5000 PW columns; eluent 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% ACN, pH 7.2; flow rate 0.5 mL/min. Free OAg is not detected by fluorescence emission

Table 1
Dilutions for preparing the Glc calibration curve for phenol sulfuric assay

Std conc. (μg/mL)	Glc 0.5 mg/mL (μL)	Water (μL)
0	0	200
62.5	25	175
125	50	150
187.5	75	125
250	100	100

- To each tube containing standards or samples add 200 μL of 5% phenol solution using the automatic pipette equipped with a 10 mL disposable Combitips syringe.
- Vortex each tube for few seconds.
- To each tube containing standards and samples, add 1 mL of sulfuric acid using the automatic pipette equipped with a disposable 25 mL Combitips syringe (avoid using normal pipettes because the sulfuric acid vapor will damage their pistons) (*see* **Note 21**).

Table 2
Dilutions for preparing the neutral sugars calibration curve for HPAEC-PAD analysis

μg/mL (each sugar)	11.25 μg/mL standards solution (μL)	Water (μL)
0	0	450
0.5	20	430
1.0	40	410
2.5	100	350
5.0	200	250
7.5	300	150
10	400	50

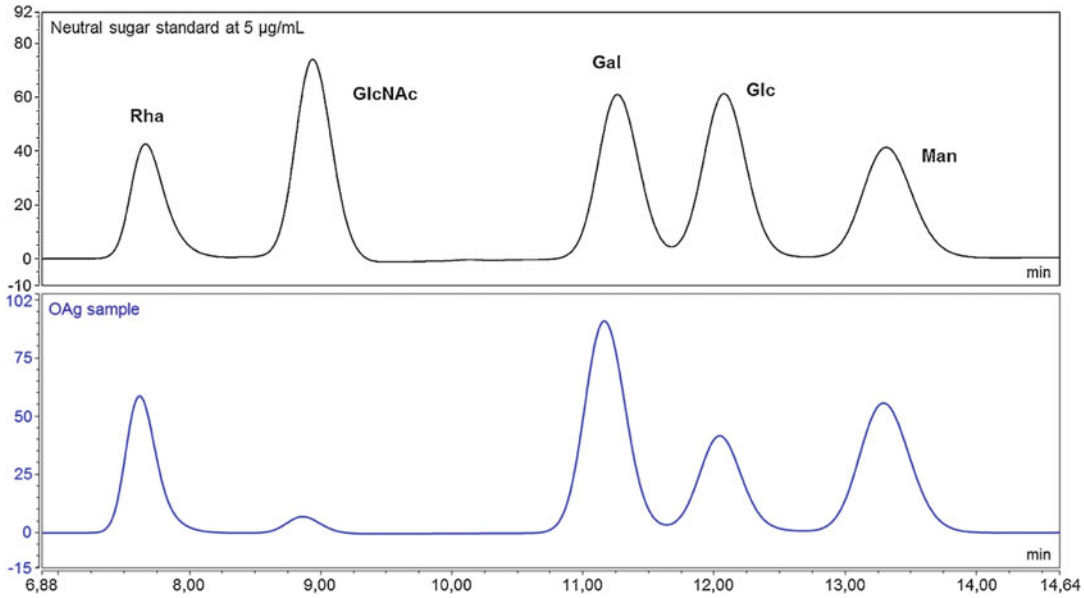


Fig. 4 HPAEC-PAD profile of *S. paratyphi* A OAg sample compared to the monosaccharide standards

- Wait 10 min keeping tubes at RT within a chemical hood.
- Vortex each tube for few seconds.
- Wait 30 min keeping tubes at RT within a chemical hood.
- Start the analyses with instrument set as reported below:
  - Spectrophotometer wavelength set at 490 nm and the lamp turned on at least 1 h prior of readings to warm up.

Table 3
Dilutions for preparing the GlcNAc calibration curve for HPAEC-PAD analysis

μg/mL (GlcNAc)	11.25 μg/mL standards solution (μL)	Water (μL)
0	0	450
0.5	20	430
1.0	40	410
2.5	100	350
5.0	200	250
7.5	300	150
10	400	50

- Read each tube UV ABS, using as blank for the spectrophotometer the 0 μg/mL standard.
- Calculate a linear regression between concentration and ABS for the standards and calculate on it the total sugar concentration of the samples.

2. HPAEC-PAD for neutral sugars quantification: Rha, Gal, Glc and Man, sugars of the OAg chain repeating unit, and GlcNAc, present as a unique sugar in the core region, are estimated by HPAEC-PAD after acid hydrolysis of unconjugated or conjugated OAg samples to release the monosaccharides. Commercial monomer sugars are used for building the calibration curves (*see* **Note 22**).

   Paratose (Par), the other monosaccharide present in *S. paratyphi* A OAg chain, could not be determined by this method as no commercially available standard exists, therefore the presence and amount of Par is determined by ^{1}H NMR.

(a) Calibration curve standard solutions.

- In 2 mL screw cap vials, prepare in duplicate the dilutions starting from the 11.25 μg/mL neutral sugar standard mix, as indicated in Table 2:

(b) Sample preparation.

- Dilute the sample with water to a suitable concentration to fit the calibration curve.
- Prepare three (in triplicate) 2 mL screw cap vials containing 450 μL of the diluted sample.

(c) Sample/standard treatment.

- To each vial containing standards or samples add 150 μL TFA 8 M (final concentration 2 M) (*see* **Note 23**), close them and vortex each vial for few seconds.
- Place all the vials in a rigid cardboard rack and incubate in a preheated oven at 100 °C for 4 h.
- After this time, allow all the tubes to cool at 2–8 °C for 30 min.
- Remove the caps and dry the samples/standards overnight in centrifugal evaporator at RT in order to remove solvent/TFA.
- Redissolve the content of each vial adding 450 μL of water and vortex accurately for few seconds.
- Filter the content of each vial in the sampler polypropylene vials using 0.45 nylon syringe (4 mm) filters.

(d) Analysis setting for Thermo Dionex ICS series instrument.

- Column System: Dionex CarboPac PA10 guard—CarboPac PA10 columns connected in series.
- Column/detector compartment: 35 °C.
- Autosampler compartment: 10 °C.
- Electrochemical detector equipped with gold working electrode, potential sets with standard carbohydrate waveform.
- Chromatographic conditions:
  - Sample injection volume: 25 μL, full loop mode.
  - Run time: 50 min.
  - Flow rate: 1 mL/min.
  - Eluent program:

    20 min, NaOH 18 mM (36% of eluent NaOH 50 mM)

    10 min NaOH 28 mM, AcONa 100 mM (36% NaOH 50 mM; 10% AcONa 1 M with NaOH 100 mM).

    20 min NaOH 18 mM (36% of eluent NaOH 50 mM).
  - Remark: after the last chromatographic analysis is completed, store the column in 18 mM NaOH solution.
- Per each sugar standard calculate a linear regression between concentration and the peak area and calculate on it the concentration of each sugar monomer in the samples (Fig. 4).

3. HPAEC-PAD for GlcNAc (*see* **Note 22**).

(a) Calibration curve standard solutions.

- In 2 mL screw cap vials, prepare in duplicate the dilutions starting from the 11.25 μg/mL GlcNAc standard (Table 3):

(b) Sample preparation.

- Dilute the sample with water to a suitable concentration to fit the calibration curve.
- Prepare three (in triplicate) 2 mL screw cap vials containing 450 μL of the diluted sample.

(c) Sample/standard treatment.

- To each vial containing standards and samples add 150 μL TFA 4 M (final concentration 1 M) (*see* **Note 23**), close them and vortex each vial for few seconds.
- Place all the vials in a rigid cardboard rack and incubate in a preheated oven at 100 °C for 6 h.
- Allow all the tubes to cool at 2–8 °C for 30 min.
- Remove the caps and dry the samples/standards overnight in centrifugal evaporator at RT in order to remove solvent/TFA.
- Redissolve the content of each vial adding 450 μL of water and vortex accurately for few seconds.
- Filter the content of each vial in the sampler polypropylene vials using 0.45 nylon syringe (4 mm) filters.

(d) Analysis setting for Thermo Dionex ICS series instrument.

- Column System: Dionex CarboPac PA10 guard—CarboPac PA10 columns connected in series.
- Column/detector compartment: 35 °C.
- Autosampler compartment: 10 °C.
- Electrochemical detector equipped with gold working electrode, potential sets with standard carbohydrate waveform.
- Chromatographic conditions:
  - Sample injection volume: 25 μL, full loop mode.
  - Run time: 50 min.
  - Flow rate: 1 mL/min.
  - Eluent program:

    Gradient from NaOH 10 mM to 18 mM in 20 min (from 20% to 36% of NaOH 50 mM eluent).

10 min NaOH 28 mM, AcONa 100 mM (36% NaOH 50 mM; 10% AcONa 1 M with NaOH 100 mM).

20 min NaOH 10 mM (20% of eluent NaOH 50 mM).

– Remark: after the last chromatographic analysis is completed, store the column in 18 mM NaOH solution.

- Calculate a linear regression for GlcNAc standard between concentration and the peak area and calculate on it the concentration of the GlcNAc in the samples.

4. HPLC-SEC.

HPLC-SEC analysis is used to estimate the molecular size distribution of OAg samples. Samples are run, without any pretreatment, diluted with water at 100–200 μg/mL OAg concentration.

1. System setting.
   (a) Column System: TSK gel G3000 PWXL column with a TSK gel PWXL guard column.
   (b) Calculate void and bed volume calibration injecting λ-DNA and NaN3, respectively. For Kd determination, the following equation was used: $Kd = (T_e - T_0)/(T_t - T_0)$ where: T_c = elution time of the analyte, T_0 = elution time of the biggest fragment of λ-DNA and T_t = elution time of NaN3.
   (c) Column compartment: 30 °C.
   (d) Autosampler compartment: 4 °C.
   (e) Detector: OAg peaks by differential refractive index (dRI). UV detection at 214 and 260 nm for following impurity reduction during the purification steps.
2. Chromatographic conditions:
   (a) Mobile phase: 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% ACN, pH 7.2.
   (b) Flow rate: 0.5 mL/min.
   (c) Run time: isocratic for 30 min.
   (d) Sample injection volume: 80 μL.

(*see* **Note 24**)

HPLC-SEC analysis is used to estimate the molecular size distribution of the glycoconjugate, in comparison with free OAg and free protein. Samples are run usually diluted in PBS at 50–100 μg/mL protein concentration.

Table 4
Dilutions for preparing the KDO calibration curve for semicarbazide/HPLC-SEC method

KDO			
μg/mL	nmol/mL	$KDONH_4$ 40 μg/mL (μL)	Water (μL)
4	15.7	10	90
8	31.4	20	80
14	54.9	35	65
20	78.4	50	50
40	156.8	100	0

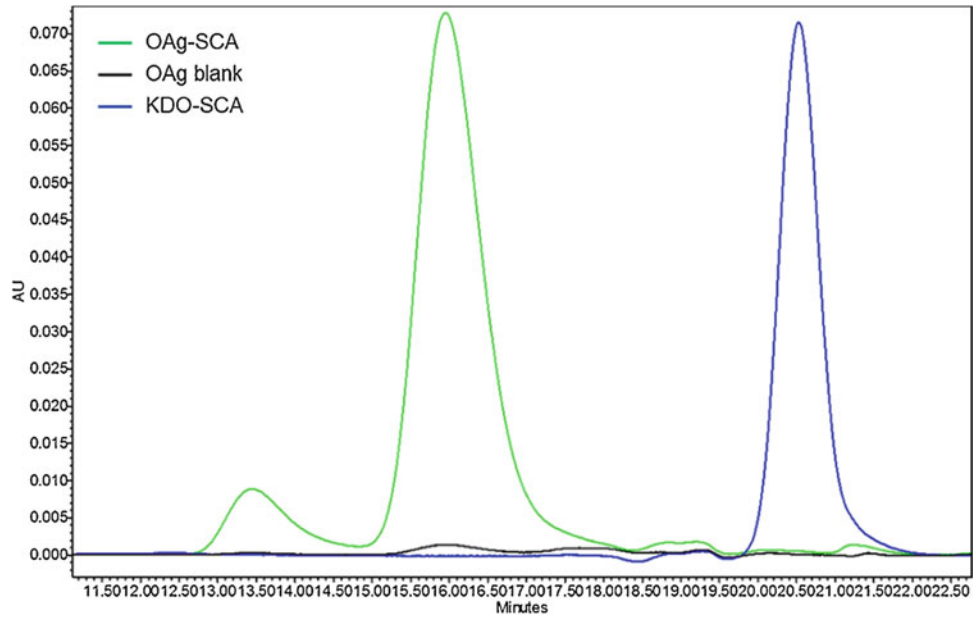


Fig. 5 HPLC-SEC profiles (ABS 252 nm) of OAg and KDO standard after derivatization with semicarbazide (SCA) and of underivatized OAg (blank). Each sample (80 μL) run on a Tosoh TSK gel 3000 PWXL column; eluent 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% ACN, pH 7.2; flow rate 0.5 mL/min

HPLC-SEC can also be used to estimate the amount of unconjugated protein in conjugate samples.

For example, the area of unreacted CRM_{197} can be quantified with respect to a calibration curve built with CRM_{197} samples in the range 5–50 μg/mL. The percentage of unconjugated CRM_{197} is calculated dividing the amount of free CRM_{197} detected by HPLC-SEC by the total amount of protein quantified in the sample by micro BCA.

Table 5
Dilutions for preparing the ADH calibration curve for TNBS method

Amino groups (nmol/mL)	ADH (nmol/mL)	ADH std 100 nmol/mL (μL)	Water (μL)
0	0	0	500
24	12	60	440
48	24	120	380
96	48	240	260
144	72	360	140
200	100	500	0

Table 6
Dilutions for preparing the ADH calibration curve for free ADH quantification by RP-HPLC

ADH			
μg/mL	nmol/mL	ADH 0.4 μg/mL (μL)	Water (μL)
0.024	0.14	30	470
0.052	0.30	65	435
0.100	0.57	125	375
0.200	1.15	250	250
0.400	2.30	500	0

Table 7
Detailed chromatographic elution conditions for free ADH quantification by RP-HPLC

Time (min)	Flow rate (mL/min)	Water (%)	ACN (%)
0.0	1	35	65
7.5	1	35	65
7.6	1	5	95
20.0	1	5	95
20.1	1	35	65
50.0	1	35	65

1. System setting.
   (a) Column System: TSK gel G6000 PW column connected in series with a TSK gel G5000 PW column and a TSK gel PWH guard column.

Table 8
Dilutions for preparing the SIDEA calibration curve for free SIDEA quantification by RP-HPLC

SIDEA (nmol/mL)	SIDEA 15 μM (μL)	Water/ACN 50% (μL)
2.5	150	750
5.0	300	600
10	600	300
15	900	0

Table 9
Summary of volumes used to prepare sample, spiked sample, and spike solutions for free saccharide quantification by deoxycholate precipitation

Sample	Conjugate solution at 100 μg/mL in NaCl 0.1 M (μL)	OAg solution 700 μg/mL (μL)	NaCl 0.1 M solution (μL)
Conjugate	1440	–	60
Spiked conjugate	1440	60	–
Spike	–	60	1380

(b) Calculate void and bed volume calibration injecting λ-DNA and NaN_3, respectively. For Kd determination, the following equation is used: $Kd = (T_e - T_0)/(T_t - T_0)$ where: T_e = elution time of the analyte, T_0 = elution time of the biggest fragment of λ-DNA and T_t = elution time of NaN3.

(c) Column compartment: 30 °C.

(d) Autosampler compartment: 4 °C.

(e) Detector: OAg peaks are detected by differential refractive index (dRI), while UV detection at 214 nm and 280 nm are used for free protein and conjugate detection. Protein and conjugate peaks are also detected using tryptophan fluorescence (emission spectrum at 336 nm, with excitation wavelength at 280 nm).

2. Chromatographic conditions:

(a) Mobile phase: 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% ACN, pH 7.2.

(b) Flow rate: 0.5 mL/min.

(c) Run time: isocratic for 30 min.

(d) Sample injection volume: 80 μL.

Remarks: Perform system equilibration with elution buffer before starting samples analysis. This equilibration step lasts 70 min if the column system must be equilibrated with a different eluent; 60 min or more are needed also to warm up the PDA lamps before the analysis. These steps can be skipped if the system (HPLC and columns) is already equilibrated with the buffer and lamps are on. After the last chromatographic analysis, store the column in 0.02% NaN_3.

5. ^1H nuclear magnetic resonance (NMR) spectroscopy

   (a) Experimental conditions.

   - Sample preparation.
     - Dry the OAg (unconjugated or conjugated) sample (2.5 mg total sugar).
   - Solubilize dried sample in 650 μL D_2O and transfer into 5-mm NMR tubes.

     System settings.
     - Acquisition temperature: 25 °C.
     - Fid acquisition time: 5 s.
     - Relaxation delay: 15 s.
     - Number of scans: 64.

   (b) OAg identity.

   NMR analysis on the liquid state is performed to confirm the identity of the OAg samples by detecting typical signals of the OAg chain, confirming the presence of the characteristic sugars [17–19].

   (c) OAg O-acetylation level.

   A first ^1H NMR spectrum is recorded to ensure the absence of impurities at the same chemical shift of the acetate anion released after de-O-acetylation of the sample that would interfere with the quantification of the O-acetyl content. O-acetylation level is quantified by comparing acetate signal (released after treatment with NaOD, at 1.91 ppm) and a known signal of the OAg RU structure (*see* **Note 25**).

   Record two 1H NMR spectra as described before:

   - the first one in D2O.
   - the second one after de-O-acetylation adding sodium deuteroxide (NaOD) to a final 200 mM concentration (35 μL NaOD 4 M directly in the tube after having recorded the first spectrum) and heat treatment (37 °C for 2 h for complete de-O-acetylation).

6. KDO quantification by semicarbazide/HPLC-SEC method.

OAg samples are analyzed by HPLC-SEC after derivatization with semicarbazide to quantify α-ketoacid present at the reducing end. This reaction is performed as a slight modification of the semicarbazide assay for α-ketoacid determination [20].

(a) Calibration curve standard solution.

- Using water, prepare in labeled tubes, in duplicate, the dilutions of KDO ammonium salt 40 μg/mL standard solution as indicated in Table 4.
- Vortex all the standard dilution tubes for few seconds.

(b) Sample preparation.

- Each sample is analyzed in duplicate.
- Label two 1.5 mL Eppendorf tubes with sample name and two 1.5 mL Eppendorf tubes with sample name + "blank."
- Gently mix the sample in order to homogenize the content. Dilute the sample with water to an appropriate concentration (to fit the calibration curve) and with a pipette transfer 100 μL of the diluted sample in each labeled Eppendorf tube.

(c) Sample/standard derivatization.

- With the automatic pipette add 100 μL of semicarbazide solution to each sample/standard tube. Close the tubes. Vortex all tubes for few seconds.
- With the automatic pipette add 100 μL of water to each blank sample tube. Close the tubes. Vortex all tubes for a few seconds.
- Keep the tubes in a 50 °C preheated bath for 50 min.
- Chill the tubes in a 2–8 °C fridge for 15 min.
- Vortex all the tubes for few seconds.
- Transfer samples, blank samples, and standards in prelabeled HPLC vials.
- Place the vials in the HPLC autosampler prechilled at 4 °C.

(d) Analysis setting.

- Column System: PW-XL guard—G3000 PWXL columns connected in series.
- Column compartment: 30 °C.
- Autosampler compartment: 4 °C.
- UV detector acquisition channel: 252 nm ABS.

- Chromatographic conditions:
  - Eluent: 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% ACN, pH 7.2 (isocratic conditions).
  - Flow rate: 0.5 mL/min.
  - Run time: 35 min.
  - Sample injection volume: 80 μL.

Remark: Perform system equilibration with elution buffer before starting the analysis. This equilibration step lasts 70 min if the column system must be equilibrated with a different eluent; 60 min or more are needed also to warm up the PDA lamps before the analysis. These steps can be skipped if the system (HPLC and columns) is already equilibrated with the buffer and lamps are on. After the last chromatographic analysis is completed, store the system in NaN_3 0.02% preservative solution.

- Quantification of reactive carbonyl groups (nmol/mL) in the samples.
  - Correct the peak area corresponding to OAg in samples derivatized with semicarbazide by subtracting the area of the corresponding blank.
  - Quantify the amount of KDO using the calibration curve built with the peak areas of derivatized KDO standard at 252 nm (Fig. 5).

7. Amino Groups Determination by Colorimetric TNBS Method [16].

(a) Calibration curve standard solutions.
- In 5 mL glass tubes, prepare in duplicate the ADH dilutions starting from 100 nmol/mL, as reported in Table 5.

(b) Sample preparation.
- Dilute the sample with water to a suitable concentration to fit the calibration curve. Prepare two 5 mL tubes containing 500 μL of the diluted sample.

(c) Sample/standard treatment.
- To each tube containing standards and samples, add 500 μL of sodium hydrogen carbonate 4% solution and vortex each tube for few seconds.
- To each tube, add then 500 μL of TNBS 0.1% solution and vortex each tube for few seconds.
- Cover tubes with aluminum foil and incubate in a preheated thermostatic bath at 40 °C for 2 h.

- Allow all the tubes to cool at RT for 10 min and then read each tube UV ABS at 335 nm, using as blank for the spectrophotometer the 0 nmol/mL standard.

(d) Calculate a linear regression between concentration and ABS for the standard and calculate on it the sample amino groups concentration.

8. Free ADH quantification by RP-HPLC [21].

(a) ADH Calibration curve.

- Prepare in labeled Eppendorf tubes the dilutions of ADH 0.4 μg/mL standard solution as indicated in Table 6.

(b) Sample/standard derivatization.

- The derivatization procedure is applied to calibration curve solutions and to samples.
- Per each standard/sample, transfer two 100 μL aliquots in different Eppendorf tubes, labeling them with their content name.
- In a 50 mL Falcon tube, add in the following order and stir after each addition:
  - 4.840 μL of PBA solution 2.5 mM.
  - 1.210 μL of pyridine 20% solution.
  - 1.210 μL of EDC 2 M solution.

  This solution must be prepared just before use.
- To each 100 μL of solution to be derivatized, add 300 μL of the solution prepared.
- Close the Eppendorf tube and incubate at 40 °C for 60 min.
- Dry them overnight (at least for 24 h) on centrifugal evaporator at 60 °C in order to remove DMSO and Pyridine.
- Redissolve each tube content in 400 μL of ACN 65% solution (the dissolution time is about half an hour) and vortex.
- Centrifuge all Eppendorf tubes for 30 min at 12,000 RCF in order to remove insoluble salts.
- Filter the upper liquid through a 0.2 μm nylon filter into different HPLC vials labeling them with content sample name.

(c) System setting:

- Column System: HPLC in line filter—C18 column connected in series.

- Column compartment: 30 °C.
- Autosampler compartment: 4 °C.
- Fluorimeter detector: excitation wavelength set at 345 nm; emission wavelength acquired at 480 nm (photomultiplier gain level set at 1) (*see* **Note 26**).
- Chromatographic conditions:
  - Separation performed in isocratic condition: eluent 65% ACN and 35% water (mixture generated by HPLC pump); after analyte peak elution, column cleaning step with 95% ACN and 5% water. Detailed elution conditions are reported in Table 7.
  - Flow rate: 1 mL/min.
  - Run time: 50 min.
  - Sample injection volume: 100 µL.

(d) Calculate percentage of free NH_2 groups as molar ratio % of free NH_2 groups (*see* **Note 27**) divided by total NH_2 groups introduced after derivatization with ADH (quantified by TNBS).

9. Total active ester group quantification by A260 [22].
   (a) Solubilize OAg-ADH-SIDEA sample in water to a sugar concentration of 5 mg/mL.
   (b) Immediately after solubilization, add 250 µL of this solution to 500 µL of water and measure A260 (as blank).
   (c) Add 250 µL of the OAg solution to 500 µL 0.1 M NH4OH to release the N-hydroxy succinimide groups and measure the absorption of the N-hydroxy succinimidate anion at 260 nm.
   (d) Build the calibration curve with 20–200 nmol/mL N-Hydroxy succinimide.
   (e) Percentage of derivatization with SIDEA is calculated as % molar ratio of linked active ester groups (subtracting the moles of free active ester groups quantified by RP-HPLC) to total NH2 groups before derivatization measured by TNBS. The ratio indicates the % in moles of NH2 groups activated as esters.
10. Free SIDEA quantification by RP-HPLC.
    (a) SIDEA Calibration curve.
    - In prelabeled Eppendorf tubes, prepare the dilutions of the SIDEA 15 µM standard solution with Water/ACN 50% and mix them by vortexing (Table 8).

Remark: As with the samples, the calibration curve solutions must also be prepared just prior to the assay.

- Filter the solutions through a 0.2 μm nylon filter into different HPLC vials labeling them with content standard name.

(b) Samples preparation.

- Weigh in an Eppendorf tube about 5 mg (total sugar) of sample and dissolve it in the suitable quantity of water/ACN 50% in order to obtain a 20 mg/mL (total sugar) solution.

Water/ACN 50%to be added (mL)
: sample weight (mg) × purity (%)/20.

- For each sample:
  - Label one Eppendorf tube with "sample name," transfer 100 μL of sample and 100 μL of water/ACN 50% performing a factor 2 dilution.
  - Filter the samples through a 0.2 μm nylon filter into different HPLC vials, labeling them with content sample name.

(c) System setting.

- Column System: HPLC in line filter—Kinetex C18 column connected in series.
- Column compartment: 30 °C.
- Autosampler compartment: 4 °C.
- UV detector: wavelength set at 195 nm.
- Chromatographic conditions:
  - Separation performed in isocratic condition: eluent 50% ACN and 50% water (mixture generated by HPLC pump).
  - Flow rate: 1 mL/min.
  - Run time: 8 min.
  - Sample injection volume: 25 μL.
  - Remarks: After the last chromatographic analysis store the column in 65% ACN and 35% water.

(d) Calculation of free active ester groups.

Quantify the amount of free SIDEA using the calibration curve built with the peak areas of SIDEA standard at 195 nm.

Calculate percentage of free active ester groups as molar ratio % of free ester groups (*see* **Note 28**) divided by total active ester groups (determined by A260 after ammonia treatment).

11. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).
    (a) Sample treatment (both, protein alone, and the conjugation mixture).
        - Use 5–20 μL of the samples with a protein content of 5–10 μg.
        - Mix the samples with 0.5 M dithiothreitol solution (1/5 v/v) and NuPAGE LDS sample buffer (1/5 v/v).
        - Spin them in the centrifuge in order to draw down all the volume in the bottom of the tube.
        - Place the tubes in a 100 °C preheated block heater and boil the samples for 1 min.
        - Spin again all samples in the centrifuge in order to draw down all the volume in the bottom of the tube.
    (b) Buffer preparation and system setup.
        - Use a NuPAGE® Novex 7% Tris-Acetate Gel (*see* **Note 29**).
        - Dilute 25 mL of Tris-Acetate running buffer 20× with 475 mL of water in a graduate glass cylinder and transfer into the middle part of the cell up to cover the gel wells and more up to 2 cm from the front bottom of the cell.
        - Insert the gel in the electrophoresis cell after having removed the strip present in the bottom of the gel.
    (c) Sample loading.
        - Remove the wells protection from the gel.
        - Use the first well to load few microliters of an appropriate marker.
        - Place all the samples in the wells noting the lanes used.
    (d) Electrophoresis run.
        - Close the cell and connect it to the power supply.
        - Set up the power supply at 200 V and 45 mA and run the gel.
        - Wait until the blue marker of the wells reaches the bottom of the gel.
    (e) Gel Staining.
        - Open the cell and remove the gel from its holder using the gel knife.

- Transfer the gel into the reservoir for staining containing 30 mL of water. Put on the gyro-rocker and leave it rocking for about 2 min.
- Remove water and add about 30 mL of Bio-Safe Coomassie (mix the tank before use), being sure to cover all the gel.
- Leave the gel on the gyro-rocker for about 30 min.
- Remove the stain solution and add 30 mL of water. Rock the gel until bands are clear.
- Collect a picture of the gel with a camera or scanner.

(f) Data evaluation.
- Check conjugate formation by the presence of the smear pattern typical of a glycoconjugate, in comparison to free protein band (Fig. 3a).

12. Free saccharide quantification.

The separation of free saccharide from conjugate is achieved by conjugate coprecipitation with deoxycholate [23]. Deoxycholate is added as sodium salt to the conjugate solution containing sodium chloride 0.1 M; then lowering the pH with HCl, the surfactant becomes insoluble and coprecipitates with the conjugate. The free saccharide is then quantified, for example, using HPAEC-PAD preparing calibration curves in NaCl 0.1 M solution to reproduce sample matrix (as it affects the detector response).

In parallel to the sample above, a sample spiked with a known amount of free OAg is assayed each time in order to verify the procedure (spike recovery needs to be in the range 80–120%)

(a) OAg solution 700 μg/mL.
- Dilute OAg to about 700 μg/mL in NaCl 0.1 M.

(b) Sample solution at 100 μg/mL in NaCl 0.1 M.
- Prepare at least 4 mL of sample solution at about 100 μg/mL of saccharide (dilute it with water).
- Label a 15 mL flacon tube with "Sample name 100 μg/mL NaCl 0.1 M."
- Weight inside the tube 24 ± 1 mg of sodium chloride.
- Transfer in the tube 4 mL of the sample solution diluted to 100 μg/mL and mix on vortex to dissolve all the salt.

(c) Free saccharide separation.
- Initial Equipment preparation.
  - Switch on the centrifuge and set the temperature to 4 °C to let it cool.

(d) Sample/spiked sample/spike preparation.

- Sample.

  This solution must undergo deoxycholate precipitation described in the following sections.

  – Label a 2 mL Eppendorf tube as "Sample."
  – Transfer 1440 µL of "Sample solution at 100 µg/mL in NaCl 0.1 M" in the tube.
  – Add to the tube 60 µL of "NaCl 0.1 M solution" and homogenize its content by vortexing.

- Spiked sample.

  This solution must undergo to deoxycholate precipitation described in the following sections.

  – Label a 2 mL Eppendorf tube as "spiked sample."
  – Transfer 1440 µL of "Sample at 100 µg/mL in NaCl 0.1 M" in the tube.
  – Add to the tube 60 µL of "OAg solution 700 µg/mL" and homogenize its content by vortexing.

- Spike.

  This solution does not have to undergo the deoxycholate precipitation but will be assayed in HPAEC-PAD to quantify the OAg spiked quantity.

  – Label a 2 mL Eppendorf tube as "Spike."
  – Transfer 1380 µL of NaCl 0.1 M solution in the tube.
  – Add to the tube 60 µL of "OAg solution 700 µg/mL" and homogenize its content by vortexing.

Summary of volumes in Table 9.

(e) Deoxycholate precipitation.

- Cool the two Eppendorf tubes "sample" and "spiked sample" in ice for 30 min.
- Add to each Eppendorf tube 0.15 mL of deoxycholate solution, vortex them for some seconds and put in ice for 30 min again.

  Remark: cooling in ice is extremely important.

- Transfer 1 mL of hydrochloric acid (HCl) 1 M solution in a 2 mL Eppendorf tube and cool it in ice for at least 10 min.

  Remark: cooling in ice is extremely important.

- Add to each Eppendorf tube 75 µL of cooled HCl 1 M solution, vortex them for some seconds and centrifuge at 12,000 RCF for 30 min at 4 °C.

Remark: make sure that the centrifuge has reached 4 °C before use.

- Transfer the supernatant of each sample in 2.0 mL Wheaton vials. Dry them overnight on centrifugal evaporator at RT in order to remove HCl.
- Redissolve the content of each Eppendorf tube in 1440 μL of water and vortex accurately.

(f) Analysis of samples.

Apply a method for saccharide quantification, for example, HPAEC-PAD for *S. paratyphi* A OAg, to the following samples:

- Sample post deoxycholate, typically undiluted. This will give the amount of free saccharide in the conjugate.
- Spiked sample post deoxycholate, typically diluted of factor 2 using NaCl 0.1 M solution. This will be used for the spike recovery.
- Spike, typically undiluted.
- Sample solution at 100 μg/mL in NaCl 0.1 M, typically diluted of factor 4 using NaCl 0.1 M solution. This will give total amount of sugar in the conjugate sample.

(g) % free OAg is calculated by dividing the amount of free OAg in the sample post deoxycholate per the total sugar content quantified in the untreated conjugate sample.

The procedure is valid if the calculated spike recovery:

$$\frac{\text{spiked sample} - \text{sample}}{\text{spike}} \times 100 \text{ is between 75 and 125\%.}$$

4 Notes

1. Add the powder slowly into the cylinder, allowing for a better solubilization.
2. To prepare buffer solutions, it could be useful to consult online buffer calculators (e.g., www.biomol.net/en/tools/buffercalculator.htm).
3. Use few microliters of NaOH 4 M or HCl 4 M to reach the required pH, if needed. If more volume of a strong acid or base is required, prepare the buffer again being more accurate with weighing.

4. For calculating the amount of water to be added, consider the real μg of sugar weighed taking account of purity and the eventual hydration water.
5. For a 2-mL scale, use 4-mL Wheaton glass vials, using either a boiling water bath or an oven; for a 2-L scale, use a heat-jacketed glass round-bottom flask connected to a condenser; for a 30-L scale, perform the hydrolysis directly in the bioreactor, maintaining the temperature constant at 100 ± 0.5 °C through the double jacket and the closed loop thermostat system.
6. Purification process described for 1 L of "post-hydrolysis supernatant."
7. During concentration and diafiltration steps, keep retentate volume constant, maintaining input pressure (Pin) at 1.8–2.0 bar and transmembrane pressure (TMP) at 1.1–1.2 bar. Membranes with a different molecular weight cut-off could be used according to the OAg size.
8. The formed precipitate during this step can be discarded, containing protein and nucleic acid impurities.
9. Further purify "post-pH 3" by cation-exchange chromatography, through a Sartobind S MA75 filter, if the residual protein content after the precipitation at pH 3 is >3% (w/w respect to total sugar content). Before proceeding with filtration, equilibrate the Sartobind S cartridge with 20 mM citrate, pH 3. Load the OAg sample and collect it in the flow through. Indicate the solution as "post-Sartobind S."
10. Or to "post-Sartobind S."
11. The formed precipitate during this step containing nucleic acids can be discarded.
12. "Post hydrolysis supernatant" and "post-EtOH/$CaCl_2$" samples are analyzed after desalting against water on a HiTrap desalting column, 5 mL, prepacked with Sephadex G-25 Superfine (GE Healthcare), to avoid interference from the matrix in the colorimetric methods.
13. This is specific for *S. paratyphi* A OAg, related to its OAg chain and core sugar composition. The same method can be applied to *S. typhimurium* and *S. enteritidis* OAg.
14. For *S. paratyphi* A OAg KDO quantification should be in good agreement with GlcNAc quantification by HPAEC-PAD confirming the presence of one α-ketoacid per OAg chain.
15. Alternatively, Speedvac can be used or the product can be lyophilized.
16. If necessary, the solubilization can be carried out at 30 °C or by mixing overnight.

17. In some cases, the total NH_2 groups also include phosphoethanolamine groups on the OAg.
18. Weigh a known amount of lyophilized OAg-ADH-SIDEA, solubilize it in water and carry out all the characterization using this solution.
19. Alternatively, other purification methods can be used (e.g., TFF, hydrophobic interaction chromatography [HIC]) [24].
20. Alternative methods can be used for free OAg quantification, for example, solid phase extraction (SPE) using a C4 cartridge (Vydac Bioselect) followed by HPAEC-PAD for quantification.
21. This step is critical for the color development. The sulfuric acid must be added quickly (automatic pipette with high drop speed) keeping the tip end about 2–3 cm above the sample solution. Moreover, the dropped volume must reach the sample/standard solution without coming into contact with the glass tube. In this way the sulfuric acid hydration is fast and highly exothermic and generates the heat necessary to drive the reaction. The color development fails if part of sulfuric acid mixed in the tube forms a two-phase system: In this case, the sample/standard tube must be discarded.
22. Note that this method can be applied on OAg containing these monosaccharides, such as *S. paratyphi* A, *S. typhimurium*, and *S. enteritidis* OAg. For any other polysaccharide, appropriate calibration curves and conditions of hydrolysis need to be identified and optimized.
23. Use an electronic positive displacement pipette as the acid can ruin a Gilson pipette.
24. Perform system equilibration with elution buffer before starting sample analysis. This equilibration step lasts 70 min if the column system must be equilibrated with a different eluent; 60 min or more are also needed to warm up the PDA lamps before the analysis. This step can be skipped if the system (HPLC and columns) is already equilibrated with the buffer and lamps are on. After the last chromatographic analysis, store the column in 0.02% NaN_3.
25. For example, for *S. paratyphi* A (or *S. typhimurium* and *S. enteritidis*) OAg, the H-6 of Rha or Paratose (or Abequose and Tyvelose, respectively) can be used for comparison with the acetate ester signal.
26. Fluorescence detector is set with excitation wavelength at 345 nm and emission wavelength at 480 nm to only see the emission of the diderivatized PBA-ADH-PBA and not the reactant PBA.
27. Amino group concentration is twice the ADH concentration.

28. Free ester groups concentration is twice the SIDEA concentration.
29. A different gel can be used based on the conjugate and carrier protein used.

Conflict of Interest

F.M., C.G., and R.D.B. are employees of GSK group of companies. F.M. is listed as an inventor on patents owned by the GSK group of companies.

References

1. Micoli F, Costantino P, Adamo R (2018) Potential targets for next generation antimicrobial glycoconjugate vaccines. FEMS Microbiol Rev 42:388–423
2. Micoli F, Del Bino L, Alfini R, Carboni F, Romano MR, Adamo R (2019) Glycoconjugate vaccines: current approaches towards faster vaccine design. Expert Rev Vaccines 18:881–895
3. Rappuoli R, De Gregorio E, Costantino P (2019) On the mechanisms of conjugate vaccines. Proc Natl Acad Sci U S A 116:14–16
4. Rappuoli R (2018) Glycoconjugate vaccines: principles and mechanisms. Sci Transl Med 10 (456):pii: eaat4615
5. MacLennan CA, Martin LB, Micoli F (2014) Vaccines against invasive Salmonella disease: current status and future directions. Hum Vaccin Immunother 10:1478–1493
6. Mani S, Wierzba T, Walker RI (2016) Status of vaccine research and development for Shigella. Vaccine 34:2887–2894
7. Sayeed MA, Bufano MK, Xu P, Eckhoff G, Charles RC, Alam MM, Sultana T, Rashu MR, Berger A, Gonzalez-Escobedo G, Mandlik A, Bhuiyan TR, Leung DT, LaRocque RC, Harris JB, Calderwood SB, Qadri F, Vann WF, Kovac P, Ryan ET (2015) A cholera conjugate vaccine containing O-specific polysaccharide (OSP) of V. cholerae O1 inaba and recombinant fragment of tetanus toxin heavy chain (OSP:rTTHc) induces serum, memory and lamina proprial responses against osp and is protective in mice. PLoS Negl Trop Dis 9: e0003881
8. Frenck RW Jr, Ervin J, Chu L, Abbanat D, Spiessens B, Go O, Haazen W, van den Dobbelsteen G, Poolman J, Thoelen S, Ibarra de Palacios P (2019) Safety and immunogenicity of a vaccine for extra-intestinal pathogenic Escherichia coli (ESTELLA): a phase 2 randomised controlled trial. Lancet Infect Dis 19:631–640
9. Westphal O, Jann K (1965) Bacterial lipopolysaccharides: extraction with phenolwater and further applications of the procedure. In: Whistler RL (ed) Methods in carbohydrate chemistry. Academic, New York, pp 83–91
10. Konadu E, Shiloach J, Bryla DA, Robbins JB, Szu SC (1996) Synthesis, characterization, and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of Salmonella paratyphi A bound to tetanus toxoid with emphasis on the role of O acetyls. Infect Immun 64:2709–2715
11. Micoli F, Rondini S, Gavini M, Pisoni I, Lanzilao L, Colucci AM, Giannelli C, Pippi F, Sollai L, Pinto V, Berti F, MacLennan CA, Martin LB, Saul A (2013) A scalable method for O-antigen purification applied to various Salmonella serovars. Anal Biochem 434:136–145
12. Micoli F, Rondini S, Gavini M, Lanzilao L, Medaglini D, Saul A, Martin LB (2012) O:2-CRM(197) conjugates against Salmonella Paratyphi A. PLoS One 7:e47039
13. De Benedetto G, Micoli F, Londero S, Salvini L, Sturiale L, Garozzo D, Ravenscroft N, Giannelli C, Cescutti P (2019) Characterization of the Salmonella Typhimurium core oligosaccharide and its reducing end 3-deoxy-d-manno-oct-2-ulosonic acid used for conjugate vaccine production. Carbohydr Res 481:43–51
14. De Benedetto G, Salvini L, Gotta S, Cescutti P, Micoli F (2018) Investigation on sugar-protein connectivity in Salmonella O-antigen glycoconjugate vaccines. Bioconjug Chem 29:1736–1747

15. Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350
16. Qi XY, Keyhani NO, Lee YC (1988) Spectrophotometric determination of hydrazine, hydrazides, and their mixtures with trinitrobenzenesulfonic acid. Anal Biochem 175:139–144
17. Micoli F, Ravenscroft N, Cescutti P, Stefanetti G, Londero S, Rondini S, Maclennan CA (2014) Structural analysis of O-polysaccharide chains extracted from different Salmonella Typhimurium strains. Carbohydr Res 385:1–8
18. Ravenscroft N, Cescutti P, Gavini M, Stefanetti G, MacLennan CA, Martin LB, Micoli F (2015) Structural analysis of the O-acetylated O-polysaccharide isolated from Salmonella paratyphi A and used for vaccine preparation. Carbohydr Res 404:108–116
19. Perepelov AV, Shekht ME, Liu B, Shevelev SD, Ledov VA, Senchenkova SN, L'Vov VL, Shashkov AS, Feng L, Aparin PG, Wang L, Knirel YA (2012) Shigella flexneri O-antigens revisited: final elucidation of the O-acetylation profiles and a survey of the O-antigen structure diversity. FEMS Immunol Med Microbiol 66:201–210
20. Macgee J, Doudoroff M (1954) A new phosphorylated intermediate in glucose oxidation. J Biol Chem 210:617–626
21. Nohta H, Sonoda J, Yoshida H, Satozono H, Ishida J, Yamaguchi M (2003) Liquid chromatographic determination of dicarboxylic acids based on intramolecular excimer-forming fluorescence derivatization. J Chromatogr A 1010:37–44
22. Miron T, Wilchek M (1982) A spectrophotometric assay for soluble and immobilized N-hydroxysuccinimide esters. Anal Biochem 126:433–435
23. Lei QP, Lamb DH, Heller R, Pietrobon P (2000) Quantitation of low level unconjugated polysaccharide in tetanus toxoid-conjugate vaccine by HPAEC/PAD following rapid separation by deoxycholate/HCl. J Pharm Biomed Anal 21:1087–1091
24. Stefanetti G, Rondini S, Lanzilao L, Saul A, MacLennan CA, Micoli F (2014) Impact of conjugation chemistry on the immunogenicity of S. Typhimurium conjugate vaccines. Vaccine 32:6122–6129

Chapter 15

Oligosaccharide Antigen Conjugation to Carrier Proteins to Formulate Glycoconjugate Vaccines

Brittany R. Smith and Zhongwu Guo

Abstract

Conjugation, that is, covalent linkage, to immunological proteins is a common strategy to address the low immunogenicity issue of carbohydrate antigens in vaccine development. This chapter describes an easy and efficient method for oligosaccharide–protein conjugation employing dicarboxylic acid linkers. In this regard, a free amino group is introduced to an oligosaccharide antigen to facilitate coupling with the bifunctional linker upon reaction with its corresponding disuccinimidyl ester. The resultant monosuccinimidyl ester of the oligosaccharide antigen then reacts with the free amino groups of a carrier protein to provide the desired oligosaccharide–protein conjugate.

Key words Oligosaccharide, Carrier protein, Dicarboxylic acid linker, Conjugation, Glycoconjugate, Vaccine

1 Introduction

Tumor-associated carbohydrate antigens (TACAs) are promising target molecules for the development of therapeutic cancer vaccines since TACAs are overexpressed on the cancer cell surface [1, 2]. However, on their own, TACAs are poorly immunogenic. To overcome this limitation, TACAs are usually conjugated to an immunostimulatory carrier molecule to improve their immunogenicity [3–5]. The carrier molecule is usually a protein, such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin (OVA), or human serum albumin (HSA) [3–5]. Alternatively, it can be a synthetic small molecule, such as lipids (e.g., monophosphoryl lipid A [MPLA] [6]).

Many methods have been developed for conjugating TACAs to carrier molecules [7, 8]. Among them, one of the most common is through alkylation and acylation of the free amino groups of carrier proteins. In this regard, the commonly utilized linkers include alkylating diethyl squarate [9, 10] and acylating dicarboxylic anhydrides (succinic anhydride, glutaric anhydride) [11] or activated

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esters of dicarboxylic acids in forms of *p*-nitrophenol [11, 12] or *N*-hydroxysuccinimide (NHS) [13, 14], such as disuccinimidyl glutarate (DSG) [15, 16], disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS3), and di-(*N*-succinimidyl) carbonate (DSC).

In this chapter, we are focused on the conjugation of synthetic oligosaccharides to carrier proteins through dicarboxylic acid linkers by using activated disuccinimidyl esters of dicarboxylic acids (Scheme 1) because these linkers have been proven to be efficient and cause less side effects [17]. Typically, an azido group, which is stable to most reactions involved in carbohydrate synthesis, is introduced to the reducing end of free oligosaccharides during their synthesis as the azido group can be readily converted into a free amino group to facilitate oligosaccharide conjugation to carrier proteins. Thus, a detailed procedure for this conjugation method starting with the reduction of the azido group in synthetic oligosaccharides [16, 18] is provided here (Scheme 1). The remaining steps of this procedure include condensation of the amino derivative of an oligosaccharide with the dicarboxylic acid linker using a bifunctional disuccinimidyl ester [15, 19], conjugation of the resultant mono-activated ester of the oligosaccharide antigen to a carrier protein, and finally analysis of the carbohydrate loading of the oligosaccharide–protein conjugate [14–16, 19–21].

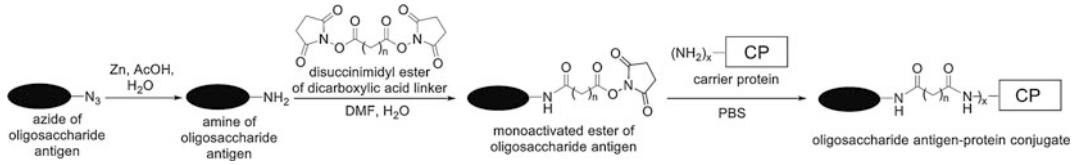
2 Materials

2.1 Reduction of the Azido Group in Oligosaccharide Antigen

1. Dram vial.
2. Magnetic stir bar.
3. Spatula.
4. Stir plate.
5. Microliter syringe.
6. Rotary evaporator.

2.2 Coupling of Amino Derivative of Oligosaccharide Antigen with Disuccinimidyl Ester of Dicarboxylic Acid Linker (See Note 1)

1. Commercial 0.1 M phosphate-buffered saline (PBS).
2. Dram vial.
3. Magnetic stir bar.
4. Spatula.
5. Stir plate.
6. Syringes (1.0 mL).
7. Buchner funnel.
8. High vacuum pump.



Scheme 1 Oligosaccharide antigen conjugation to carrier proteins via dicarboxylic acid linkers

2.3 Conjugation of Mono-activated Ester of Oligosaccharide Antigen to Carrier Protein

1. Dram vial.
2. Magnetic stir bar.
3. Stir plate.
4. Bio-gel A 0.5 m gel.
5. Separation column.
6. 0.1 M PBS.
7. Test tubes (13 × 100 mm).
8. Dialysis tube or bag (with molecular cut of 5–10 kDa).
9. Bicinchoninic acid (BCA).
10. 15% (v/v) H_2SO_4 in EtOH.
11. Freeze dryer.

2.4 Analysis of Carbohydrate Loading of the Resultant Glycoconjugate

1. Commercial monosaccharides appropriate for the oligosaccharide antigen (*see* **Note 2**).
2. Dry test tubes (10 mL).
3. Cuvettes (1 mL).
4. Microliter pipette and tips.
5. UV-Vis spectrometer.
6. 96% H_2SO_4 (concentrated).
7. 4% phenol (aq. solution).
8. Blank carrier protein.

3 Methods

3.1 Reduction of the Azido Group in Oligosaccharide Antigen

1. Dissolve the azido derivative of oligosaccharide antigen (50 μmol) in distilled water (3.0 mL).
2. Add zinc powder (164 mg, 2.5 mmol) (*see* **Note 3**) to the above solution.
3. Add acetic acid (29 μL).
4. Stir the reaction mixture at ambient temperature for 24 h.
5. Add *N,N*-diisopropylethylamine (DIPEA) (*see* **Note 4**) to neutralize the reaction.

6. Filter the reaction mixture through a Celite pad and wash the pad with water.
7. Combine the filtrates and concentrate them in vacuo.
8. Use the resultant crude amine product in the next reaction without further purification.

3.2 Coupling of Amino Derivative of Oligosaccharide Antigen with Disuccinimidyl Ester of Dicarboxylic Acid Linker

1. Dissolve the above-obtained amino derivative of oligosaccharide antigen (50 μmol) in a 4:1 mixture of *N,N*-dimethylformamide (DMF) and 0.1 M PBS (0.5 mL) (*see* **Note 5**).
2. Add the disuccinimidyl ester of a dicarboxylic acid, such as disuccinimidyl glutarate (DSG) (245 mg, 750 μmol) (*see* **Note 6**).
3. Stir the reaction mixture at ambient temperature for 4 h.
4. Remove solvents in vacuo.
5. Wash the resultant mono-activated ester with ethyl acetate ten times (10 × 1.0 mL) (*see* **Note 7**).
6. Dry the solid product under high vacuum.
7. Use the mono-activated ester in the next step without further purification (*see* **Note 6**).

3.3 Conjugation of Mono-activated Ester of Oligosaccharide Antigen to Carrier Protein

1. Dissolve the carrier protein (33 μmol) and the above-obtained mono-activated ester of antigen (50 μmol) in 0.1 M PBS (0.5 mL) (*see* **Note 6**).
2. Stir the solution at ambient temperature for 3 days.
3. Apply the solution to a Bio-gel A 0.5 column (1 × 15 cm) and elute the column with 0.1 M PBS to separate the conjugate from the unconjugated oligosaccharide (*see* **Note 8**).
4. Combine fractions that contain both the protein indicated by the BCA assay (*see* **Note 9**) and the carbohydrate indicated by staining with 15% (v/v) H_2SO_4 in EtOH (*see* **Note 10**).
5. Dialyze the combined fractions against distilled water for 2 days.
6. Lyophilize the solution to afford the conjugate as a white solid.

3.4 Analysis of Carbohydrate Loading of the Resultant Glycoconjugate

3.4.1 Colorimetric Method

Preparation of calibration curve

1. Mix stoichiometric amounts of monosaccharides contained in the oligosaccharide antigen (*see* **Note 2**).
2. Use this mixture to prepare a standard stock solution (1.0 mg/mL) in distilled water.
3. Transfer the standard stock solution to ten different test tubes in 5.0 μL increments, ranging from 5.0 to 50.0 μL.
4. Add 500 μL of 4% phenol (aq. solution) and 2.5 mL of 96% H_2SO_4 in each test tube (*see* **Note 11**).

5. Prepare a negative control containing the free carrier protein (50–100 μg) (*see* **Note 12**) by the same protocol and use it to blank the UV-Vis spectrometer.
6. Transfer the content in each test tube to a cuvette.
7. Measure the A_{490} value of each sample with a UV-Vis spectrometer.
8. Use the A_{490} and sample weight (μg) data to plot calibration curve.

Measurement of carbohydrate loading of glycoconjugate

1. Add 50–100 μg of accurately weighed conjugate, 500 μL of 4% phenol, and 2.5 mL of 96% H_2SO_4 to a test tube (*see* **Note 11**).
2. Transfer the content of the test tube to a cuvette.
3. Measure the A_{490} value of the sample with a UV-Vis spectrometer.
4. Use the calibration curve to determine the weight of carbohydrates in the conjugate sample.
5. Calculate the carbohydrate loading of the conjugate based on the following equations:

$$\%\text{carbohydrate loading by weight} = \frac{\text{weight (μg) of carbohydrates}}{\text{weight (μg) of glycoconjugate sample}} \times 100$$

$$\text{carbohydrate loading by glycans per carrier} = \frac{\text{weight (μg) of carbohydrates}}{[\text{weight (μg) of glycoconjugate} - \text{weight (μg) of carbohydrates}]} \times \frac{\text{protein MW (Da)}}{\text{antigen MW (Da)}}$$

3.4.2 MALDI-TOF Mass Spectrometric Method

For glycoconjugates of smaller proteins, such as OVA (42.7 kDa), BSA (66.5 kDa), and HSA (66.5 kDa), MALDI-TOF MS can be utilized to measure the molecular mass (M. mass) of the conjugate, which is then used to calculate the carbohydrate loading or the average number of antigens loaded onto each carrier protein using the following equations:

$$\%\text{carbohydrate loading by weight} = \frac{[\text{M.mass (Da) of glycoconjugate} - \text{M.mass (Da) of protein}]}{\text{M.mass (Da) of glycoconjugate}} \times 100$$

$$\text{carbohydrate loading by glycans per carrier} = \frac{[\text{M.mass (Da) of glycoconjugate} - \text{M.mass (Da) of protein}]}{\text{M.mass (Da) of oligosaccharide antigen}}$$

3.4.3 Electrophoresis Method

The conjugation of carbohydrate antigens to carrier proteins, including larger carriers such as KLH (390 kDa), can be further confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the resultant glycoconjugate, which shows an increased molecular size and mass compared to the free protein (*see* **Note 13**).

4 Notes

1. Dicarboxylic acid anhydrides can also be utilized in place of disuccinimidyl esters. In this case, an additional step to transform the resulting carboxylic acid derivatives into activated esters is necessary before the conjugation to carrier proteins.
2. The monosaccharide samples are used to prepare standard stock solutions for obtaining the calibration curve in the analysis of carbohydrate loading of the conjugate. The monosaccharides used should be the same as those in the carbohydrate antigen and in the same molar ratio equivalent. For example, the Globo-H antigen is composed of one Fuc unit, three Gal units, one GalNAc unit, and one Glc unit; thus the monosaccharide mixture should be made of Fuc, Gal, GalNAc, and Glc in a molar ratio of 1:3:1:1 [15, 20].
3. Commercial zinc powder should be activated before use by treatment with diluted HCl aq. solution (0.1 N), which is followed by thorough washing with distilled water until the washing becomes neutral.
4. Other tertiary amines, such as trimethyl and triethyl amines, can be used to neutralize the reaction. However, the use of primary and secondary amines as bases should be avoided because these compounds can interfere with the acylation reaction [7].
5. DMF is added as a cosolvent to help dissolve disuccinimidyl dicarboxylic acid ester as it is not soluble in water or PBS.
6. N-Hydroxysuccinimide esters can be hydrolyzed slowly in water. Thus, they should be stored as dry solids before being added to reaction solutions and the activated intermediates should be applied to the next step as soon as possible.
7. The product should be washed with ethyl acetate thoroughly to remove any excessive dicarboxylic acid disuccinimidyl ester because its presence can affect subsequent antigen conjugation with carrier protein.
8. The antigen–protein conjugate elutes much earlier from the size-exclusion column than the unconjugated oligosaccharide antigen because of the large difference between their molecular sizes.
9. A spectrophotometer in combination with a Pierce BCA protein assay kit can be used for the detection of proteins.
10. Staining of carbohydrates with 15% (v/v) H_2SO_4 in EtOH: Apply the eluted fractions onto a silica gel TLC plate, and then dip the plate in the 15% (v/v) H_2SO_4 in EtOH solution. Wipe the back of the plate clean and char it on a hot plate until the

plate background becomes slightly yellowish. Carbohydrates are stained in dark brown or black color.

11. Addition of sulfuric acid to the aqueous solution will generate heat. Allow the sample to cool gradually to ambient temperature. Wait 20 min or until complete color change is seen before transferring samples to a cuvette.
12. The addition of carrier protein in the blank aims to eliminate the potential influence of protein on the carbohydrate analysis of glycoconjugate.
13. This method can only provide qualitative results confirming the conjugation.

References

1. Monzavi-Karbassi B, Pashov A, Kieber-Emmons T (2013) Tumor-associated glycans and immune surveillance. Vaccine 1:174–203
2. Hakomori SI (2001) Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv Exp Med Biol 491:369–402
3. Danishefsky S, Allen J (2000) From the laboratory to the clinic: a retrospective on fully synthetic carbohydrate-based anticancer vaccines. Angew Chem Int Ed Engl 39:836–863
4. Feng D, Shaikh AS, Wang F (2016) Recent advance in tumor-associated carbohydrate antigens (TACAs)-based antitumor vaccines. ACS Chem Biol 11:850–863
5. Guo Z, Wang Q (2009) Recent development in carbohydrate-based cancer vaccines. Curr Opin Chem Biol 13:608–617
6. Moyle PM, Toth I (2008) Self-adjuvanting lipopeptide vaccines. Curr Med Chem 15:506–516
7. Hermanson GT (2013) The reactions of bioconjugation. In: Audet J, Preap M (eds) Bioconjugate techniques. Elsevier, Rockford, pp 229–258
8. Gamblin DP, Scanlan EM, Davis BG (2009) Glycoprotein synthesis: an update. Chem Rev 109:131–163
9. Jacques S, Rich JR, Ling C et al (2006) Chemoenzymatic synthesis of GM 3 and GM 2 gangliosides containing a truncated ceramide functionalized for glycoconjugate synthesis and solid phase applications. Org Biomol Chem 3:142–154
10. Andersen SM, Ling C-C, Zhang P et al (2004) Synthesis of ganglioside epitopes for oligosaccharide specific immunoadsorption therapy of Guillian-Barré syndrome. Org Biomol Chem 2:1199–1212
11. Wang Q, Xue J, Guo Z (2009) Synthesis of a monophosphoryl lipid A derivative and its conjugation to a modified form of a tumor-associated carbohydrate antigen GM3. Chem Commun 5536–5537
12. Liao G, Zhou Z, Suryawanshi S et al (2016) Fully synthetic self-adjuvanting α-2,9-oligosialic acid based conjugate vaccines against group C meningitis. ACS Cent Sci 2:210–218
13. Toyokuni T, Dean B, Cai S et al (1994) Synthetic vaccines: synthesis of a dimeric Tn antigen-lipopeptide conjugate that elicits immune responses against Tn-expressing glycoproteins. J Am Chem Soc 116:395–396
14. Wang Q, Ekanayaka SA, Wu J et al (2008) Synthetic and immunological studies of 5′-N-phenylacetyl sTN to develop carbohydrate-based cancer vaccines and to explore the impacts of linkage between carbohydrate antigens and carrier proteins. Bioconjug Chem 19:2060–2067
15. Zhou Z, Liao G, Mandal SS et al (2015) A fully synthetic self-adjuvanting globo H-based vaccine elicited strong T cell-mediated antitumor immunity. Chem Sci 6:7112–7121
16. Liao G, Zhou Z, Burgula S et al (2015) Synthesis and immunological studies of linear oligosaccharides of β-glucan as antigens for antifungal vaccine development. Bioconjug Chem 26:466–476
17. Buskas T, Li Y, Boons G (2004) The immunogenicity of the tumor-associated antigen Lewis (y) may be suppressed by a bifunctional cross-linker required for coupling to a carrier protein. Chem Eur J 10:3517–3524
18. Burgula S, Swarts BM, Guo Z (2012) Total synthesis of a glycosylphosphatidylinositol

anchor of the human lymphocyte CD52 antigen. Chem Eur J 18:1194–1201

19. Liao G, Zhou Z, Guo Z (2015) Synthesis and immunological study of α-2,9-oligosialic acid conjugates as anti-group C meningitis vaccines. Chem Commun 51:9647–9650
20. Fournier E (2001) Colorimetric quantification of carbohydrates. In: Wrolstad RE, Acree TE, Decker EA et al (eds) Handbook of food analytical chemistry. Wiley, New York, pp E1.1.1–E1.1.8
21. Wu J, Guo Z (2006) Improving the antigenicity of sTn antigen by modification of its sialic acid residue for development of glycoconjugate cancer vaccines. Bioconjug Chem 17:1537–1544

Chapter 16

Exploitation of Capsule Polymerases for Enzymatic Synthesis of Polysaccharide Antigens Used in Glycoconjugate Vaccines

Christa Litschko, Insa Budde, Monika Berger, and Timm Fiebig

Abstract

The exploitation of recombinant enzymes for the synthesis of complex carbohydrates is getting increasing attention. Unfortunately, the analysis of the resulting products often requires advanced methods like nuclear magnetic resonance spectroscopy and mass spectrometry. Here, we use the capsule polymerases Cps4B and Cps11D from *Actinobacillus pleuropneumoniae* serotypes 4 and 11, respectively, as examples for the in vitro synthesis of capsule polymers similar to those used in glycoconjugate vaccine formulations. We demonstrate how substrate turnover in an enzymatic reaction can be analyzed by HPLC-based anion exchange chromatography and provide the protocol for separation and detection of UV-active polymer. Moreover, we describe how UV-inactive polymer can be separated and visualized using polyacrylamide gel electrophoresis followed by combined alcian blue–silver staining.

Key words In vitro vaccine production, Capsule polymerase, Capsular polysaccharide, Enzymatic synthesis, Glycoconjugate vaccine, HPLC, PAGE, Alcian blue–silver staining, *Actinobacillus pleuropneumoniae*

1 Introduction

Capsular polysaccharides belong to the main virulence factors of bacterial pathogens by providing protection against host immune defense mechanisms [1]. However, since they represent the outermost layer of the cell and thus the interface to the host immune system, they can also be used as antigens in glycoconjugate vaccine formulations. Glycoconjugate vaccines have been tremendously effective in combatting diseases caused by various bacterial pathogens [2]. Through coupling of the saccharide antigen to a carrier protein, a T-cell dependent immune response is achieved, including immunologic memory, immunoglobulin M to immunoglobulin G isotype switching, and protection of infants [3]. The conventional production of glycoconjugate vaccines includes the purification of the capsule polymer from pathogen cultures (Fig. 1). This

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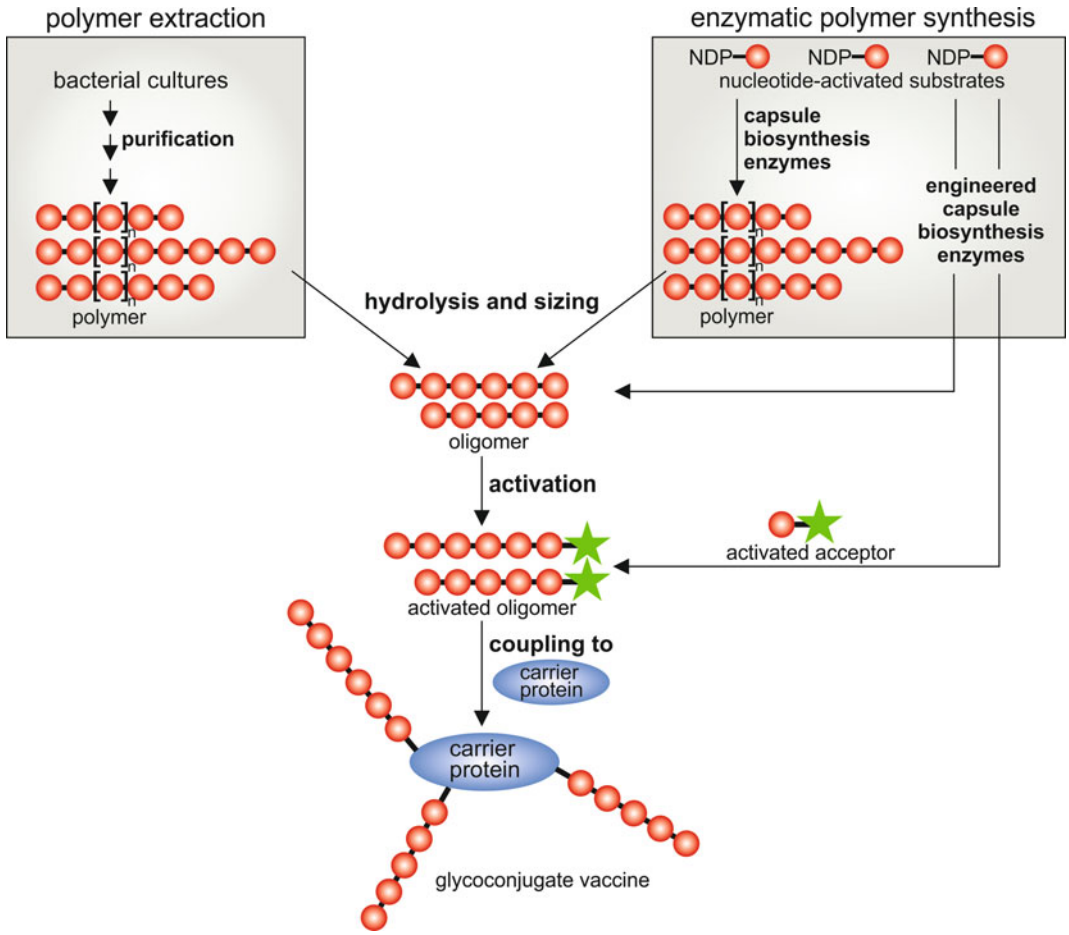

Fig. 1 Conventional and enzymatic glycoconjugate synthesis. Traditionally, polymers are purified from bacterial cultures based on their physicochemical properties. Enzymatic synthesis entirely avoids pathogen culture and polymer synthesis starts from highly pure, nucleotide-activated monosaccharides using recombinant enzymes as catalysts. Engineering the enzyme properties can facilitate the synthesis protocol by enabling control over the product length or allowing the incorporation of functional groups necessary for saccharide-protein coupling

biohazardous step essentially depends on the high-tech infrastructure of modern production plants, which require high biosafety standards [4], making glycoconjugate vaccines expensive, not sufficiently accessible to low-income countries, and, with regard to animal husbandry, where they could reduce/avoid the exuberant use of antibiotics, not sufficiently cost-effective [5–7].

Alternative means for the provision of the polymer antigen are chemical and enzymatic synthesis [8, 9]. With *Neisseria meningitidis* serogroup X, we and other groups have shown that capsule polymerases, the enzymes that generate the capsule polymer in vivo, can be expressed as soluble constructs [10, 11], can be engineered to produce tailored oligosaccharides [12], allow the

introduction of functional groups in their substrates facilitating downstream coupling of oligosaccharides to the carrier protein [13, 14], and can be exploited for the generation of functional glycoconjugates, using standard, low-cost lab equipment (Fig. 1) [14, 15].

In the herein presented protocol, we use the capsule polymerases Cps4B and Cps11D of *Actinobacillus pleuropneumoniae* (*App*) serotypes 4 and 11, respectively, to illustrate how enzymatic synthesis of capsule polymer can be achieved in vitro. *App* is a pig specific pathogen that causes huge economic losses in animal husbandry, and although effective glycoconjugate vaccines have been generated using polymer from bacterial cultures [16, 17], presumably the high costs for their production have hindered further efforts to bring them to market [7]. Both Cps4B and Cps11D are part of an ATP-transporter dependent assembly system in vivo, also known as group 2 capsule biosynthesis complex [1]. Group 2 polymerases, though believed to be membrane associated, are not integral membrane proteins, allowing soluble expression and purification of recombinant constructs with good yields. In addition, all group 2 polymerases generate negatively charged polymers with good solubility in water, and most enzymes can initiate polymerization in vitro also in the absence of their priming acceptor [18], solely requiring nucleotide activated substrates for polymer production [19–21].

Cps4B and Cps11D belong to the recently described TagF-like capsule polymerase family that consists of multidomain enzymes generating phosphate-containing, teichoic acid-like capsule polymers [20]. Cps4B assembles a [→6)-β-Glc-(1 → 3)-GalNAc-(1-PO_4^-] repeating unit using UDP-Glucose (UDP-Glc) and UDP-N-acetylgalactosamine (UDP-GalNAc) as donor substrates, whereas Cps11D requires UDP-galactose (UDP-Gal) and CDP-glycerol (CDP-Gro) for the generation of a [→4)-α-Gal-(1 → 2)-Gro-(3-PO_4^-] repeating unit (Fig. 2) [22, 23]. Due to the fact that nucleotides absorb light at 260 nm

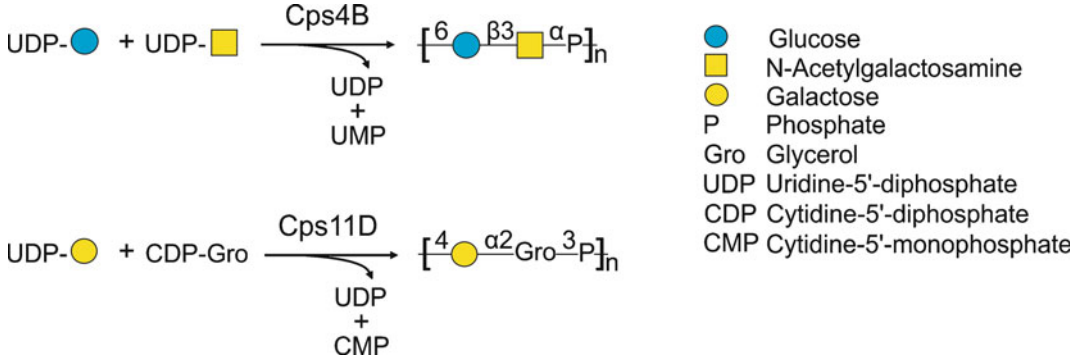

Fig. 2 Reactions catalyzed by the enzymes Cps4B and Cps11D

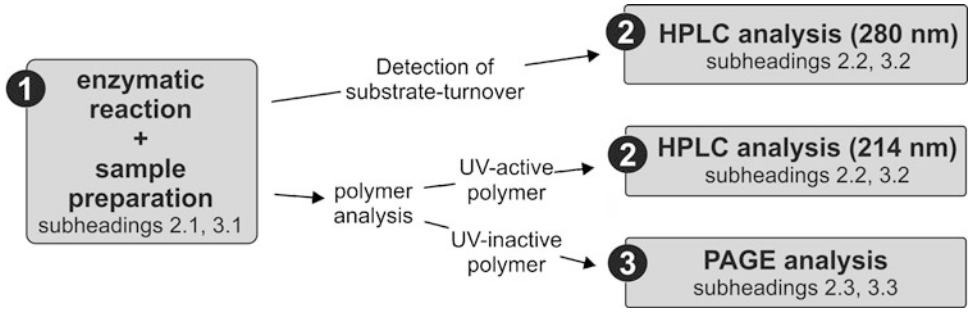

Fig. 3 Flowchart showing the different steps and subsections described in this chapter

and carry negative charges introduced by phosphate groups, substrate turnover in a group 2 capsule polymerase reaction can be readily analyzed by high-performance liquid chromatography (HPLC) coupled to UV-detection, using an anion exchange chromatography (AEC) resin as the basis for separation. UV-detection of the polymer products is possible if UV-active groups (e.g., N-acetyl groups) are present; whereas, polymers lacking these groups can be visualized using an alcian blue/silver stained PAGE.

This chapter is divided into three sections as follows:

Method 1: Enzymatic synthesis of capsule polymer.

Method 2: HPLC-AEC analysis of nucleotide substrates (280 nm) and UV-active polymer (214 nm).

Method 3: Separation and visualization of UV-inactive capsule polymer by high-percentage polyacrylamide gel electrophoresis (Fig. 3).

2 Materials

Prepare all solutions with ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (22 °C), unless indicated otherwise.

2.1 Enzymatic Activity Assays

1. Incubator allowing a constant temperature of 37 °C (*see* **Note 1**).
2. Purified proteins in a concentration ranging from 3 to 15 mg/mL. Store at −80 °C.
3. 100 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.0). For a 10× stock solution (1 M Tris–HCL pH 8.0), weigh 60.57 g of Tris in a 500 mL volumetric flask and add approx. 350 mL of water. After the salt is completely dissolved, adjust the pH to 8.0 with HCl. Add water to a volume of 500 mL, sterile-filter (22 μm) the buffer and transfer it to a 500 mL flask for autoclavation. Prepare 1× working solution from the 10× stock with water. For a 10 mL working solution,

mix 1 mL of 1 M Tris–HCL (pH 8.0) with 9 mL of water. Gently mix.

4. 25 mM dithiothreitol (DTT) (*see* **Note 2**). For 10 mL of a 40× stock (1 M DTT) dissolve 1.54 g of DTT in 10 mL of water. Prepare 0.5 mL to 1 mL aliquots for storage at −20 °C. Dilute 25 µL of 1 M DTT with 975 µL of water to obtain a 25 mM DTT working solution. Gently mix. Use the freshly prepared DTT for the preparation of the assay buffer. Use it only once and discard the solution after usage.
5. 100 mM nucleotide activated substrates. Weigh an appropriate amount of nucleotide activated substrate and dissolve in an appropriate amount of water to obtain a 100 mM stock solution (*see* **Note 3**). Prepare aliquots for the storage at −80 °C. UDP-Glc/UDP-GalNAc and UDP-Gal/CDP-Gro are substrates of Cps4B and Cps11D, respectively (*see* Fig. 2).
6. 50 mM magnesium chloride ($MgCl_2$) (*see* **Notes 4** and **5**). For 100 mL of a 20× stock solution (1 M $MgCl_2$) weigh 20.3 g of $MgCl_2$ (*$6H_2O$) in a 100 mL volumetric flask and add water to the graduation mark. Dissolve the salt, sterile-filter (22 µm) the buffer and transfer it to a 100 mL flask for autoclavation. Dilute 50 µL of the 1 M $MgCl_2$ stock solution with 950 µL of water for the preparation of 1× $MgCl_2$ (50 mM).

2.2 HPLC Assay

Prepare all solutions with ultrapure water and analytical grade reagents. The method requires an HPLC system equipped with a UV detector allowing wavelengths of 280 nm and 214 nm and corresponding software for data analysis. The column oven needs to be equipped with a column suitable for anion exchange chromatography. Results were obtained with a Prominence UFLC-XR (Shimadzu) equipped with a SPD-20AV detector (Shimadzu) (*see* **Note 6**) and a CarboPac™ PA-100 column 2 × 250 mm + guard column (Dionex™) (*see* **Note 7**).

1. 1 M Tris–HCl (pH 8.0). Weigh 121.14 g of Tris in a 1 L volumetric flask and add 600 mL of water. After the salt is completely dissolved, adjust the pH to 8.0 with HCl. Add water to a volume of 1 L. Filter the buffer and transfer it to a 1 L flask for autoclavation.
2. 5 M NaCl. Weigh 292.2 g of NaCl in a 1 L volumetric flask and add ~950 mL of water. Dissolve the salt using a magnetic stirrer. Add water to a volume of 1 L after NaCl is completely dissolved. Filter the buffer and transfer it to a flask for autoclavation.
3. Preparation of mobile phase 1: 20 mM Tris–HCl (pH 8.0). Transfer 20 mL of the 1 M Tris–HCl buffer to a 1 L volumetric flask and add water to the graduation mark. Filter the buffer and transfer it to a flask for autoclavation (*see* **Note 8**).

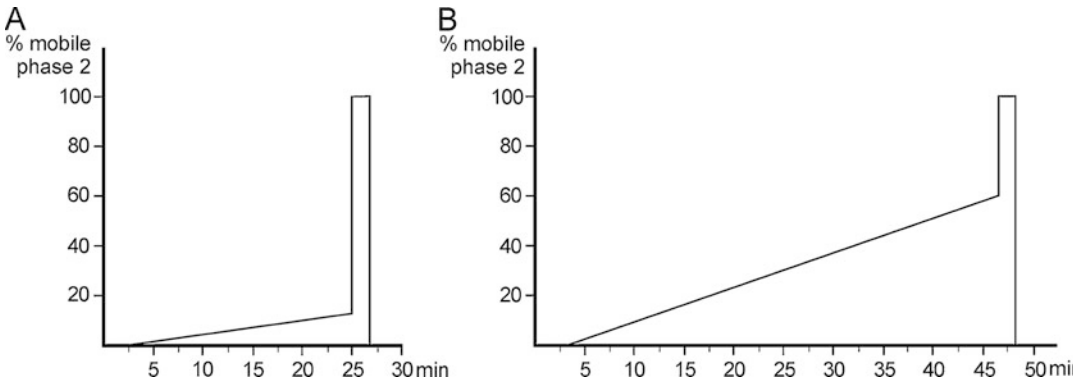

Fig. 4 Elution gradient for (**a**) nucleotide analysis and (**b**) separation of polymer

4. Preparation of mobile phase 2: 20 mM Tris–HCl (pH 8.0), 1 M NaCl. Transfer 20 mL of the 1 M Tris–HCl buffer and 200 mL of the 5 M NaCl solution to a 1 L volumetric flask and add water to the graduation mark. Filter the buffer and transfer it to a 1 L flask for autoclavation (*see* **Note 8**).
5. Prepare an HPLC instrument method for nucleotide analysis: Nucleotides are separated using a linear elution gradient from 0% to 14% mobile phase 2 over 23 min and the column is washed with 100% mobile phase 2 for 2 min. Allow pre- and postequilibration of the column with 100% mobile phase 1 for 2–3 min (*see* Fig. 4). Set a flow rate of 0.6 mL/min and a column temperature of 50 °C and record absorption at 280 nm (*see* **Note 9**).
6. Prepare an HPLC instrument method for the separation of capsule polymer. Polymers are separated using a linear elution gradient from 0% to 60% mobile phase 2 over 44 min and the column is washed with 100% mobile phase 2 for 2 min. Allow pre- and postequilibration of the column with 100% mobile phase 1 for 2–3 min (*see* Fig. 4). Set a flow rate of 0.6 mL/min and a column temperature of 50 °C and record absorption at 214 nm.
7. Standards containing nucleotide substrates and products (UDP-GalNAc, UDP, etc.) should be prepared using buffer conditions and concentrations identical to those used in the reaction mix.

2.3 Visualization of Polymer in a High-Percentage Polyacrylamide Gel Electrophoresis (PAGE)

The combined alcian blue and silver staining protocol is a sensitive method for the detection of carbohydrate containing polymers and based on a protocol published previously [24]. It requires no labeling or pretreatment of sample. Densitometry of stained bands is possible for quantitative analysis (shown in [24]) but not routinely used in our laboratory and thus not part of this protocol.

Prepare all solutions with ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Solutions containing volatile solvents need to be prepared in a fume hood. Follow all waste disposal regulations. The method requires a gel chamber that enables the constant cooling of the system during electrophoresis and that allows for the installation of gel cassettes of sufficient size (*see* **Note 10**). Electrophoresis was performed using the vertical protein electrophoresis chamber from Biometra with an included built-in water-cooling system used in combination with the minichiller from Huber.

1. 1× TBE (100 mM Tris, 100 mM boric acid, 2.5 mM EDTA). For 1 L of a 10× TBE stock solution (1 M Tris, 1 M boric acid, 25 mM EDTA), weigh 121.14 g of Tris, 61.85 g of boric acid, and 9.3 g of EDTA in a 1 L volumetric flask. Add about 600 mL of water and dissolve the salts. Subsequently add water to a volume of 1 L and autoclave the buffer. For 1× TBE, dilute 10× TBE 1:10 with water.
2. 40% acrylamide solution (commercially available). Store at 4 °C.
3. 2% bisacrylamide (commercially available). Store at 4 °C.
4. 10% ammonium persulfate (APS): Weigh 1 g APS in a 10 mL volumetric flask and add water to the graduation mark. Store at 4 °C.
5. *N,N,N′,N′*-tetramethylethylenediamine (TEMED). Store at 4 °C.
6. Loading buffer: 2 M sucrose in 10× TBE. To obtain 50 mL, dissolve 34.23 g of sucrose in 10× TBE.
7. Polysaccharide marker (dissolved 1:2 in loading buffer): 0.05% trypan blue (corresponds to polySia-DP100), 0.02% xylene cyanol (corresponds to polySia-DP52), 0.02% bromophenol blue (corresponds to polySia-DP19), 0.02% bromo cresol purple (corresponds to polySia-DP11), 0.02% phenol red (corresponds to polySia-DP6) (*see* **Note 11**).
8. Fixing solution: 40% ethanol, 5% acetic acid. For 100 mL of fixing solution, mix 40 mL of ethanol and 5 mL of acetic acid and add water to a volume of 100 mL. Should be prepared freshly for each use (*see* **Note 12**).
9. Alcian blue solution: 0.5% alcian blue, 3% acetic acid. Weigh 500 mg of alcian blue, add 3 mL of acetic acid, and add water to a volume of 100 mL. Filter the solution. The staining solution can be reused at least five to ten times (*see* **Note 12**).

10. Oxidizing solution: 0.7% periodic acid, 40% ethanol, 5% acetic acid. For 100 mL, weigh 0.7 g periodic acid, add 40 mL of ethanol and 5 mL of acetic acid and fill with water to a volume of 100 mL. Mix properly by shaking (*see* **Note 13**).
11. Silver nitrate solution (*see* **Note 14**): 0.6% silver nitrate, 0.4% ammonia, 10 mM NaOH. To obtain a ~100 mL solution, weigh 0.6 g of silver nitrate and properly dissolve the salt in 98 mL of water. After the silver nitrate is completely dissolved, add 1.2 ml of 33% ammonia and 1 mL of 2 M NaOH.
12. Formaldehyde solution: 0.05% formaldehyde, 240 μM citric acid. For a 100 mL solution, mix 135 μL of 37% formaldehyde with water, add 5 mg of citric acid and water up to a volume of 100 mL (*see* **Note 14**).
13. Stop solution: 5% acetic acid. Add 5 mL of acetic acid to a 100 mL volumetric flask and make up to 100 mL with water (*see* **Note 15**).

3 Methods

3.1 Enzymatic Activity Assays

The reaction mix is prepared at room temperature. The reaction components are stored on ice during preparation. A standard reaction mix has a volume of 75 μL and contains 0.1–0.3 nmol of purified protein. The final concentration for nucleotide activated substrates usually ranges from 2 to 10 mM, and $MgCl_2$ is added at concentrations from 5 to 20 mM. Two examples are given in Table 1.

Table 1
Reaction mixtures

Cps4B				Cps11D			
Component	**Stock con.**	**Final con.**	**μL**	**Component**	**Stock con.**	**Final con.**	**μL**
Tris–HCl pH 8.0	100 mM	20 mM	15	Tris–HCl pH 8.0	100 mM	20 mM	15
$MgCl_2$	50 mM	10 mM	15	DTT	25 mM	1 mM	3
DTT	25 mM	1 mM	3	UDP-Gal	100 mM	4 mM	3
UDP-Glc	100 mM	4 mM	3	CDP-Gro	100 mM	4 mM	3
UDP-GalNAc	100 mM	4 mM	3	Cps11D	0.047 mM	2.5 μM	4
Cps4B	0.047 mM	2.5 μM	4	H_2O			47
H_2O			32			Total vol.	75
		Total vol.	75				

1. Add all components of the reaction mixture to a tube, gently mix. Start the reaction by adding the capsule polymerase (*see* **Note 16**).
2. Incubate the reaction mixture overnight at 37 °C (*see* **Notes 17** and **18**).
3. Stop reaction by heat shock at 60 °C for 5 min (*see* **Note 19**).
4. Centrifuge sample at 20,000 × *g* for 2 min to spin down precipitated protein and potential aggregates prior to analysis by HPLC.

3.2 HPLC Assay

When using substrate concentrations as shown in Table 1, 3–5 μL of sample is required for the detection of nucleotides at 280 nm and 40–50 μL for the detection of capsule polymer at 214 nm. The samples are cooled to 15 °C in the autosampler to avoid spontaneous hydrolysis of sample ingredients and evaporation. The column oven is heated to 50 °C and the flow rate is set to 0.6 mL/min.

1. Transfer the supernatant of the centrifuged sample to a tube suitable for your HPLC device and autosampler. Leave some of the supernatant in the tube to avoid unnecessary stirring of precipitated protein. 65 μL of supernatant will suffice to perform the analysis. Cool the autosampler to 15 °C. Use tube seals with precut slits to avoid evaporation of the sample in the autosampler.
2. Heat the column oven to 50 °C (*see* **Note 20**).
3. Equilibrate the system and column with mobile phase 1.
4. Start the instrument method for the analysis of nucleotides with an absorption wavelength of 280 nm. Load a sample volume of 3–5 μL (*see* Subheading 2.2, **item 5**).
5. For the analysis of UV-active polymer, start the instrument method with an absorption wavelength of 214 nm and load 40–50 μL of sample (*see* Subheading 2.2, **item 6**).
6. Analyze the chromatograms. Identify the sample constituents by comparison with the standards (Figs. 5 and 6).

3.3 Visualization of Polymer in a High-Percentage PAGE

1. Prepare the acrylamide–bisacrylamide solution according to the table below (Table 2). A separating gel is sufficient for this method, no stacking gel is needed.
2. After the addition of TEMED, immediately pour the unpolymerized gel solution between the glass plates and insert a comb without introducing air bubbles.
3. Allow the polymerization of the gel for a minimum of 30 min.
4. Insert the gel cassette into the gel chamber and fill the chamber with 1× TBE.

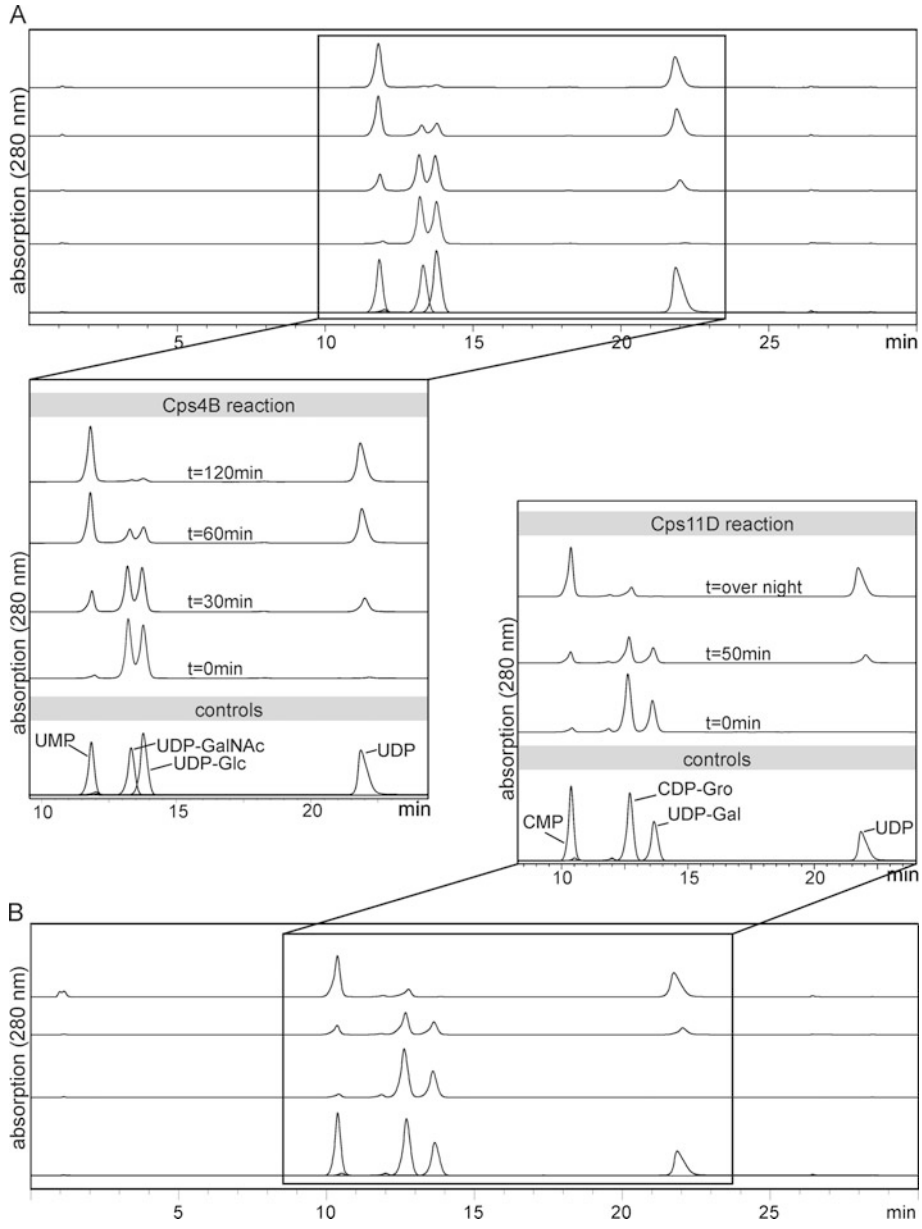

Fig. 5 HPLC-AEC analyses of (**a**) Cps4B and (**b**) Cps11D reactions. The assay allows the separation and detection (280 nm) of nucleotide activated donor substrates and released nucleotide products. Substrates and products were monitored without enzyme ($t = 0$) and at the indicated time points after the start of the reaction. The chromatograms demonstrate that Cps4B consumes its donor substrates UDP-GalNAc and UDP-Glc and simultaneously produces UMP and UDP. Cps11D consumes its donor substrates CDP-Gro and UDP-Gal and produces CMP and UDP (*see* Fig. 2)

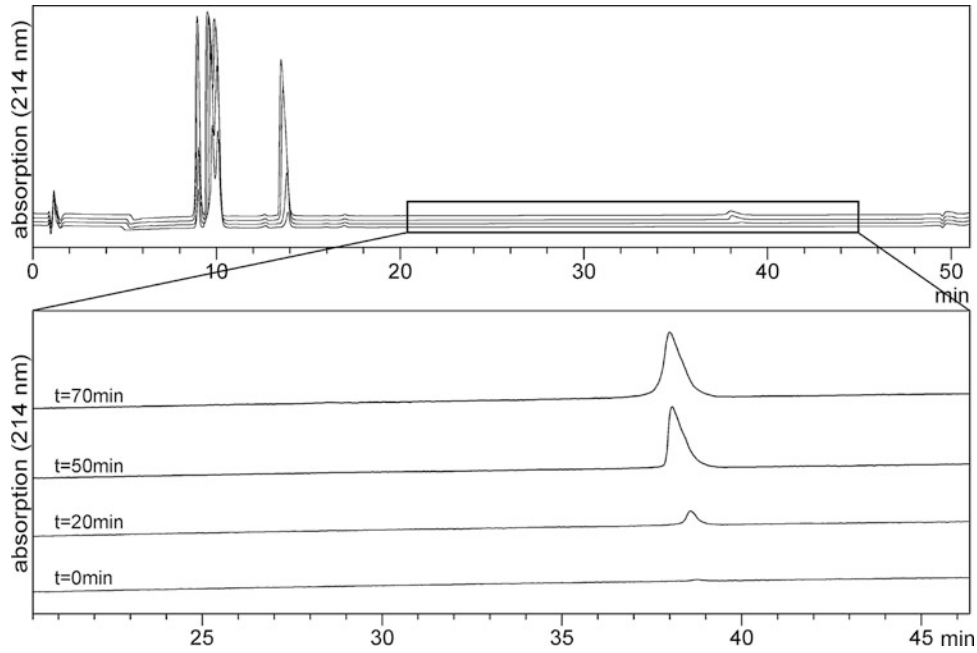

Fig. 6 HPLC-AEC analysis of polymer synthesized by Cps4B (214 nm channel). The production of polymer was monitored at time points 20, 50, and 70 min after the start of the reaction. The reaction mixture prior to the addition of enzyme ($t = 0$) was analyzed as a control. The signal intensity of the polymer eluting after 38 min is low if compared to the signal intensity of the nucleotides eluting during the first 15 min of the run

Table 2
Acrylamide mix

	15%	**25%**
40% acrylamide	6 mL	10 mL
2% bisacrylamide	1 mL	1.6 mL
10× TBE	1.6 mL	1.6 mL
H_2O	7.4 mL	2.8 mL
10% APS	80 µL	80 µL
TEMED	12 µL	12 µL
Total volume	ca. 16 mL	ca. 16 mL

5. Carefully remove the gel comb and control the wells and remove polymerized gel fragments that block the sample wells.
6. Allow the chamber to cool down and the gel to equilibrate for 30 min at 4 °C and 300 V (*see* **Note 21**).
7. Prepare samples by mixing samples 1:1 with loading buffer.
8. Slowly load samples into the wells (*see* **Note 22**).

9. Load 5 μL of the polysaccharide marker or any other suitable marker for your application.
10. Run the gel at 400 V and 4 °C (*see* **Note 21**).
11. Do not touch the gel from now on. Touching the gel might cause marks that will be stained.
12. Stop electrophoresis. Carefully remove gel cassette and gel.
13. Incubate gel for at least 1 h in freshly prepared fixing solution.
14. Incubate gel for 30 min in alcian blue staining solution. The alcian blue solution can be reused for staining up to ten gels.
15. To remove remaining alcian blue, wash the gel in water. Repeat this step three to four times. Leaving the gel in water overnight might reduce unwanted background staining.
16. Remove the water and gently shake the gel in oxidizing solution for 5 min.
17. Wash three times for 15 min with 500 mL of water.
18. Incubate for 10 min in silver nitrate solution.
19. Wash three times for 10 min with water to minimize the aggregation of insoluble silver salts.
20. Gently shake the gel in 100 mL formaldehyde solution. Carefully observe the staining progress and stop the staining reaction with stop solution when signal intensities are sufficient.
21. The gel can be stored in water or dried after incubation for at least 4 h in drying solution.

4 Notes

1. We recommend using an incubator instead of a heating block to prevent the condensation of water inside the tube lid.
2. Capsule polymerases are cytosolic enzymes and DTT is added to mimic the cytosolic reducing environment.
3. Nucleotide activated substrates are freeze-dried with different counter ions depending on the method of preparation and the supplier. Dissolve the substrates according to the molecular weight given by the supplier. We recommend to either weigh in amounts >5 mg or dissolve the entire batch of material to get precise results.
4. Cps4B consists of two domains. The N-terminal domain adopts a glycosyltransferase A (GT-A) fold and the C-terminal domain adopts a phosphotransferase fold similar to the glycosyltransferase B (GT-B) fold (details regarding the domain organization, cloning, and purification of Cps4B and Cps11D and the purification and NMR analysis of their reaction

products can be found elsewhere [20, 23]). The active site of a GT-A fold contains a conserved motif (DXD) that interacts primarily with the phosphate groups of the nucleotide donor through the coordination of a divalent cation [25]. Thus, divalent cations are usually added to the reaction buffer. We observed sufficient Cps4B activity in the presence of 10 mM $MgCl_2$. However, other cations (e.g., Mn^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}) have been reported as essential cofactors for enzyme activity of other GT-A folded or non-GT-A folded proteins [10, 25, 26] and should be considered as additives in the reaction.

5. It is of note that Cps4B and Cps11D synthesize the polymer de novo in the absence of an acceptor substrate. However, many polymerases need a priming acceptor (such as hydrolyzed polymer fragments) to initiate polymer synthesis [1].
6. The signals obtained in the HPLC-AEC assay result from the absorption of light by the nucleobases (absorption maximum approx. 260 nm) that are part of the nucleotides (e.g., CMP, UMP, UDP) and nucleotide activated substrates (e.g., UDP-Gal, CDP-Gro). The signals obtained for (UV-active) polymers result from the absorption of light by the N-acetyl groups at 214 nm, which are present in the polymer generated by Cps4B and lacking in the polymer generated by Cps11D (*see* Fig. 2).
7. CarboPac™ columns are intended to be used for the separation of charged and noncharged carbohydrates under high pH conditions. However, the separation of negatively charged molecules under neutral pH conditions is possible as well.
8. The filtered and autoclaved buffer can be used for several measurements. Please check the clarity and purity before using the buffer in the HPLC assay.
9. Nucleotides and nucleotide sugars are detected at 280 nm, which deviates from the wavelength of maximum absorption (approx. 260 nm for most nucleotides), leading to a lower UV-signal intensity and thus allowing a direct injection of sample into the HPLC system without prior dilution of the reaction mixture. If nucleotide concentrations far below 1 mM are required in the enzymatic reaction, changing the detection wavelength to 260 nm should be considered to increase the signal intensity.
10. Since fragments of group 2 polymers have the same charge-to-mass ratio, separation occurs on the basis of fragment size [24]. Consequently, improved resolution can be achieved by increasing the gel length. The gel shown in Fig. 7 measures ~13.5 cm (height) × ~11 cm (width). In addition, decreasing the sample load also contributes to better resolution of single

Fig. 7 Separation of polymers generated by Cps4B and Cps11D using high percentage PAGE (15%) followed by a combined alcian blue–silver staining. The amounts of polymer (in μg) loaded are indicated above each well. The gel illustrates nicely that the different composition and/or lengths of the analyzed polymers affect their electrophoretic mobility. Thus, conclusions regarding the lengths of the analyzed polymers should only be drawn when comparing polymers of identical chemical composition

species by minimizing band diffusion and overlap [24]. Because the polymers shown in Fig. 7 are considerably long, achieving single species resolution was beyond the scope of the experiment, but example gels are shown in the original protocol [24] and other publications [12].

11. Different tracking dyes can be used as markers. The electrophoretic mobility of a polymer is influenced by its chemical and physical properties. The polysialic acid (PolySia) size marker of defined degree of polymerization mentioned in this method can be used to estimate the electrophoretic mobility of polymers carrying one charge per monomeric repeating unit. If necessary, experimentally compare the electrophoretic mobility of different dyes with polymers of known length to establish a suitable tracking dye mix for your application.

12. Both acetic acid and alcian blue (a cationic dye for the staining of acidic polysaccharides such as polysialic acid, glycosaminoglycans, and phosphate-containing polymers) fixate the polymers in the gel and prevent smaller species from diffusing from the gel [24, 27]. In our experience, oligosaccharides ≥4 monomeric repeating units can be visualized using this protocol. It is recommended to use alcian blue in acetic acid rather than in water, because acetic acid enhances the solubility of the dye, prevents precipitation and thus allows the reuse of the staining solution [24]. If the alcian blue solution is used without subsequent silver staining, only large polymer fragments are visualized.
13. The periodic acid oxidizes 1,2-diol groups of monosaccharide subunits of the polymers to aldehydes that can be revealed by silver staining performed in the following step [27].
14. Positively charged silver ions bind to negative groups in the polymer (e.g., phosphate groups, carboxyl groups). Formaldehyde reduces the silver ions to silver, leading to the brown/black colour.
15. The addition of acetic acid changes the pH which stops the staining process.
16. The HPLC method for nucleotide separation allows the analysis of substrate turnover. If the polymerase precipitates before all substrates are used up, remove precipitates by centrifugation (20,000 × *g*) and add additional polymerase to the reaction until substrate consumption is completed and synthesis yield is maximized. In the long run, buffer conditions should be optimized for each individual polymerase and might deviate from the conditions used in this protocol for Cps4B and Cps11D.
17. Some nucleotide activated substrates are less stable under certain conditions. In our experience, UDP-GlcNAc is stable in the above-listed reaction buffer overnight at 37 °C, whereas, for example, UDP-Gal quickly breaks down to UMP and Gal-1P [21], especially when magnesium ions are replaced by manganese ions. To minimize unwanted hydrolysis, reaction time can be shortened or the enzyme concentration can be increased to maximize substrate uptake.
18. The temperature optimum for most polymerases is 37 °C. However, many polymerases are also active at room temperature. In some cases (e.g., upscaled reactions [15], solid-phase coupling of enzymes [12]), decreasing the reaction temperature might facilitate the experimental setup.
19. If necessary, minimize the duration and temperature of the heat shock since temperature-sensitive substrates or polymers might degrade.

20. The column oven is heated to decrease the viscosity of the mobile phases and thus the backpressure of the column. This enables higher flow rates, shorter run times and it results in narrower peaks with higher separation efficiencies. Since the column temperature finally influences the retention time of the analyzed eluting components, make sure column temperature is constant at all times. A non-preheated column oven and varying temperatures during liquid chromatography result in inconsistent elution times and thus inconclusive chromatograms. The CarboPac™ PA-100 column 2 × 250mm + guard column (Dionex™) used in this protocol generates a backpressure of 200 bar at a flow rate of 0.6 mL/min.
21. Cooling is required because the high voltage applied during electrophoresis generates considerable heat that might lead to degradation of the sample. Since the analyzed polymers are negatively charged, make sure that they migrate toward the positive electrode. The runtime depends on the application. The electrophoretic mobility of a polymer is dependent on its physical and chemical properties (e.g., size, charge density) and should be determined experimentally. The gel shown in Fig. 7 was run for approx. 2 h and was stopped when bromophenol blue reached the bottom of the gel.
22. The sucrose solution is very viscous and hence difficult to pipette. The loading buffer is colorless. To minimize mistakes during loading, mark slots with a permanent marker on the gel cassette. Cautiously load the samples to avoid contaminating neighboring wells. It might be beneficial to only fill 50–75% of the wells and to only load every second well.

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References

1. Willis LM, Whitfield C (2013) Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. Carbohydr Res 378:35–44. https://doi.org/10.1016/j.carres.2013.05.007
2. Vella M, Pace D (2015) Glycoconjugate vaccines: an update. Expert Opin Biol Ther 15:529–546. https://doi.org/10.1517/14712598.2015.993375
3. Berti F, Adamo R (2013) Recent mechanistic insights on glycoconjugate vaccines and future perspectives. ACS Chem Biol 8:1653–1663. https://doi.org/10.1021/cb400423g
4. Costantino P, Rappuoli R, Berti F (2011) The design of semi-synthetic and synthetic glycoconjugate vaccines. Expert Opin Drug Discov 6:1045–1066. https://doi.org/10.1517/17460441.2011.609554

5. LaForce FM, Konde K, Viviani S, Préziosi M-P (2007) The meningitis vaccine project. Vaccine 25(Suppl 1):A97–A100. https://doi.org/10.1016/j.vaccine.2007.04.049
6. Xie O, Pollard AJ, Mueller JE, Norheim G (2013) Emergence of serogroup X meningococcal disease in Africa: need for a vaccine. Vaccine 31:2852–2861. https://doi.org/10.1016/j.vaccine.2013.04.036
7. Ramjeet M, Deslandes V, Gouré J, Jacques M (2008) Actinobacillus pleuropneumoniae vaccines: from bacterins to new insights into vaccination strategies. Anim Health Res Rev 9:25–45. https://doi.org/10.1017/S1466252307001338
8. Micoli F, Del Bino L, Alfini R et al (2019) Glycoconjugate vaccines: current approaches towards faster vaccine design. Expert Rev Vaccines 18:1–15. https://doi.org/10.1080/14760584.2019.1657012
9. Adamo R (2017) Advancing homogeneous antimicrobial glycoconjugate vaccines. Acc Chem Res 50:1270–1279. https://doi.org/10.1021/acs.accounts.7b00106
10. Fiebig T, Berti F, Freiberger F et al (2014) Functional expression of the capsule polymerase of Neisseria meningitidis serogroup X: a new perspective for vaccine development. Glycobiology 24:150–158. https://doi.org/10.1093/glycob/cwt102
11. Muindi KM, McCarthy PC, Wang T et al (2014) Characterization of the meningococcal serogroup X capsule N-acetylglucosamine-1-phosphotransferase. Glycobiology 24:139–149. https://doi.org/10.1093/glycob/cwt091
12. Fiebig T, Litschko C, Freiberger F et al (2018) Efficient solid-phase synthesis of meningococcal capsular oligosaccharides enables simple and fast chemoenzymatic vaccine production. J Biol Chem 293:953–962. https://doi.org/10.1074/jbc.RA117.000488
13. Ming SA, Cottman-Thomas E, Black NC et al (2018) Interaction of Neisseria meningitidis Group X N-acetylglucosamine-1-phosphotransferase with its donor substrate. Glycobiology 28:100–107. https://doi.org/10.1093/glycob/cwx100
14. Oldrini D, Fiebig T, Romano MR et al (2018) Combined chemical synthesis and tailored enzymatic elongation provide fully synthetic and conjugation-ready Neisseria meningitidis serogroup X vaccine antigens. ACS Chem Biol 13:984–994. https://doi.org/10.1021/acschembio.7b01057
15. Fiebig T, Romano MR, Oldrini D et al (2016) An efficient cell free enzyme-based total synthesis of a meningococcal vaccine candidate. NPJ Vaccines 1:16017. https://doi.org/10.1038/npjvaccines.2016.17
16. Byrd W, Harmon BG, Kadis S (1992) Protective efficacy of conjugate vaccines against experimental challenge with porcine Actinobacillus pleuropneumoniae. Vet Immunol Immunopathol 34:307–324. https://doi.org/10.1016/0165-2427(92)90172-M
17. Byrd W, Kadis S (1992) Preparation, characterization, and immunogenicity of conjugate vaccines directed against Actinobacillus pleuropneumoniae virulence determinants. Infect Immun 60:3042–3051
18. Willis LM, Stupak J, Richards MR et al (2013) Conserved glycolipid termini in capsular polysaccharides synthesized by ATP-binding cassette transporter-dependent pathways in Gram-negative pathogens. Proc Natl Acad Sci U S A 110:7868–7873. https://doi.org/10.1073/pnas.1222317110
19. Fiebig T, Freiberger F, Pinto V et al (2014) Molecular cloning and functional characterization of components of the capsule biosynthesis complex of Neisseria meningitidis serogroup A: toward in vitro vaccine production. J Biol Chem 289:19395–19407. https://doi.org/10.1074/jbc.M114.575142
20. Litschko C, Oldrini D, Budde I et al (2018) A new family of capsule polymerases generates teichoic acid-like capsule polymers in gram-negative pathogens. MBio 9:e00641–e00618. https://doi.org/10.1128/mBio.00641-18
21. Litschko C, Romano MR, Pinto V et al (2015) The capsule polymerase CslB of Neisseria meningitidis serogroup L catalyzes the synthesis of a complex trimeric repeating unit comprising glycosidic and phosphodiester linkages. J Biol Chem 290:24355–24366. https://doi.org/10.1074/jbc.M115.678094
22. Perry MB, Altman E, Brisson JR et al (1990) Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of Actinobacillus (Haemophilus) pleuropneumoniae strains. Serodiagn Immunother Infect Dis 4:299–308. https://doi.org/10.1016/0888-0786(90)90018-J
23. Fiebig T, Litschko C, Gerardy-Schahn R et al (2017) Means and methods for producing phosphate containing capsular polysaccharides. LU100349

24. Min H, Cowman MK (1986) Combined alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: application to electrophoretic analysis of molecular weight distribution. Anal Biochem 155:275–285. https://doi.org/10.1016/0003-2697(86)90437-9
25. Breton C, Snajdrová L, Jeanneau C et al (2006) Structures and mechanisms of glycosyltransferases. Glycobiology 16:29R–37R. https://doi.org/10.1093/glycob/cwj016
26. Lairson LL, Henrissat B, Davies GJ, Withers SG (2008) Glycosyltransferases: structures, functions, and mechanisms. Annu Rev Biochem 77:521–555. https://doi.org/10.1146/annurev.biochem.76.061005.092322
27. Dubray G, Bezard G (1982) A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. Anal Biochem 119:325–329. https://doi.org/10.1016/0003-2697(82)90593-0

Chapter 17

Attenuation Methods for Live Vaccines

Dipasree Hajra, Akshay Datey, and Dipshikha Chakravortty

Abstract

Vaccination was developed by Edward Jenner in 1796. Since then, vaccination and vaccine development research has been a hotspot of research in the scientific community. Various ways of vaccine development are successfully employed in mass production of vaccines. One of the most successful ways to generate vaccines is the method of virulence attenuation in pathogens. The attenuated strains of viruses, bacteria, and parasites are used as vaccines which elicit robust immune response and confers protection against virulent pathogens. This chapter brings together the most common and efficient ways of generating live attenuated vaccine strains in viruses, bacteria, and parasites.

Key words Live attenuated strains, Gene inactivation, Mutagenesis, Irradiation, microRNA

1 Introduction

Live attenuation of pathogens is one of the major modes of vaccine development strategies. Pathogen attenuation refers to inactivation of the virulence factors which leads to loss of disease-causing ability [1]. However, the ability of the pathogen to induce a potent immune response is highly conserved. A perfect live attenuated vaccine is the one which has completely lost its virulence but has all the pathogen associated molecular patterns (PAMPs) intact. Once administered, these live attenuated strains of bacteria, virus, or parasites do not cause a disease but elicit a strong immune response as a consequence to its intact PAMPs. The immune response can either be humoral or cell mediated. Both the responses generate a memory response which serves as a protective mechanism against invading pathogens.

However, they retain their capacity of replication and transient growth within the host. Attenuation is mostly achieved by growing the organism under unnatural growth conditions for prolonged periods so that it becomes better suited to grow in the abnormal

Dipasree Hajra and Akshay Datey contributed equally to this work.

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culture conditions rather than its natural conditions [2]. The ability to cause transient infection without causing disease mount effective long-lasting immune response against the pathogen. However, this vaccination method is not fool proof. It has its own share of advantages and disadvantages. Because of their potential to replicate, these vaccines confer prolonged protective immunity and produce long lasting memory cells. Consequently, a single immunization dose is sufficient to provide protection without the need of subsequent booster doses. On the other hand, the major disadvantage of such vaccines include their ability to revert back to the virulent form. Sequential passaging of the virulent bacterial strain in unnatural host or in hostile conditions generate alterations in gene sequences which lead to attenuation of the bacterial strain. For example, in vitro passaging of *Mycobacterium bovis* for multiple rounds on potato slices cooked in bile beef led to elimination of the deletion region 1 (RD 1). Chemical mutagenesis is another method of gene disruption in virulent bacterial strains. In pathogenic *Salmonella* Typhi strain Ty2, a distinctive mutation caused inactivation of *galE* gene and Vi polysaccharide synthesis. This resulted in an impressive attenuation of the bacterial strain [3]. M01ZH09 is an *S. typhi* strain Ty2 derivative with deletion mutations in *aroC* and *ssaV* [4]. Ty800, a Ty2 derivative deleted in *phoP/phoQ* is another safe and immunogenic single-dose vaccine for typhoid patients [5]. On the other hand, targeted deletion of the *ctxA* gene encoding the toxic A subunit (CTA) of the cholera toxin (CT) led to the development of *Vibrio cholerae* vaccine strain CVD 103-HgR in the 1980s [6].

Viral inactivation is also performed by similar methods of passaging in animals, eggs, or cell culture or by sequential passaging in cold-adapted conditions. Alternatively, mutagenesis is induced either by ultraviolet radiation or by chemical means until successful attenuation. Genetically attenuated parasitic strains developed by targeted gene disruption is one of the major methods of development of parasite attenuated virus. A major advantage of genetically attenuated parasites is that they exhibit a homogeneous population with defined genetic constitution and identical attenuated phenotypes. Genetically attenuated parasite (GAP) was developed in *Plasmodium falciparum* by disruption of the *p52* gene by single crossover recombination through plasmid integration at targeted sites. The most recent *P. falciparum* GAP constitutes deletion of two tandem-arranged genes, *p52* and *p36*, that results in severe growth defects in hepatocyte infection. Irradiated sporozoites of *Plasmodium berghei* confers protective immunity against subsequent infection of viable sporozoites [7]. The subsequent section lists the various methods of attenuation with emphasis on specific case studies.

2 Materials

2.1 Bacterial Virulence Attenuation by Passaging [8]

1. Potato slices.
2. Beef bile.
3. Glycerol.
4. *Mycobacterium bovis*.

2.2 Bacterial Virulence Attenuation by Mutagenesis [9]

1. *S. typhi*, strain Ty 2 (obtained from the World Health Organization).
2. Brain heart infusion (BHI) broth.

2.2.1 Isolation of galE Mutants

3. Endo agar: Bacto peptone 10 g, galactose 10 g, K2HPO4 3.5 g, sodium sulfite 2.5 g, basic fuchsin 0.4 g, and agar 5 g dissolved in 1 L of distilled water.
4. N-methyl-N′-nitro-N-nitrosoguanidine (NG).
5. JCR-170 (obtained from Dr. H.J. Creech—Chemotherapy Laboratory, Institute for Cancer Research, Philadelphia, PA) rIO], or ultraviolet light.

2.2.2 Stability Test

1. Swiss white mice.
2. 0.85% NaCl.
3. Potter-Elvehjem homogenizer (Arthur H. Thomas, Philadelphia, PA).
4. Endo Agar (*see* Subheading 2.2.1).

2.2.3 Galactose-Induced Bacteriolysis

1. Infusion broth (Baltimore Biological Laboratories, Baltimore, MD).
2. Shaking incubator.
3. Spectrophotometer.
4. Galactose (0.1%).

2.2.4 Determination of Galactose Within Bacteria

1. [14C]-galactose (18000 counts per min/mg).
2. *S. typhi galE* mutants.
3. BHI broth, centrifuge.
4. 0.85% NaCl.
5. Glass beads (0.17–0.18 mm).
6. Sorvall Omni-Mixer (DuPont, Norwalk, CT).
7. 1 N $HClO_4$.
8. Ion exchange chromatography with Dowex 1.

2.2.5 Mouse Virulence

1. 0.85% NaCl.
2. 5% mucin.

2.2.6 Mouse Protection Test

1. 0.2 ml of a bacterial suspension containing 10^7 live Ty 21a/ml.
2. 0.2 ml of a suspension containing 10^8 viable Ty 21a cells.

2.3 Viral Attenuation by Sequential Passaging [13]

2.3.1 Vaccine Preparation

1. Oka strain varicella virus.
2. Human embryonic lung (HEL) cells and GPE cells.
3. Human diploid cells (WI-38) cells (Flow Laboratories).
4. Roux bottles.
5. Skin and muscle tissues from 3 to 4-week-old guinea pig embryos.
6. Phosphate-buffered saline solution (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g $NaHPO_4$, and 0.24 g KH_2PO_4 dissolved in 800 ml. pH is adjusted to 7.4. Volume is made up to 1 L (*see* **Note 1**).
7. Edetic acid treatment.
8. Eagle's minimum essential medium.
9. 20KC sonifier.
10. Centrifuge.
11. 5% sugar.

2.4 MicroRNA-based Viral Attenuation [14]

2.4.1 Eggs and Cell Culture

1. Embryonated chicken eggs purchased from Charles River Laboratories, CT. MDCK cells (ATCC, #CCL-34), MEM (Sigma).
2. 10% FBS (Gibco, NY).
3. 50 μg/ml gentamicin.
4. 1 mM sodium pyruvate.
5. HEK293T (ATCC, #CRL-11268), MEF (ATCC, #CRL-2214), MEF Dicer−/− (provided by Dr. Wu).
6. DF1 (ATCC, #CRL-12203).
7. A549 (ATCC, #CCL-185) cells.
8. DMEM (Gibco, NY).
9. 1% penicillin.
10. 1 μg/ml streptomycin (Gibco, NY).

2.4.2 Artificial microRNA Design and Expression

1. miR-93 cassette with a scrambled control sequence, the miR-93 locus, and amiR-93NP synthesized by GenScript.
2. microRNA-expressing plasmid pLL3.7.
3. 293T cells.

2.4.3 Virus Design, Rescue, and Titration

1. Designed—wild-type PR8 (PR8-wt), PR8-control (PR8-ctl), PR8-miR-93, and PR8-amiR-93NP.
2. PBS (*see* Subheading 2.3.1, **item 6**).

2.4.4 Mammalian Cell Infection

1. Dulbecco's phosphate-buffered saline (DPBS) supplemented with Ca^{2+}/Mg^{2+} 0.3% BSA.

Tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma).

2.4.5 Northern Blot Analysis

1. miRNeasy Mini Kit (Qiagen).
2. Probes U6 (5′-CACGAATTTGCGTGTCATCCTT-3′).
3. miR-93 (5′-CTACCTGCACGAACAGCACTTTG-3′).
4. amiR-93NP (5′-GAGGCTTCTTTATTCTAGG-3′).
5. Highly Sensitive miRNA Northern Blot Assay Kit (Signosis).
6. Chemiluminescent HRP substrate (Takara Bio).
7. Image Quant LAS400 (GE Healthcare).

2.4.6 Western Blot Analysis

1. 10% SDS-PAGE.
2. Nitrocellulose membrane.
3. Semidry transblot apparatus (Bio-Rad).
4. PBS with 1% Tween (PBST), 5% nonfat milk.
5. Anti-NP monoclonal antibody (Abcam).
6. Alkaline phosphatase–conjugated goat anti-mouse IgG antibody (Cell Signaling).
7. Chemiluminescent HRP substrate.

2.4.7 Virulence test in vivo

1. Mice (6–8-week-old) purchased from Jackson Laboratory.
2. DPBS.
3. Ketamine and xylazine as anaesthetics.

2.5 Exploiting Codon Usage Bias for Viral Attenuation [15]

1. Influenza A/Brisbane/59/2007 (H1N1) (BR59).
2. GenScript.

2.5.1 Development of a Human Influenza Virus Having Avian Influenza Virus-like Codon Bias Sequences

2.5.2 Cells and Viral Maintenance

1. MDCK, 293T, DF1, and A549 cells.
2. Minimum essential medium (MEM) (FOR MDCK, 293T, and A549 cells).
3. Dulbecco's modified Eagle medium (DMEM) (FOR DF1 cells).
4. 1% penicillin and streptomycin (P/S).
5. 10% fetal bovine serum (FBS).
6. Humidified incubator with 5% CO_2 at 37 °C.
7. Virus strain: A/HK/1/68 clone, MA20C (HK68-MA20C; H3N2), from E. Brown (University of Ottawa, Ottawa, Ontario, Canada), WT BR59 virus.
8. Hemagglutinin (HA) and neuraminidase (NA) from A/Puerto Rico/8/34 (H1N1) (PR8) or A/HK/1/68 (H3N2) (HK68).
9. Embryonated eggs.

2.5.3 Evaluation of Viral Growth Kinetics

1. 1% P/S.
2. Tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (1 μg/ml for MDCK cells, 0.5 μg/ml for A549 cells).

2.5.4 In Vivo Mouse Experiments

1. Specific-pathogen-free 4- to 9-week-old female BALB/c mice.

2.5.5 Immune Response Assays

1. MiniCollect tubes (Greiner Bio-One).
2. Monoclonal antibodies (all from BioLegend):
   (a) F4/80-phycoerythrin (PE).
   (b) I-AE–peridinin chlorophyll protein (PerCP)–Cy5.5.
   (c) CD11b-allophycocyanin (APC)-Cy7.
   (d) Gr1-PE-Cy7.
   (e) IA8-APC.
   (f) CD11c-fluorescein isothiocyanate (FITC).
   (g) DAPI (4′,6-diamidino-2-phenylindole).
   (h) CD3-APC.
   (i) CD4-APC-Cy7.
   (j) CD8-PerCP-Cy5.5.
   (k) Dx5-FITC.
   (l) γδT-PE.
   (m) B220-PE-Cy7.
3. A549 cells.
4. RNeasy minikit (Qiagen).

5. Turbo DNA-free DNase treatment and removal reagents (Ambion).
6. oligo(dT) primer and SuperScript II reverse transcriptase (Life Technologies).
7. Gene-specific primers for qPCR of beta-interferon [21] and β-actin [37].

2.5.6 Vaccine Efficacy Study

1. 12 Female BALB/c Mice.
2. Phosphate-buffered saline (PBS). (*see* Subheading 2.3.1, **item 6**) (*see* **Note 1**).
3. MA-WT virus, PR8, HK68-MA20C [33].
4. 10% formalin for fixation.
5. Monoclonal antibody against the NP (clone HB65; ATCC) of influenza A virus.

2.5.7 Protein Expression Studies Using Western Blotting

1. 12% SDS–polyacrylamide gels.
2. Primary antibodies.
   (a) PB2 (catalog number vN19; Santa Cruz).
   (b) PB1 (catalog number F5-19; BEI Resources).
   (c) PA (a generous gift from Ervin Fodor, University of Oxford), HA (catalog number ab91531; Abcam).
   (d) NP (catalog number C43; Abcam).
   (e) NA (catalog number ab21304; Abcam).
   (f) M1 (catalog number GA2B; Santa Cruz).
   (g) NS1 (catalog number 23-1; Santa Cruz).
   (h) β-actin (catalog number C4; Santa Cruz).
3. IRDye-conjugated secondary antibodies (LI-COR).
4. Odyssey CLx infrared imaging system.

2.6 Transposon Mutagenesis [16]

2.6.1 Cell Culture

1. *Cell line*: HEK293T, MDCK cells.
2. DMEM.
3. 5% FBS.
4. Penicillin/streptomycin (100 U/mL and 50 μg/mL, respectively).
5. 1 mM sodium pyruvate.

2.6.2 Generation of M Gene Segment Mutant Plasmid Library and Functional Profiling

1. 15-nt sequence (5′-NNNNNTGCGGCCGCA-3′; N = duplicated 5 nucleotides from target DNA).
2. (MGS kit, Finnzymes).
3. *E. coli* DH10B.
4. ElectroMax DH10B, Invitrogen.

5. HEK293T cells, MDCK cells.
6. TRIzol reagent (Invitrogen).
7. iScript cDNA Synthesis kit (Bio-Rad).
8. Primer Sequences.
   (a) 5′-AGCAAAAGCAGGTAGATATT-3′.
   (b) 5′-GGGGCCAAAGAAATAGCACT-3′.
   (c) 5′-TCCTAGCTCCAGTGCTGGTC-3′.
   (d) Vic-labeled insertion-specific mini-primer (5-′-TGCGGCCGCA-3′).
9. KOD Hot-Start polymerase (Novagen).
10. Liz-500 size standard (Applied Biosystems).
11. 96-capillary genotyper (3730xl DNA Analyzer, Applied Biosystems).
12. ABI Software.
13. PCR.
14. Ultracentrifuge.

2.6.3 Virus Strains

1. Eight plasmids harboring the cDNA of A/WSN/33[12] (a gift from Dr. Yuying Liang at Emory University).
2. HEK293T with TranwsIT LT-1 (Panvera).
3. Influenza virus A/Victoria/3/75 (seasonal A/H3N2 virus).
4. A/Wisconsin/65/05 (seasonal A/H3N2 virus).
5. A/Hongkong/68 (seasonal A/H3N1 virus) (gifts from Dr. Ioanna Skountzou at Emory University).
6. Influenza virus A/Cambodia/P0322095/05.

2.6.4 Virus Titration

1. Dilution buffer: PBS (*see* Subheading 2.6.1, **item 6**) (*see* **Note 1**), 10% BSA, $CaCl_2$, 1% DEAE-dextran, and $MgCl_2$.
2. 6-well plates.
3. Growth medium: 1% low-melting agarose, TPCK-treated trypsin (0.7 μg/mL).
4. Plaque Staining: 1% crystal violet, 20% ethanol, PBS (*see* Subheading 2.6.1, **item 6**) (*see* **Note 1**).
5. DMEM.
6. *Gaussia* luciferase (gLuc) reporter system (*see* Subheading 3.6, **step 4**).
7. *Renilla* luciferase substrate (Promega).

2.6.5 Animals

1. Adult Mice: Female C57BL/6 mice (6–8 weeks old) (purchased from the Jackson Laboratory).

2. Neonatal Mice: BALB/c mice (15 day old) (Vital River Beijing) of weight 6–9 g.
3. Ferrets: Healthy young adult outbred female ferrets (Mustela putorius furo; between 4 and 5 months of age) (purchased from a commercial breeder (Wuxi)).

2.6.6 Mouse Immunization and Challenge

1. Mouse Pan T Cell Isolation Kit and MS columns (Miltenyi Biotec).

2.6.7 In Vivo Challenge Using HPAI Virus H5N1

1. Female BALB/c mice (*Mus musculus*) aging of 6–8 weeks (purchased from Charles River Laboratories).
2. Microisolator cages with ventilation facility having negative pressure with HEPA-filtered air and a 12:12 h light–dark cycle.
3. BSL3 facilities at the Pasteur Institute of Cambodia.
4. Pentobarbital sodium (75 mg/kg; Sigma).

2.7 Genetic Modifications in Parasites [17]

2.7.1 Generation of the p36p⁻ Parasite Lines

1. Vector harboring the pyrimethamine-resistant *Toxoplasma gondii* (*tg*) *dhfr/ts* gene, human (*h*) *dhfr* selectable marker, and *gfp* placed under the control of the constitutive *pbef-1aa* promoter [18–20] and a 2-kb fragment of the D-type small subunit (*dssu*) rRNA gene of *Plasmodium berghei* [21].
2. *P. berghei* wild type (clone 15cy1; ANKA strain).
3. WR99210 (16 mg/kg bodyweight) [20].
4. Primer Pairs Used.
   (a) WT L1362 5′-CCGCTCGAGACCTTAGGACACTTTG AAATTTG-3′.
   (b) L1363 5′-CCGCTCGAGCTACTCATAATAAGAAGAA GAGGTAC-3′.
   (c) Disrupted L1389 5′-ATTTTGCAACAATTTTATTCTT GG-3′.
   (d) L313 5′-ACGCATTATATGAGTTCATTTTAC-3′.
   (e) WT L270 5′-GTGTAGTAACATCAGTTATTGTGTG-3′.
   (f) L271 5′-CTTAGTGTTTTGTATTAATGACGATTTG-3′.
   (g) Disrupted *cssu* L270 and L635 5′-TTTCCCAGTCACG ACGTTG-3′.
5. RNA Isolation and RT-PCR (Invitrogen).
6. Primers for amplification of cDNA derived from the *p36p*⁻ or *circumsporozoite* (CS) gene.
   (a) L1425 (5′-GAAATGAATATGTCGGTACTATG-3′).
   (b) L1363 (5′-CCGCTCGAGCTACTCATAATAAGAAG AAGAGGTAC-3′).

(c) L1502 (5′-AGTCAACAGATTATTGCCGATG-3′).
(d) L1503 (5′-TACAAATCCTAATGAATTGCTTAC-3′).

2.7.2 Analysis of the p36p⁻ Parasite Phenotype During Blood Stage and Mosquito Stage Development

1. Swiss mice.
2. *Anopheles stephensi*.
3. 300 µl of PBS (*see* Subheading 2.3.1, **item 6**) (*see* **Note 1**).
4. Cell counters.

2.7.3 Analysis of Characteristics of the Infectivity of p36p⁻ Sporozoites

1. BALB/c and C57BL6 females of weight 15–20 g.
2. 5×10^4 purified sporozoites.
3. Giemsa stain.
4. Cell counter.
5. Centrifuge.
6. Glass coverslips.
7. 0.02% gelatin in water.
8. Anti-CS 3D11 antibody (Ab).
9. HepG2 cells.
10. MEM medium [22].
11. Rhodamine-dextran (1 mg/ml).
12. Anti-PbEXP-1.
13. Anti-HSP90 or anti-HSP70 [23].
14. DAPI.

2.7.4 In Vitro Analysis of Apoptosis in RAS and p36p⁻ Parasite-Invaded Hepatocytes

1. 3×10^4 sporozoites (WT, *p36p* ⁻, or RAS).
2. Gamma source, 16 Krad [24].
3. 2×10^5 HepG2 cells.
4. DMEM.
5. 10% FCS.
6. 1% penicillin/streptomycin.
7. 1 mM glutamine.
8. Anti-HSP70 Ab [23].
9. Active caspase-3 detection kit (Promega).
10. DAPI stain.

2.7.5 In Vivo Analysis of Apoptosis in p36p⁻ Parasite-Invaded Hepatocytes

1. C57BL/6 mice.
2. 5×10^5 sporozoites of *p36p*⁻ or WT.
3. Anti-HSP70.
4. DAPI stain.

2.7.6 Analysis of the Immunization Potential of p36p⁻ Sporozoites

1. BALB/c mice.
2. C57BL6 mice.
3. *p36p*⁻ sporozoites or RAS or PBS.
4. Giemsa-stain.
5. Real-time PCR machine.
6. A-type 18S ribosomal RNA primers.

2.8 Gamma Irradiation for Mutagenesis in Parasites [25]

2.8.1 Dissection of Sporozoites

1. 50% Human plasma diluted in saline.
2. X-ray beam of a Picker Vanguard teletherapy unit.
3. A/J mice.
4. Injection needle.
5. Hamster.
6. *Plasmodium berghei*.

3 Methods

3.1 Bacterial Virulence Attenuation by Passaging [8]

M. bovis strain is subjected to culturing on potato slices for 13 years. These potato slices are cooked in beef bile with glycerol supplementation. The strain obtained after passaging is found to lose virulence. This strategy was employed in the development of the BCG vaccine against tuberculosis.

3.2 Bacterial Virulence Attenuation by Mutagenesis

1. Isolation of *galE* mutants: *S. typhi*, strain Ty 2 is grown in brain heart infusion (BHI) broth. Exponential growth phase culture is plated onto Endo agar where lactose is replaced by galactose by either directly or after mutagenic treatment with JCR-170, N-methyl-N′-nitro-N-nitrosoguanidine (NG) or ultraviolet light.
2. Stability Test: The stability of the mutants is tested by plating on Endo agar either directly or after treatment with JCR-170 or NG. For in vivo stability test, Swiss white mice are inoculated with 10^7 cells intraperitoneally. After 5–10 days, mice are euthanized, and their liver and spleen are harvested. The harvested liver and spleen are homogenized in 0.85% NaCl in a Potter-Elvehjem homogenizer. The homogenate is plated onto galactose-Endo agar.
3. Galactose-induced bacteriolysis: Cells are grown in infusion broth at 37 °C on a rotating shaker at 142 rpm. Growth is monitored by measurement of the optical density at 550 nm. Galactose (0.1%) is added at the moment of inoculation or when the culture is in the logarithmic phase of growth.
4. Enzyme assays: The procedures for the assays of galactose-permease and galactose-l-phosphate-uridyl transferase are as

described previously [9]. Briefly, UDP-galactose-4-epimerase is determined by the procedure of Nikaido [26]. For the determination of galactokinase, the method of Wilson and Hogness is used [11]. Protein is measured by the method of Lowry et al. [12]. Specific enzyme activities were expressed as units/mg of protein.

5. Determination of galactose within bacteria: [14C] galactose (18000 counts per min/mg) is incorporated to a log-phase culture of *S. typhi galE* mutants in BHI broth. After every 30-min interval, five 2-L aliquots are harvested. They are centrifuged and washed twice in 0.85% NaCl. The bacteria are lysed with glass beads (0.17–0.18 mm) in a Sorvall Omni-Mixer (DuPont, Norwalk, Conn.), and the suspension is centrifuged. The supernatant (SN 1) is recovered. The pellet is resuspended in distilled water. The cell wall components are separated from the glass beads by repeated decantation. The cell wall suspension is then centrifuged at 40,000 $\times$ *g* for 20 min, and the supernatant (SN 2) is pooled with the supernatant of the first centrifugation. The pellet is suspended in distilled water and centrifuged at 2000 $\times$ *g* for 20 min. The supernatant is then spun down at 40,000 $\times$ *g* and the pellet is suspended in water and used for determinations of carbon-14. The cytoplasmic fraction is removed of protein contamination by precipitation with 1 N $HClO_4$ and then is used for determinations of total [14C] galactose. In this fraction, the concentrations of free galactose, galactose-I-phosphate, and UDP galactose are determined after their separation by ion exchange chromatography with Dowex 1.
6. Determination of mice virulence: Live bacteria are suspended in 0.85% NaCl or in 5% mucin and injected intraperitoneally into female mice of average weight around 18–20 g. Survival studies are conducted for around seven days. The median lethal dose (LDso) is estimated by the method of Reed and Muench [27].
7. Mouse protection test: Female mice weighing 18–20 g are subjected to immunization by intraperitoneal inoculation of 0.2 ml of a bacterial suspension containing 10^7 live Ty 21 a/ml, or by subcutaneous inoculation of 0.2 ml of a suspension containing 10^8 viable Ty 21a cells. The elimination of the vaccine strain is determined by serial bacterial counts in liver and spleen. Four weeks postimmunization, the mice are subjected to secondary challenge of 10^6 viable cells by intraperitoneal injection or by intravenous inoculation of 5×10^7 viable cells of *S. typhi* Ty 2. The degree of protection is determined by the number of mice alive 10 days after challenge.

3.3 Viral Attenuation by Sequential Passaging [13]

1. Vaccine Preparation: Varicella (Oka strain) virus is serially grown in human embryonic lung (HEL) cells for eleven times and then in GPE cells. After a twelfth passage in GPE cells, the virus is subsequently passaged in human diploid cells (WI-38) cells (Flow Laboratories). GPE cells are obtained by trypsinization of skin and muscle tissues from 3- to 4-week-old guinea pig embryos. Passage of virus is carried out by transfer of infected cells. Cell-free varicella virus is obtained essentially by the method of Caunt and Taylor-Robinson and Brunell [28, 29]. The tissue-culture fluid is removed, and infected cells are washed with phosphate-buffered saline solution (PBS) and collected by edetic acid treatment. Harvested cells are suspended in 3 ml of Eagle's minimum essential medium and lysed by sonication by a 20KC sonifier for 30–40 s. The disrupted cells are centrifuged for 15 min at 4000 r.p.m. and the supernatant is collected. The collected supernatant is used as an, experimental vaccine after addition of a suitable stabilizer like 5% sugar. The vaccines are prepared from the virus at the sixth or seventh passage in GPE cells and second passage in WI-38 cells. They are examined for the presence of bacteria and mycoplasmas. Absence of viruses other than varicella virus is confirmed by electron microscopy.

3.4 MicroRNA-Based Viral Attenuation

1. Eggs and Cell culture: Embryonated eggs are incubated at 37.5 °C for up to 9 days. MDCK cells are cultured in MEM supplemented with 10% FBS, 50 μg/ml gentamicin, and 1 mM sodium pyruvate. HEK293T, MEF, MEF Dicer−/−, DF1, and A549 cells are cultured in DMEM supplemented with 10% FBS, 1% penicillin, and 1 μg/ml streptomycin.
2. Artificial microRNA design and expression: The miR-93 cassette with a scrambled control sequence, the miR-93 locus, and amiR-93NP are synthesized by GenScript and cloned into the microRNA-expressing plasmid pLL3.7 [30]. For transfection, 8×10^5 293T cells are seeded per well into 6-well plates. The following day, the cells are transfected with 1 μg plasmid pcDNA-NP with 1 μg pLL3.7, pLL3.7-ctl, pLL3.7-miR-93, or pLL3.7-amir-93NP. After 24 h of transfection, cells are harvested and lysed. Expression of NP and amiR-93NP are detected by western blot and northern blot, respectively.
3. Virus design, rescue, and titration: Modified NS gene segments with miR-93 locus and amir-93NP cassette insertions are synthesized by GenScript. Reorganization of the NS gene segment is done as described [31]. Viruses are rescued using a plasmid-based rescue system [32]. The viruses designed were wild-type PR8 (PR8-wt), PR8-control (PR8-ctl), PR8-miR-93, and PR8-amiR-93NP. Viral stocks are titrated in chicken eggs and expressed as EID50. Briefly, tenfold serial dilutions of

viruses are prepared in PBS. Each egg is inoculated with a 100 μl dilution. Virus from allantoic fluid is tested by hemagglutination (HA) assay, and the titer is calculated according to the Reed and Muench method [27].

4. Mammalian cell infection: Cells are seeded in different culture vessels 1 day prior to infection. For the infection, cells are washed with Dulbecco's phosphate-buffered saline (DPBS) supplemented with Ca^{2+}/Mg^{2+} and infected with influenza virus at specific MOIs diluted in fresh medium without serum. After 1-h incubation, cells are washed with DPBS again supplemented with Ca^{2+}/Mg^{2+} before adding culture medium supplemented with 0.3% BSA. Cells are harvested according to assay-dependent requirements. For infection in MDCK cells, culture medium is supplemented with tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma).
5. Northern blot analysis: RNAs are isolated from different cell lines using the miRNeasy Mini Kit (Qiagen) and stored at −80 °C.Probes for U6 (5′-CACGAATTTGCGTGTCATCCTT-3′), miR-93 (5′-CTACCTGCACGAACAGCACTTTG-3′), and amiR-93NP (5′-GAGGCTTCTTTATTCTAGG-3′) are used (*see* **Note 2**). Northern blot experiments are performed using the Highly Sensitive miRNA Northern Blot Assay Kit as per the manufacturer's protocol (Signosis). Membranes are developed with chemiluminescent HRP substrate (T akara Bio). Images are acquired using the Image Quant LAS400 (GE Healthcare).
6. Western blot analysis: Lysed MDCK cell samples are loaded and separated on 10% SDS-PAGE. Using a semidry transblot apparatus (Bio-Rad), proteins are transferred onto a nitrocellulose membrane. Blocking of the membrane is performed in PBS with 1% Tween (PBST) and 5% nonfat milk for 1 h. Primary antibody probing is done with an anti-NP monoclonal antibody (Abcam) at 4 °C overnight. After washing with PBST, the membrane is incubated with alkaline phosphatase–conjugated goat anti-mouse IgG antibody (Cell Signaling) at room temperature for 1 h. After PBST washing, the membrane is developed with chemiluminescent HRP substrate before imaging. Finally, expression of NP protein is normalized with β-actin (actin).
7. Virulence test in vivo: Mice (6- to 8-week-old) are divided randomly into groups of four mice. For determination of the MLD50, virus is serially diluted in DPBS, and 50 μl is intranasally inoculated into mice anesthetized by injection with ketamine and xylazine. The MLD50 is calculated according to the method of Reed and Muench [27]. After infection, mice are monitored daily for clinical symptoms, weight loss, and death.

8. Humoral immune response and protective immunity: Mice (6- to 8-week-old) are randomly divided into groups and intranasally inoculated with 50 μl of diluted influenza virus. Mouse blood is collected on days 15 and 29, and serum is isolated for analysis by microneutralization assay and ELISA for anti-HA responses. For testing the IgG antibody concentration in mouse serum, plates are coated with HA of the PR8 virus, and specific IgG, IgG1, and IgG2a are measured in the sera of immunized mice. After the last bleeding, mice are challenged with 100× LD50 mouse adapted PR8 (H1N1), A/California/04/2009(H1N1) (CA09), or 104 PFU A/Hong HK/1/68 (H3N2) (HK68) influenza virus. The challenged mice are monitored for clinical symptoms and survival.

3.5 Exploiting Codon Usage Bias for Viral Attenuation

1. Development of a human influenza virus having avian influenza virus-like codon bias sequences [15]: Codon usage study is performed using the Influenza A/Brisbane/59/2007 (H1N1) (BR59) strain. Number of mutations required to be introduced to alter the codon bias from human to avian like influenza virus are selected by comparing the codon usage frequency of wild-type (WT) BR59 with that of the frequency of avian influenza virus. Introduction of such mutations into the viral genome led to the emergence of the required mutant with the avian virus–like codon usage. The minimum free energy of the mutated gene sequences and their nucleotide usage frequencies are more identical to that of the avian influenza virus [34]. Synthesis of the RNA segments of the wild type and mutated virus are then performed using GenScript.
2. Cells and Viral Maintenance: MEM and DMEM are used to maintain MDCK, 293T, A549 cells and DF1 cells, respectively. Supplementation of both MEM and DMEM is done with 1% P/S and 10% fetal bovine serum FBS. All cells are incubated at 37 °C humidified incubator with 5% CO_2. WT BR59 virus, 8 segment mutated virus (8-mut), and 1–4 segment mutated virus are generated by reverse genetics techniques [32]. BR59 wild-type or mutant genes and HA and NA (previously stated in Subheading 2.5.2) containing recombinant viruses are also rescued. Mouse-adapted WT (MA-WT) BR59 is rescued by introduction of three site directed mutations that are known to enhance virus pathogenicity in mice (T89I, N125T, and D221G in the HA gene) [35]. Reverse genetics approach is used to rescue PR8 [32]. Heterosubtypic virus challenge is performed with a mouse-adapted A/HK/1/68 clone, MA20C (HK68-MA20C; H3N2) [36]. Amplification of all the viruses are performed in embryonated eggs or MDCK

cells. Identity of the viruses is confirmed by sequencing. Viral titers are determined by standard plaque assay on MDCK cells.

3. Evaluation of Viral Growth kinetics: Multiplicity of infection (MOI) of 0.001 and 0.01 is chosen for MDCK cells and A549 cells respectively. Cells are maintained in infection medium supplemented with 1% P/S and tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (1 μg/ml for MDCK cells, 0.5 μg/ml for A549 cells) at 37 °C. The supernatant is collected after first, second, and third day of infection. Inoculation is done with 100 PFU of viruses into allantoic fluid in experiments using embryonated eggs and the allantoic fluid is collected after first, second, and third day of infection. Plaque assays on MDCK cells are performed to determine the viral titers.
4. In vivo mouse experiments: Specific pathogen-free female BALB/c mice aged 4–5 weeks are subjected to intranasal infection. Monitoring of mice body weights are done daily and mice showing weight loss of more than 25% are euthanized. Lung tissues are harvested at third and seventh of infection. Harvested tissues are homogenized for titration by the 50% tissue culture infective dose ($TCID_{50}$) assay using MDCK cells. Determination of the measure of the numbers of $TCID_{50}$s per gram is performed using the Reed and Muench method [27].
5. Estimation of Immune responses: Twenty-eighth day postinfection, serum is analyzed for the titer of neutralizing antibodies. Heat inactivation of serum is performed at 56 °C for 0.5 h. MDCK cells are used for microneutralization assay with 100 $TCID_{50}$s of viruses [37]. Bronchoalveolar lavage (BAL) fluid is collected at seventh day postinfection. Profiling of immune cells isolated from Bronchoalveolar lavage (BAL) fluid is performed as described previously [38]. Briefly, after blocking Fc receptor with Fc blocker, cells are subjected to staining with the following two cocktails of monoclonal antibodies (all from BioLegend) to detect innate and adaptive immune cells. Cocktail 1 comprises F4/80-phycoerythrin (PE), I-AE-peridinin chlorophyll protein (PerCP)-Cy5.5, CD11b-allophycocyanin (APC)-Cy7, Gr1-PE-Cy7, IA8-APC, CD11c-fluorescein isothiocyanate (FITC), and DAPI (4′,6-diamidino-2-phenylindole). Cocktail 2 consists of CD3-APC, CD4-APC-Cy7, CD8-PerCP-Cy5.5, Dx5-FITC, γδT-PE, B220-PE-Cy7, and DAPI. Subsequently, samples are run through flow cytometry analysis.

   The beta interferon mRNA expression is evaluated using quantitative PCR. An MOI of 5 is chosen for viral infection to A549 cells and the experiment is conducted in triplicate. Total RNA extraction is done after 4 h and 8 h of infection followed

by DNase treatment and reverse transcription (*see* **Notes 2** and **3**). Expression levels of cDNAs of beta interferon [39] and β-actin [40] is determined by real-time qPCR. For beta interferon, template is denatured initially at 95 °C for 20 s, followed by denaturation for 3 s at 95 °C and annealing for 30 s at 60 °C for 40 cycles. In case of β-actin, initial denaturation of 20 s at 95 °C is performed, followed by denaturation of 3 s at 95 °C and annealing for 30 s at 64 °C for 40 cycles. A melting curve analysis of the amplicons is done from 60 °C to 95 °C at the rate of 0.1 °C/s to determine the specificity of the assay. Normalization of beta interferon mRNA expression is done with respect to the level of β-actin expression.

6. Vaccine efficacy evaluation: 12 female BALB/c mice are subjected to intranasal infection. Mock vaccination is performed with phosphate-buffered saline (PBS) in another group of 12 mice. Infected and mock treated mice are challenged with MA-WT virus, PR8, HK68-MA20C [36], or PBS on 28th day postvaccination. Loss in body weight is monitored daily for a period of 14 days. Lungs are harvested from each group for viral titration and immunohistochemistry analysis after third and seventh day of viral challenge. Harvested lung tissues are homogenized for titration by the $TCID_{50}$ assay using MDCK cells. Fixation of tissues is done in 10% formalin and processed further as described previously [41] for immunohistochemical studies. Staining of the tissues is done with monoclonal antibody against the NP (clone HB65; ATCC) of influenza A virus.
7. Protein expression Studies Using Western blotting: Infection to A549 and DF1 cells is done at an MOI of 5. Whole-cell lysates are harvested at indicated time points postinfection. Protein samples are run in 12% SDS-polyacrylamide gels. Western blotting is performed using the following primary antibodies: PB2, PB1, PA, HA, NP, NA, M1, NS1, and β-actin (as previously mentioned in Subheading 2.5.7). β-Actin is used as loading controls. Detection of primary antibody is performed using corresponding IRDye-conjugated secondary antibodies.

3.6 Transposon Mediated Mutagenesis [16]

1. Creation of M Gene Segment Mutant Plasmid Library: A stretch of 15-nucleotide long sequence 5′-NNNNNTGCGG CCGCA-3′ is inserted at random region by Mu-transposon-mediated mutagenesis (MGS kit, Finnzymes) [16] (*see* **Notes 4–6**). The M gene mutant is electroporated into *E. coli* DH10B at 2.0 kV, 200 Ω, 25 μF (ElectroMax DH10B, Invitrogen). Transfection of HEK293T cells is done with the M gene mutant plasmid and seven other wild type plasmids for generation of virion particles. The supernatant is harvested at third day posttransfection and transferred to MDCK cells for

propagation. After collection of virus after 2 days, viral particles are subjected to either storage or propagation (up to four passages). RNA extraction is carried out using the TRIzol reagent (Invitrogen) followed by cDNA synthesis with the iScript cDNA Synthesis kit (Bio-Rad) (*see* **Notes 2** and **3**). The 15-nucleotide long insert is amplified using three gene-specific forward primers (*see* Subheading 2.6.2) and Vic-labeled insertion-specific mini-primer (*see* Subheading 2.6.2) with the help of KOD Hot-Start polymerase (Novagen). The PCR reaction is set to the initial denaturation at 95 °C for 10 min and at 95 °C for 45 s for 30 cycles. Annealing is performed at 52 °C for 30 s, and extension is carried at 72 °C for 90 s for 30 cycles; with a final extension at 72 °C for 10 min for 1 cycle. Analysis of the fluorescent-labeled PCR products is done in duplicate with a Liz-500 size standard (Applied Biosystems) using a 96-capillary genotyper (3730xl DNA Analyzer, Applied Biosystems). Sequencing data is analyzed using ABI software to remove nonspecific data and the background noise. To study in vivo infection scenario, the mutant virus is subjected to titration and concentration by ultracentrifugation. Retitrated viral pool is used to inject mice. Lungs are harvested 2 days postinfection. Homogenized tissues are dissolved in TRIzol for RNA extraction. PBS or WSN-infected mice are treated as controls (*see* **Notes 2** and **3**).

2. Virus strain generation: Generation of A/H1N1 virus is performed by Influenza A/WSN/1933 by reverse genetics approach[27]. HEK293T cells are transfected with the eight plasmids containing the cDNA of A/WSN/33 (Subheading 2.6.2) using TransIT LT-1 (Panvera) following the manufacturer's protocol. Serial passaging of the virus is performed in MDCK cells thrice to obtain a final titer of $10^{7.4}$ PFU/ml. Determination of the MLD_{50} of both strains is done in C57BL/6 mice. Amplification of Influenza virus A/Victoria/3/75 (seasonal A/H3N2 virus), A/Wisconsin/65/05 (seasonal A/H3N2 virus), and A/Hongkong/68 (seasonal A/H3N1 virus) is performed in MDCK cells for two to three passages to a final titer of $10^{5.5}$ PFU/mL, $10^{5.4}$ PFU/mL, and 10^{7} PFU/mL, respectively. For the MLD_{50} determination, C57BL/6 and BALB/c mice are used. Influenza virus A/Cambodia/P0322095/05 [42] is propagated in MDCK cells. The supernatants containing the virion particles are pooled, concentrated by centrifugation, and stored at −80 °C. MDCK cells and BALB/c mice are used for the determination of the $TCID_{50}$ and the MLD_{50} of the viruses, respectively, and the calculation is performed as described previously [43].
3. Virus Titrations: MDCK cells are used to carry out plaque assay and calculated as PFU/μL of supernatant. Serially diluted viral

samples (in dilution buffer previously mentioned in Subheading 2.6.4) are added to MDCK cells and are incubated for 1 h at 37 °C. It is then supplemented with growth medium (Subheading 2.6.4). Staining solution (Subheading 2.6.4) is used to stain infected cells after second day for visualization of the plaques. End-point titration is performed in MDCK cells to measure virus titer. Inoculation in MDCK cells is performed with tenfold serial dilutions of the virus. One hour postinoculation PBS wash is given once and cells are allowed to grow for 2 days in DMEM to visualize cell viability. Luminescence assay or plaque assay is used to determine the viral titre. In vitro growth analysis of individual mutants is performed using *Gaussia* luciferase (gLuc) reporter system responsive to influenza virus. Briefly, the gLuc coding region is incorporated between a human RNA polymerase I promoter and a murine RNA polymerase I terminator in the reverse-sense orientation [16]. The gLuc coding sequence is flanked by the UTRs from the PA segment of influenza virus A/WSN/33 strain to enable influenza virus infection dependent gLuc expression [16]. HEK293Ts are transfected with the gLuc reporter for a day. Then the supernatants comprising the mutant or WT influenza viruses are added. gLuc gets successfully released into the supernatant upon active infection. gLuc can be quantified with *Renilla* luciferase substrate (Promega).

4. Mouse Immunization and Challenge: Groups of female C57BL/6 and BALB/c mice are subjected to intranasal or intratracheal inoculation with either PBS or W7-791. Each group consisted of five to six mice. Prior to intratracheal injection, anesthetization of the mice is performed intraperitoneally with a ketamine–xylazine mixture. Subsequently, the trachea is exposed surgically and a solution of 30 μL is injected directly with a sterile 27G needle [44]. All mice are challenged after four weeks of immunization intranasally or intratracheally with the following influenza strain: A/WSN/1933 (H1N1) at 4 MLD_{50}, A/Puerto Rico/8/1934 (H1N1) at 4 MLD_{50}, A/Cambodia/P0322095/05 (HPAI-H5N1) at 2 MLD_{50}, or A/Victoria/3/75 (H3N2) at 2 MLD_{50}. Daily monitoring of the mice is performed to detect symptoms of illness. Weight loss of 30% or more is reported and the mice are euthanized. Female C57BL/6 mice are randomly divided into two groups of vaccinated or unvaccinated mice for the adoptive-transfer experiment. Unvaccinated groups are mock immunized. On the other hand, the vaccinated group is immunized with a single dose of W7-791 at 10^6 PFU/mouse. Cells are harvested from one set of mice from each group after 4 weeks of vaccination for the transfer experiment. The other set served as a vaccinated, nontransferred, control. Isolation of the total

CD4+ and CD8+ T cells from the harvested spleens of the vaccinated and the unvaccinated mice is performed by using the Mouse Pan T Cell Isolation Kit and MS columns (Miltenyi Biotec). The cells from the same group are pooled on the same day, and ~$10^{6.3}$ T cells/mouse are administered via the retro-orbital route to a new set of naive female C57BL/6 mice. In a similar fashion, sera from either the vaccinated or unvaccinated groups and matching groups are collected and pooled together. 100 μL/mouse of the pooled serum is injected retro-orbitally to a new set of naive female C57BL/6 mice. After 1 day of adoptive transfer, the mice in all groups are challenged with 2 MLD_{50} of WSN or 2 MLD_{50} of HK68/H3 intranasally.

3.7 Genetic Modifications in Parasites

1. Disruption of the *p36p* to create *p36p−/−* Parasite Lines: A p36p replacement vector containing pyrimethamine-resistant *Toxoplasma gondii* (*tg*) *dhfr/ts* and *dhfr* selectable marker is introduced in vector $b3D.D_T.\wedge H.\wedge D_b$ in order to disrupt the *p36p* locus (*see* **Note 7**). The vector also comprises *gfp* placed under the control of the constitutive *pbef-1aa* promoter [18–20] and a fragment of 2 kb of the D-type small subunit (*dssu*) rRNA gene of *P. berghei* [21]. The linearized vector has the propensity to integrate in C-type small subunit (*cssu*) and/or *dssu*. Integration into *cssu* cause no change to the parasitic phenotype [45]. Wild-type (WT) *P. berghei* ANKA strains (clone 15cy1) are used to generate $p36p^{-}$ parasites. KO1 $p36p^{-}$ parasites are cotransfected with the *gfp* vector to create *gfp*-expressing $p36p^{-}$ mutants [46]. Transformed parasites are selected by treating infected animals with WR99210 (16 mg/kg bodyweight) [20]. KOGFP clone containing *gfp* at the site of *cssu* is selected. Confirmation of correctly integrated constructs is analyzed by RT-PCR and Southern analysis [46]. PCR amplification of WT and $p36p^{-}$ parasites is performed by using primers specific for the WT ((L1362) (*see* Subheading 2.7.1) and L1363 (*see* Subheading 2.7.1)) and disrupted ((L1389) (*see* Subheading 2.7.1) and L313 (*see* Subheading 2.7.1)). WT and $p36p^{-}$:*gfp* parasites are amplified by using primers specific for WT ((L270) (*see* Subheading 2.7.1) and L271 (*see* Subheading 2.7.1)) and disrupted *cssu* ((L270 and L635 (*see* Subheading 2.7.1)). Primers (1389 and 313) produced the fragment of 1.0 kb in KOGFP parasites with disrupted *p36p* locus. RT-PCR is performed on WT and $p36p^{-}$ sporozoites RNA as described by Invitrogen. In order to amplify cDNA derived from the $p36p^{-}$ or *circumsporozoite* (CS) gene, primers (L1425 (*see* Subheading 2.7.1) and L1363 (*see* Subheading 2.7.1)), and (L1502 (*see* Subheading 2.7.1) and L1503 (*see* Subheading 2.7.1)) are used.

2. Analysis of the *p36p*$^{-}$ Parasite Phenotype During Blood Stage and Mosquito Stage Development: The analysis of blood stage development phenotype is undertaken in "asynchronous infections" in Swiss mice and during "standardized synchronized development in vivo and in vitro" as described [47]. In vitro studies pertaining to gamete generation, ookinete formation and fertilization is performed as described [48]. Formation of oocyst and development of sporozoites are investigated using *Anopheles stephensi*. The salivary glands of ten infected mosquitoes are mixed in 300 μl of PBS and the number of sporozoites per salivary gland is determined after counting the numbers of sporozoites in duplicate in a cell counter.
3. Analysis of Characteristics of the Infectivity of *p36p*$^{-}$ Sporozoites: Female BALB/c and C57BL6 mice of weight 15–20 g are infected through infected mosquito bites or through intravenous injection of 5×10^4 purified sporozoites. Sporozoites are collected after dissection of infected mosquito salivary glands [49]. Each mouse is allowed to be fed by around 20–40 infected mosquitoes for 20 days. Monitoring of the Blood stage infections is done in Giemsa-stained blood smears or by analyzing tail blood infected with *p36p*$^{-}$:*gfp* parasites through FACS [18] 4–14 day postinfection. Count of the average circular movement performed by a sporozoite helped in the estimation of the gliding motility [50]. A total of 4×10^4 sporozoites are centrifuged at $1800 \times g$ for 10 min onto glass coverslips coated with 0.02% gelatin in water. Following 2 h incubation at 37 °C staining was performed with anti-CS 3D11 antibody (Ab) for sporozoite and trail visualization. Quantification is done taking into account three independent coverslips by counting the average number of circles covered by 100 sporozoites. Studies of hepatocyte invasion and traversal are performed in vitro by addition of purified sporozoites to HepG2 cells grown in MEM medium as described [22]. By using a cell-impermeant fluorescent tracer, rhodamine-dextran (1 mg/ml) [23] parasite-wounded hepatocytes are counted for quantification of cell traversal. The percentage of sporozoites inside dextran-negative cells is used to determine hepatocyte invasion as described [51]. Determination of sporozoite development within HepG2 cells is performed in vitro by staining cells with different antibodies: anti-PbEXP-1 (detection of a PVM-resident protein) and anti-HSP90 or anti-HSP70 [52] (detection of the parasite cytoplasmic heat shock protein 90 or 70). DAPI staining is done to visualize the nuclei. Quantification of trophozoite development is done by counting the number of trophozoites present in a whole coverslip 24 h postinvasion.

4. Analysis of Apoptosis in RAS and *p36p*$^{-}$ Parasite-Invaded Hepatocytes (In vitro): Detection of apoptosis is done by active caspase-3 detection [52] and nucleus is stained by DAPI as described [52]. 3×10^4 sporozoites of WT, *p36p*$^{-}$, or RAS, gamma source, 16 Krad [24], are added to 2×10^5 HepG2 cells [23, 24] and incubated for 6 h before staining with anti-HSP70 Ab [23] for detection of parasites. Apoptotic cells are detected by using an active caspase-3 detection kit (Promega) and DAPI staining after 6 h of infection. Infectious and apoptotic cells are evaluated by counting the number of parasite-infected cells and apoptotic parasite-infected cells per coverslip in triplicate, respectively.
5. Analysis of Apoptosis in *p36p*$^{-}$ Parasite-Invaded Hepatocytes (In Vivo): Intravenous injection containing 5×10^5 *p36p*$^{-}$ or WT sporozoites is administered to two groups of C57BL/6 mice. Livers are extracted 6 h postinfection. Parasites are detected using anti-HSP70 [23] in the tissue histological sections of each mouse. Nuclear morphology is visualized by DAPI staining. Sections are examined for the presence of infected cells with apoptotic signs.
6. Analysis of the Immunization Potential of *p36p*$^{-}$ Sporozoites: BALB/c and C57BL6 mice are subjected to intravenous injection of *p36p*$^{-}$ sporozoites or RAS [24] or PBS. Parasitemia is detected in Giemsa-stained blood smears. Mice are evaluated for blood stage parasitemia in alternate day from day 3 to day 21 after challenge with varying dosage of WT sporozoites at different time points. Euthanization is performed 40 h after parasitic challenge. Liver is extracted and quantified for infection by real-time PCR quantification [39].

3.8 Gamma Irradiation for Mutagenesis in Parasites [25]

1. Collection of Sporozoites: Sporozoites are collected by dissecting the salivary glands of laboratory bred *Anopheles stephensi*. Prior to dissection, *A. stephensi* are fed on a hamster infected with *Plasmodium berghei* for 14 days.
2. Homogenization of salivary glands: Isolated salivary gland is homogenized in 50% human plasma diluted in saline.
3. X-ray irradiation: The homogenized sporozoites are subjected to irradiation of 280 kVp X-ray beam of a Picker Vanguard teletherapy unit. The radiation range varied from 2 to 15 krads.
4. Protective Immunity Assessment: A/J mice are injected intraperitoneally with approximately 5000–7000 sporozoites contained within 0.2 ml injection volume. Same-sex and age-matched A/J control mice are subjected to injection containing same volume nonirradiated sporozoites. Vaccinated and control mice are subjected with secondary challenge of viable sporozoites (1000/mouse) about 2 weeks after injection of the irradiated sporozoites.

4 Notes

1. pH must be adjusted to the correct pH of 7.4 before use.
2. Integrity and quality of RNA must be checked before further downstream processes by either NanoDrop or Qubit or by agarose gel imaging.
3. Control PCR must be done with DNase treated sample before and after cDNA conversion for detection of successful reverse transcription.
4. Mutations are required to be introduced at sites not involved in packaging and splicing of viral RNP [53–55].
5. Incorporation of mutations must be at sites exhibiting higher than 99% conservation at amino acid sequence but not at nucleotide level in order to avoid the risk of adding mutations at "mutational hot spots" or causing loss of conserved codon positions serving as "potential critical RNA signals" [34–36].
6. Out-of-frame ORFs must be excluded from mutagenesis.
7. A major advantage of creating of genetically attenuated parasite vaccine is that they exhibit identical genetic identity by existing in a homogeneous population. However, attenuation by a single crossover event can lead to reversion to wild type parasite. Thus, double crossover recombination can prevent such problems of reversion [7].

References

1. Badgett MR, Auer A, Carmichael LE, Parrish CR, Bull JJ (2002) Evolutionary dynamics of viral attenuation. J Virol 76 (20):10524–10529. https://doi.org/10.1128/jvi.76.20.10524-10529.2002
2. Ebert D (1998) Experimental evolution of parasites. Science 282(5393):1432–1435. https://doi.org/10.1126/science.282.5393.1432
3. Dragunsky EM, Rivera E, Donald Hochstein H, Levenbook IS (1990) In vitro characterization of Salmonella typhi mutant strains for live oral vaccines. Vaccine 8 (3):263–268. https://doi.org/10.1016/0264-410X(90)90056-R
4. Kirkpatrick BD, McKenzie R, O'Neill JP, Larsson CJ, Bourgeois AL, Shimko J, Bentley M, Makin J, Chatfield S, Hindle Z, Fidler C, Robinson BE, Ventrone CH, Bansal N, Carpenter CM, Kutzko D, Hamlet S, Lapointe C, Taylor DN (2006) Evaluation of Salmonella enterica serovar Typhi (Ty2 aroC-ssaV-) M01ZH09, with a defined mutation in the Salmonella pathogenicity island 2, as a live, oral typhoid vaccine in human volunteers. Vaccine 24(2):116–123. https://doi.org/10.1016/j.vaccine.2005.08.008
5. Hohmann EL, Oletta CA, Killeen KP, Miller SI (1996) phoP/phoQ-deleted Salmonella typhi (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. J Infect Dis 173(6):1408–1414. https://doi.org/10.1093/infdis/173.6.1408
6. Kaper JB, Michalski J, Ketley JM, Levine MM (1994) Potential for reacquisition of cholera enterotoxin genes by attenuated Vibrio cholerae vaccine strain CVD 103-HgR. Infect Immun 62(4):1480–1483
7. Butler NS, Schmidt NW, Vaughan AM, Aly AS, Kappe SHI, Harty JT (2011) Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites. Cell Host Microbe 9(6):451–462. https://doi.org/10.1016/j.chom.2011.05.008
8. Ravenel MP (1928) La Vaccination Préventive Contre la Tuberculose par le "BCG.". Am J

Public Health Nations Health 18(8):1075. https://doi.org/10.2105/ajph.18.8.1075-a

9. Germanier R, Fürer E (1971) Immunity in experimental salmonellosis. II. Basis for the avirulence and protective capacity of gal E mutants of Salmonella typhimurium. Infect Immun 4(6):663–673
10. Wilson DB, Hogness DS (1964) The enzymes of the galactose operon in Escherichia coli. I. Purification and characterization of uridine diphosphogalactose 4-epimerase. J Biol Chem 239:2469–2481
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurements with de folin phenol reagent. Readings 193(1):265–275. https://doi.org/10.1016/0304-3894(92)87011-4
12. Takahashi M, Otsuka T, Okuno Y, Asano Y, Yazaki T, Isomura S (1974) Live vaccine used to prevent the spread of varicella in children in hospital. Lancet 2(7892):1288–1290. https://doi.org/10.1016/S0140-6736(74)90144-5
13. Li J, Arévalo MT, Diaz-Arévalo D, Chen Y, Choi JG, Zeng M (2015) Generation of a safe and effective live viral vaccine by virus self-attenuation using species-specific artificial microRNA. J Control Release 207:70–76. https://doi.org/10.1016/j.jconrel.2015.04.001
14. Fan RLY, Valkenburg SA, Wong CKS, Li OTW, Nicholls JM, Rabadan R, Peiris JSM, Poon LLM (2015) Generation of live attenuated influenza virus by using codon usage bias. J Virol 89(21):10762–10773. https://doi.org/10.1128/jvi.01443-15
15. Wang L, Liu SY, Chen HW, Xu J, Chapon M, Zhang T, Zhou F, Wang YE, Quanquin N, Wang G, Tian X, He Z, Liu L, Yu W, Sanchez DJ, Liang Y, Jiang T, Modlin R, Bloom BR, Li Q, Deng JC, Zhou P, Qin FXF, Cheng G (2017) Generation of a live attenuated influenza vaccine that elicits broad protection in mice and ferrets. Cell Host Microbe 21(3):334–343. https://doi.org/10.1016/j.chom.2017.02.007
16. Van Dijk MR, Douradinha B, Franke-Fayard B, Heussler V, Van Dooren MW, Van Schaijk B, Van Gemert GJ, Sauerwein RW, Mota MM, Waters AP, Janse CJ (2005) Genetically attenuated P36p-deficient malarial sporozouites induce protective immunity and apoptosis of infected liver cells. Proc Natl Acad Sci U S A 102(34):12194–12199. https://doi.org/10.1073/pnas.0500925102
17. Franke-Fayard B, Trueman H, Ramesar J, Mendoza J, Van Der Keur M, Van Der Linden R, Sinden RE, Waters AP, Janse CJ (2004) A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Mol Biochem Parasitol 137(1):23–33. https://doi.org/10.1016/j.molbiopara.2004.04.007
18. De Koning-Ward TF, Sperança MA, Waters AP, Janse CJ (1999) Analysis of stage specificity of promoters in Plasmodium berghei using luciferase as a reporter. Mol Biochem Parasitol 100(1):141–146. https://doi.org/10.1016/S0166-6851(99)00042-0
19. De Koning-Ward TF, Fidock DA, Thathy V, Menard R, Van Spaendonk RML, Waters AP, Janse CJ (2000) The selectable marker human dihydrofolate reductase enables sequential genetic manipulation of the Plasmodium berghei genome. Mol Biochem Parasitol 106(2):199–212. https://doi.org/10.1016/S0166-6851(99)00189-9
20. Stowers A, Carter R (2001) Current developments in malaria transmission-blocking vaccines. Expert Opin Biol Ther 1(4):619–628
21. Carrolo M, Giordano S, Cabrita-Santos L, Corso S, Vigário AM, Silva S, Leirião P, Carapau D, Armas-Portela R, Comoglio PM, Rodriguez A, Mota MM (2003) Hepatocyte growth factor and its receptor are required for malaria infection. Nat Med 9:1363–1369. https://doi.org/10.1038/nm947
22. Mota MM, Pradel G, Vanderberg JP, Hafalla JCR, Frevert U, Nussenzweig RS, Nussenzweig V, Rodriguez A (2001) Migration of Plasmodium sporozoites through cells before infection. Science 291(5501):141–144. https://doi.org/10.1126/science.291.5501.141
23. Orjih AU, Cochrane AH, Nussenzweig RS (1982) Comparative studies on the immunogenicity of infective and attenuated sporozoites of Plasmodium berghei. Trans R Soc Trop Med Hyg 76(1):57–61. https://doi.org/10.1016/0035-9203(82)90019-0
24. Nussenzweig RS, Vanderberg J, Most H, Orton C (1967) Protective immunity produced by the injection of X-irradiated sporozoites of Plasmodium berghei. Nature 216(5111):160–162
25. Fukasawa T, Nikaido H (1961) Galactose-sensitive mutants of Salmonella II. Bacteriolysis induced by galactose. Biochim Biophys Acta 48:470–483. https://doi.org/10.1016/0006-3002(61)90045-2
26. Reed LJ, Müench H (1938) A simple method of estimating 50 percent end-points. Am. J Hyg 27:493–497
27. Caunt AE, Taylor Robinson D (1964) Cell-free varicella-zoster virus in tissue culture. J Hyg

(Lond) 62:413–424. https://doi.org/10.1017/S0022172400040158

28. Grose C, Perrotta DM, Brunell PA, Con Smith G (1979) Cell-free varicella-zoster virus in cultured human melanoma cells. J Gen Virol 43(1):15–27. https://doi.org/10.1099/0022-1317-43-1-15
29. Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Zhang M, McManus MT, Gertler FB, Scott ML, Van Parijs L (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet 33(3):401–406. https://doi.org/10.1038/ng1117
30. Manicassamy B, Manicassamy S, Belicha-Villanueva A, Pisanelli G, Pulendran B, García-Sastre A (2010) Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. Proc Natl Acad Sci U S A 107(25):11531–11536. https://doi.org/10.1073/pnas.0914994107
31. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A 97(11):6108–6113. https://doi.org/10.1073/pnas.100133697
32. Li J, Arévalo MT, Chen Y, Posadas O, Smith JA, Zeng M (2014) Intranasal immunization with influenza antigens conjugated with cholera toxin subunit B stimulates broad spectrum immunity against influenza viruses. Hum Vaccines Immunother 10(5):1211–1220. https://doi.org/10.4161/hv.28407
33. Priore SF, Moss WN, Turner DH (2012) Influenza A virus coding regions exhibit host-specific global ordered RNA structure. PLoS One 7:e35989. https://doi.org/10.1371/journal.pone.0035989
34. Xu L, Bao L, Li F, Lv Q, Ma Y, Zhou J, Xu Y, Deng W, Zhan L, Zhu H, Ma C, Shu Y, Qin C (2011) Adaption of seasonal H1N1 influenza virus in mice. PLoS One 6(12):e28901. https://doi.org/10.1371/journal.pone.0028901
35. Brown EG, Liu H, Chang Kit L, Baird S, Nesrallah M (2001) Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: Identification of functional themes. Proc Natl Acad Sci U S A 98(12):6883–6888. https://doi.org/10.1073/pnas.111165798
36. Poon LLM, Leung YHC, Nicholls JM, Perera P-Y, Lichy JH, Yamamoto M, Waldmann TA, Peiris JSM, Perera LP (2009) Vaccinia virus-based multivalent H5N1 avian influenza vaccines adjuvanted with IL-15 confer sterile cross-clade protection in mice. J Immunol 182(5):3063–3071. https://doi.org/10.4049/jimmunol.0803467
37. Valkenburg SA, Li OTW, Mak PWY, Mok CKP, Nicholls JM, Guan Y, Waldmann TA, Peiris JSM, Perera LP, Poon LLM (2014) IL-15 adjuvanted multivalent vaccinia-based universal influenza vaccine requires CD4+ T cells for heterosubtypic protection. Proc Natl Acad Sci U S A 111:5676–5681. https://doi.org/10.1073/pnas.1403684111
38. Wen X, Huang X, Mok BW-Y, Chen Y, Zheng M, Lau S-Y, Wang P, Song W, Jin D-Y, Yuen K-Y, Chen H (2014) NF90 exerts antiviral activity through regulation of PKR phosphorylation and stress granules in infected cells. J Immunol 192(8):3753–3764. https://doi.org/10.4049/jimmunol.1302813
39. Blaschke V, Reich K, Blaschke S, Zipprich S, Neumann C (2000) Rapid quantitation of proinflammatory and chemoattractant cytokine expression in small tissue samples and monocyte-derived dendritic cells: Validation of a new real-time RT-PCR technology. J Immunol Methods 246(1-2):79–90. https://doi.org/10.1016/S0022-1759(00)00304-5
40. Nicholls JM, Wong LPW, Chan RWY, Poon LLM, So LKY, Yen HL, Fung K, Van Poucke S, Peiris JSM (2012) Detection of highly pathogenic influenza and pandemic influenza virus in formalin fixed tissues by immunohistochemical methods. J Virol Methods 179(2):409–413. https://doi.org/10.1016/j.jviromet.2011.11.006
41. Buchy P, Mardy S, Vong S, Toyoda T, Aubin JT, Miller M, Touch S, Sovann L, Dufourcq JB, Richner B, Van Tu P, Tien NTK, Lim W, Peiris JSM, Van der Werf S (2007) Influenza A/H5N1 virus infection in humans in Cambodia. J Clin Virol 39(3):164–168. https://doi.org/10.1016/j.jcv.2007.04.010
42. Ding H, Tsai C, Gutiérrez RA, Zhou F, Buchy P, Deubel V, Zhou P (2011) Superior neutralizing antibody response and protection in mice vaccinated with heterologous DNA prime and virus like particle boost against HPAI H5N1 virus. PLoS One 6(1):e16563. https://doi.org/10.1371/journal.pone.0016563
43. Shahangian A, Chow EK, Tian X, Kang JR, Ghaffari A, Liu SY, Belperio JA, Cheng G, Deng JC (2009) Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. J Clin Invest 119(7):1910–1920. https://doi.org/10.1172/JCI35412
44. Van Spaendonk RML, Ramesar J, Van Wigcheren A, Eling W, Beetsma AL, Van Gemert GJ, Hooghof J, Janse CJ, Waters AP

(2001) Functional equivalence of structurally distinct ribosomes in the malaria parasite, Plasmodium berghei. J Biol Chem 276 (25):22638–22647. https://doi.org/10.1074/jbc.M101234200

45. Ménard R, Janse C (1997) Gene targeting in malaria parasites. Methods 13(2):148–157. https://doi.org/10.1006/meth.1997.0507
46. Janse CJ, Waters AP (1995) Plasmodium berghei: The application of cultivation and purification techniques to molecular studies of malaria parasites. Parasitol Today 11 (4):138–143
47. Van Dijk MR, Janse CJ, Thompson J, Waters AP, Braks JAM, Dodemont HJ, Stunnenberg HG, Van Gemert GJ, Sauerwein RW, Eling W (2001) A central role for P48/45 in malaria parasite male gamete fertility. Cell 104 (1):153–164. https://doi.org/10.1016/S0092-8674(01)00199-4
48. Ozaki LS, Gwadz RW, Godson GN (1984) Simple centrifugation method for rapid separation of sporozoites from mosquitoes. J Parasitol 70(5):831–833. https://doi.org/10.2307/3281779
49. Stewart MJ, Vanderberg JP (1988) Malaria sporozoites leave behind trails of circumsporozoite protein during gliding motility. J Protozool 35(3):389–393. https://doi.org/10.1111/j.1550-7408.1988.tb04115.x
50. Silvie O, Rubinstein E, Franetich JF, Prenant M, Belnoue E, Rénia L, Hannoun L, Elings W, Levy S, Boucheix C, Mazier D (2003) Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity. Nat Med 9(1):93–96. https://doi.org/10.1038/nm808
51. Leirião P, Albuquerque SS, Corso S, van Gemert GJ, Sauerwein RW, Rodriguez A, Giordano S, Mota MM (2005) HGF/MET signalling protects Plasmodium-infected host cells from apoptosis. Cell Microbiol 7 (4):603–609. https://doi.org/10.1111/j.1462-5822.2004.00490.x
52. Moss WN, Priore SF, Turner DH (2011) Identification of potential conserved RNA secondary structure throughout influenza A coding regions. RNA 17(6):991–1011. https://doi.org/10.1261/rna.2619511
53. Gog JR, Dos Santos Afonso E, Dalton RM, Leclercq I, Tiley L, Elton D, von Kirchbach JC, Naffakh N, Escriou N, Digard P (2007) Codon conservation in the influenza A virus genome defines RNA packaging signals. Nucleic Acids Res 35(6):1897–1907. https://doi.org/10.1093/nar/gkm087
54. Lamb RA, Takeda M (2001) Death by influenza virus protein. Nat Med 7(12):1306–1312

Chapter 18

Surface Modification of Adenovirus Vector to Improve Immunogenicity and Tropism

Yasmine Gabal and Joshua D. Ramsey

Abstract

Although adenovirus is a popular vector for delivering genes, there are several drawbacks that limit its effectiveness, including tropism and both the innate and adaptive immune responses. One approach that has been used to ameliorate these drawbacks is PEGylation of the virus with subsequent modification to add functional moieties for the purpose of cell targeting or enhancing infection. Here, we describe a general approach for PEGylating adenovirus and conjugating cell-penetrating peptides to the surface of the virus to impart the ability to transduce CAR-negative cells.

Key words Gene delivery, Gene vector, Adenovirus, Polyethylene glycol, PEGylation, Cell-penetrating peptides

1 Introduction

Adenovirus (Ad) is one of the most widely used gene delivery vectors in gene therapy clinical trials [1] and has been proposed as a vector capable of delivering DNA vaccines [2]. Most people, however, possess neutralizing antibodies that inactivate the virus [3]. In addition, systemic administration of high doses of Ad triggers an inflammatory immune response resulting in rapid clearance of vector DNA from transduced tissues [4, 5]. Further, Ad relies on interaction of the virus fiber and knob with the coxsackie-adenovirus receptor (CAR) to initiate cellular attachment and ultimately infection. This reliance on the virus receptor limits the ability of Ad to deliver genes to CAR-negative cells such cancer cells, endothelial cells, epithelial cells, and smooth muscle cells [6, 7].

For these reasons there is considerable interest in modifying adenovirus to overcome these drawbacks. Approaches are typically classified as either genetic or chemical modification of the virus. For example, a common genetic approach to reduce immunogenicity is to genetically replace Ad-5 (i.e., serotype 5) viral proteins, such as

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the hexon, fiber, or knob, with similar proteins from other virus serotypes, species, or families. Wu et al. demonstrated that exchanging Ad-5 and Ad-3 hexon proteins produced a virus capable of evading neutralizing antibodies that recognize the Ad-5 vector [8]. Similarly, replacing the Ad-5 knob with that of Ad-3 was shown to alter virus tropism and enhance binding and entry into melanoma cells [9].

Chemical modification of the virus includes covalently or non-covalently complexing polymers to the surface of the virus. For example, the surface of Ad-5 has been chemical modified using polymers such as polyethylene glycol (PEG), poly-N-(2-hydroxypropyl) methacrylamide (pHPMA), poly(ethylenimine) (PEI), poly (L-lysine) (PLL), and chitosan [10–14]. PEGylating adenovirus has been shown to prolong systemic circulation, reduce liver transduction, and reduce the innate immune response [15, 16]. PEGylation, however, reduces infection efficiency of Ad through steric hindrance [17, 18].

An approach that our group has taken, which has shown success in addressing some of the aforementioned drawbacks, is to PEGylate the surface of the virus and subsequently attach peptides to the virus to improve uptake or target desired cells (Fig. 1) [19, 20]. The protocol outlined here describes our approach for PEGylating adenovirus and attaching cell-penetrating peptides (CPPs). PEGylation reduces the immunogenicity of the virus and also improves its resistance to inactivation by neutralizing antibodies. PEGylation, however, typically inhibits uptake of particles and so the addition of CPPs is necessary to restore infectivity or impart infectivity on CAR-negative cells.

The approach described here can be extended to a variety of polymers and distal moieties (antibodies, peptides, aptamers, etc.). The general approach is to use a heterobifunctional PEG, maleimide-PEG-hydroxysuccinimide ester, to PEGylate the virus though a reaction with the terminal amine group within the capsid lysine residues. The thiol-reactive maleimide end of the polymer is subsequently reacted with a cysteine residue added to the end of the CPP. The details of this approach are presented below.

2 Materials

Work with adenovirus must be carried out in a class II biological safety cabinet. All pipette tips, microcentrifuge tubes, and 15 ml conical centrifuge tubes should be sterile. Lab personnel should wear proper PPE including a laboratory coat, safety glasses, and gloves. Virus-containing material should be autoclaved or disinfected and disposed of as biohazardous waste.

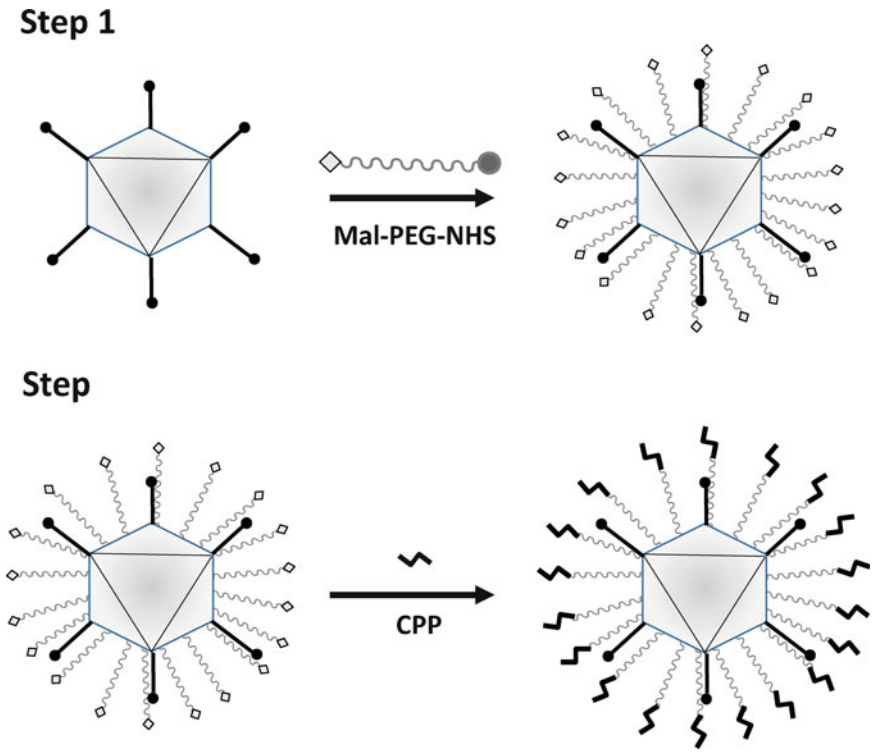

Fig. 1 Approach for producing CPP-PEG-Ad particles. In Step 1, the N-hydroxyl succinimidyl ester (NHS) chemical group on the heterobifunctional MAL-PEG-NHS reacts with lysine residues in the fiber and capsid protein of the native adenovirus particle to produce PEGylated virus. In Step 2, the thiol-reactive maleimide (MAL) reacts with the cysteine sulfhydryl group on the CPP to produce CPP-PEG-Ad

2.1 Adenovirus

Purified adenovirus at 1×10^{10} infectious unit/ml (IU/ml) (*see* **Notes 1** and **2**).

2.2 Polyethylene Glycol

Heterobifunctional PEG (MAL-PEG-NHS) (Fig. 2) with a PEG molecular weight of 5 kDa can be purchased from CreativePEG-works (Cat. PHB-952) (*see* **Note 3**).

2.3 Cell-Penetrating Peptides

The cell-penetrating peptide, Pen (RQIKIWFQNRRMKWKKC) has a cysteine residue added to the C-terminus end of the peptide.

2.4 Cells

1. NIH/3T3 cells.
2. The complete growth medium is Dulbecco's Modified Eagle's Medium (DMEM) with bovine calf serum added to a final concentration of 10%.

2.5 Buffers

1. 100 mM HEPES buffer: Dissolve 2.38 g of HEPES (free acid) in 80 ml of deionized (DI) water. Bring the volume up to 100 ml with DI water. Filter through a 0.22 μm filter to sterilize. Store at 4 °C for up to 4 months.

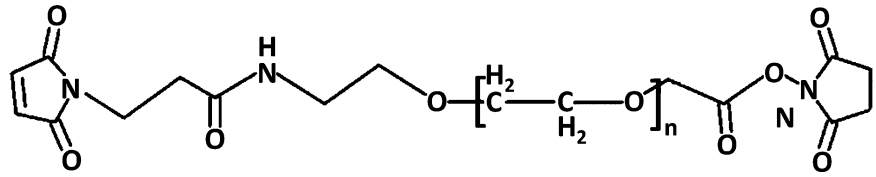

Fig. 2 Chemical structure of MAL-PEG-NHS

2. 50 mM HEPES (pH 8.0): Take 50 ml from the stock solution (100 mM) and increase the volume to 80 ml with DI water. Adjust the pH to 8.0 using NaOH before increasing the final volume to 100 ml with DI water. Filter the buffer to sterilize and store at 4 °C.
3. 20 mM HEPES buffer (pH 7.0): Take 20 ml from the stock solution (100 mM) and increase the volume to 80 ml with DI water. Adjust the pH to 7.0 using NaOH before increasing the final volume to 100 ml with DI water. Filter the buffer to sterilize and store at 4 °C.

2.6 Equipment and Instruments

1. Cell culture incubator (high humidity, 37 °C, 5% CO_2).
2. Class II biological safety cabinet.
3. Vortex mixer.
4. Water bath at 37 °C.
5. Protein Concentrators (PES, 10 kDa MWCO, 0.5 ml).
6. Microcentrifuge.
7. 1000, 200, and 10 μl pipettes.

3 Methods

3.1 Preparing Plates for Transduction

1. Trypsinize NIH/3T3 cells from a 90% confluent T-25 flask and resuspend in 8–10 ml of medium.
2. Determine the cell concentration using a hemocytometer and plate 5×10^4 cells in 500 μl of cell growth medium per well of a 24 well plate.
3. After 18–24 h, the cells should appear 50–60% confluent and are ready for transduction. (*See* Table 1 for different seeding densities and vessel size options).

3.2 Preparing Adenoviral Stock

1. Decide the desired multiplicity of infection (MOI) for transduction. You will need to know the approximate number of cells seeded. Refer to Table 1 for seeding densities and volumes of medium for each well based on the size of the plate (*see* **Note 4**).

Table 1
Seeding density and volume of medium for each well in different plate sizes

Tissue culture plate	Cell seeding density[a] (cells/well)	Medium volume (ml/well)	Transduction medium volume (ml/well)
48-well plate	2.5×10^4	0.3	0.15
24-well plate	5×10^4	0.5	0.25
12-well plate	10×10^4	1.0	0.50
6-well plate	30×10^4	3.0	1.50

[a]The values reported in table are example seeding values and may need to be adjusted based on a particular cell line or desired cell density during transduction

2. Verify that the virus titer is known, which is needed to calculate the volume of virus required to produce the desired MOI.

General formulae:

$$\text{Virus}\left(\frac{\text{IU}}{\text{well}}\right) = \text{seeding density}\left(\frac{\text{cells}}{\text{well}}\right) \times \text{MOI}\left(\frac{\text{IU}}{\text{cell}}\right)$$

$$\text{Vol.of Virus}\left(\frac{\mu\text{l}}{\text{well}}\right) = \frac{\text{Virus (IU/well)}}{\text{Viral titer (IU/}\mu\text{l)}}$$

Example: Calculating the amount of virus required in a 24-well plate.

I. Amount of virus needed for transduction:

$$\text{Seeding density} = 5 \times 10^4\left(\frac{\text{cells}}{\text{well}}\right); \text{MOI} = 200$$

$$\text{Virus}\left(\frac{\text{IU}}{\text{well}}\right) = 5 \times 10^4\left(\frac{\text{cells}}{\text{well}}\right) \times 200\left(\frac{\text{IU}}{\text{cell}}\right) = 1 \times 10^7\left(\frac{\text{IU}}{\text{well}}\right)$$

II. Transforming IU into volume of virus needed per well:

$$\text{Viral titer} = 1 \times 10^{10}\left(\frac{\text{IU}}{\text{ml}}\right) = 1 \times 10^7\left(\frac{\text{IU}}{\mu\text{l}}\right);$$

$$\text{Virus}\left(\frac{\text{IU}}{\text{well}}\right) = 1 \times 10^7\left(\frac{\text{IU}}{\text{well}}\right)$$

Therefore,

$$\text{Vol.of Virus}\left(\frac{\mu\text{l}}{\text{well}}\right) = \frac{1\times10^7\ \left(\frac{\text{IU}}{\text{well}}\right)}{1\times10^7\ \left(\frac{\text{IU}}{\mu\text{l}}\right)} = 1\left(\frac{\mu\text{l}}{\text{well}}\right)$$

As a result, 1 μl of the viral stock is needed to transduce 5×10^4 cells with a MOI of 200.

3.3 PEGylating Adenovirus

The following procedure is designed to produce 10^7 CPP-PEG-Ad particles, which in our case is suitable for transducing one well of a 24-well plate. The reaction can be scaled to transduce a greater number of wells or a larger plate.

1. Dilute 1 μl of virus from the purified viral stock into 99 μl of 50 mM HEPES buffer (pH 8.0) to reach 100 μl. Keep the virus on ice until used.
2. Prepare 1 ml of stock solution of 1 mg/ml MAL-PEG-NHS (5 kDa) in 50 mM HEPES buffer (pH 8.0) (*see* **Notes 5** and **6**).

3. Dilute 40 μl of the PEG stock solution with 10 μl of 50 mM HEPES buffer (pH 8.0).
4. Dropwise, add 50 μl of the diluted PEG solution to the side of the microcentrifuge tube containing the virus while gently vortexing so that the final concentration of PEG is 4 μg/10^6 vp.
5. Incubate for 45 min at room temperature while pulse vortexing every 7–10 min.
6. *See* **Note 7** for details on how to determine the extent of the reaction between PEG and Ad.

3.4 Formation of CPP-PEG-Adenovirus

1. Prepare an 800 μl stock solution of 2 mg CPP/ml in 20 mM HEPES buffer (pH 7.0) (*see* **Note 5**).
2. Dilute 31.25 μl of the CPP stock solution into 18.75 μl of 20 mM HEPES buffer (pH 7.0).
3. Dropwise, add 50 μl of the CPP solution (from **step 2**) onto the side of the microcentrifuge tube containing the PEGylated virus while gently vortexing so that the final concentration of CPP is 6.25 μg/10^6 vp.
4. Incubate for 45 min while pulse vortexing every 7–10 min.
5. Purify the CPP-PEG-Ad particles by removing unreacted peptide and polymer using a Pierce protein concentrator with 10 kDa MWCO, as follow:
   (a) Place the sample into the concentrator sample chamber.
   (b) Place the cap and the sample chamber and insert the sample chamber into a collection tube.
   (c) Place the concentrator assembly into the microcentrifuge with a proper counterbalance and centrifuge at 500 × *g* until the sample remaining in the sample chamber is half the original volume.
   (d) Add approximately 100 μl of 20 mM HEPES buffer (pH 7.0) to the sample chamber to bring the volume back to 200 μl. Repeat **steps 5b–d** twice to further purify the sample.
   (e) After purification with the protein concentrator, return the volume to 200 μl with 20 mM HEPES buffer (pH 7.0).
6. *See* **Note 8** for details on how to determine the extent of the reaction between the PEGylated virus and the CPP.

3.5 Transduction of NIH/3T3 Cells

For our purposes, we expose cells to a PBS control that allows us to measure background gene expression levels. Also, additional Ad virus is needed if one is interested in comparing the efficiency of

the CPP-PEG-Ad vector with unmodified Ad vector. Each sample is evaluated on a minimum of three duplicate wells.

1. In a 37 °C water bath, warm enough DMEM medium for the sample and control wells.
2. Carefully remove the cell culture medium (DMEM +10% CS) from the wells (*see* **Notes 9** and **10**).
3. Gently add the transduction medium (i.e., DMEM) to each well. The volume of transduction medium is half the normal volume of medium used for culturing the cells (Table 1).
4. Dropwise, pipet the prepared CPP-PEG-Ad particles (Subheading 3.4, **step 5**), controls, or additional samples onto each respective well.
5. Return the tissue culture plates to the incubator (humidified atmosphere, 5% CO_2, 37 °C) and incubate for 4 h.
6. After 4 h, aspirate the serum-free medium and replace with the appropriate volume of DMEM supplemented with 10% CS (Table 1). Return the cell culture plates to the incubator and store at 5% CO_2 at 37 °C.
7. After 36 h, assay for reporter gene expression to assess the effectiveness of the CPP-PEG-Ad vector.

4 Notes

1. Ad with an E1/E3 gene deletion and packaging the *LacZ* reporter gene under the control of a cytomegalovirus (CMV) promoter is purchased from Capital Biosciences (Rockville, MD) and amplified using an HEK-293 cell line. Once the cytopathic effect is observed, cells are collected and subjected to three freeze/thaw cycles to lyse the cells. The virus is purified from the cell lysate using a Vivapure Adenopack (Sartorius Stedim, Arvada, Colorado). The virus is exchanged into a physiological buffer, such as phosphate buffered saline (PBS), before use in cell-based assays.
2. The virus is freshly purified on the day of the experiment and can be stored in aliquots at 20 °C for up to 1 week before use.
3. One terminal end of the PEG polymer has a thiol-reactive, maleimide (MAL) group, and the other terminal end has an amine-reactive, N-hydroxyl succinimidyl ester (NHS) group. NHS and MAL enable conjugation of PEG to Ad and subsequently to the peptide, respectively. Maleimide contains a reactive C=C double bond and is light and oxygen sensitive. Store at 4 °C in an opaque container and under a nitrogen atmosphere until use.

4. The MOI describes the number of virus particles used to infect one cell. The probability of infecting a cell, however, is subject to the statistical Poisson distribution. For example, an MOI of 100 indicates that 100 virus particles are needed for one cell to be infected efficiently. In principle, the MOI for Ad particles ranges from 10 to 1000, depending on the particular cell line.
5. Stability of NHS esters are compared by their hydrolysis half-life ($t_{1/2}$). The hydrolysis half-life of the PEG-SCM is 1–5 min. Thus, dissolve PEG in buffer just before adding to the virus. Avoid frequent freeze/thaw cycles.
6. A 1 ml stock solution (1 mg/ml) of MAL-PEG-NHS and an 800 μl of stock solution (2 mg/ml) of CPP will be sufficient to prepare enough PEN-PEG-Ad solution to transduce a 24-well plate.
7. As PEG molecules conjugate to the surface of the virus, they mask protein residues that decrease the overall charge while increasing the particle size of Ad [21, 22]. Thus, initial characterization of PEGylated adenoviruses is to assess the physicochemical properties of the virus such as particle size and zeta potential. The amount of PEG conjugated to Ad is estimated using a fluorescamine assay [23], in which fluorescamine reacts with free lysine on the virus capsid to estimate the degree of PEGylation (DOP). Briefly, 50 μl of fluorescamine (Sigma, St. Louis, MO), at a concentration of 0.6 mg/ml in acetone, is added to serial dilutions of modified and unmodified virus samples at room temperature and left for 15 min. Fluorescence measurements are then measured using a PTI fluorometer (Photon Technologies International, Edison, NJ), with an excitation wavelength of 390 nm and an emission wavelength of 475 nm. Fluorescence measurements are plotted against virus concentration, and the amount of PEG conjugated is determined by comparing the slopes of the modified to the unmodified virus sample [20].
8. An Ellman's assay is used to quantify the amount of free thiol, corresponding to unreacted cysteine residues of CPPs that are not conjugated to the PEG-Ad particles [19]. The Ellman's reagent, 5,5′-dithio-*bis*-2-nitrobenzoic acid (DTNB), has an oxidizing disulfide bond that is reduced in the presence of free thiols forming a mixed disulfide and releasing one molecule of 5-thio-2-nitrobenzoic acid (TNB). TNB is a yellow product that can be quantified by measuring absorbance at 412 nm. The amount of free CPP in the sample is estimated by comparing the sample absorbance to a standard curve composed of known concentrations of a sulfhydryl-containing compound (DNTB thiol).

9. When aspirating and replacing medium, aspirate three wells at a time and immediately replace the medium with serum-free medium. This approach minimizes the time the cells are allowed to dry, which can lead to the cells detaching from the plate.
10. The protocol describes transduction of a CAR-negative cell line, NIH/3T3. Depending on the particular cell line used, it may be necessary to vary the cell culture medium, cell seeding density, and MOI.

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References

1. Ginn SL et al (2018) Gene therapy clinical trials worldwide to 2017: an update. J Gene Med 20 (5):e3015
2. Tatsis N, Ertl HC (2004) Adenoviruses as vaccine vectors. Mol Ther 10(4):616–629
3. Wohlfart C (1988) Neutralization of adenoviruses: kinetics, stoichiometry, and mechanisms. J Virol 62(7):2321–2328
4. Nayak S, Herzog RW (2010) Progress and prospects: immune responses to viral vectors. Gene Ther 17(3):295
5. Worgall S et al (1997) Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. Hum Gene Ther 8(1):37–44
6. Sharma A et al (2009) Adenovirus receptors and their implications in gene delivery. Virus Res 143(2):184–194
7. Bergelson JM et al (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275 (5304):1320–1323
8. Wu H et al (2002) Construction and characterization of adenovirus serotype 5 packaged by serotype 3 hexon. J Virol 76 (24):12775–12782
9. Volk AL et al (2003) Enhanced adenovirus infection of melanoma cells by fiber-modification: incorporation of RGD peptide or Ad5/3 chimerism. Cancer Biol Ther 2 (5):511–515
10. Fisher K et al (2001) Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. Gene Ther 8(5):341
11. O'Riordan C, Lachapelle A, Delgado C, Parkes V (1999) Wadsworth SC Smith AE Francis GE PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. Hum Gene Ther 10:1349–1358
12. Baker A et al (1997) Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery. Gene Ther 4(8):773
13. Kawamata Y et al (2002) Receptor-independent augmentation of adenovirus-mediated gene transfer with chitosan in vitro. Biomaterials 23(23):4573–4579
14. Fasbender A et al (1997) Complexes of adenovirus with polycationic polymers and cationic lipids increase the efficiency of gene transfer in vitro and in vivo. J Biol Chem 272 (10):6479–6489
15. Croyle MA et al (2001) "Stealth" adenoviruses blunt cell-mediated and humoral immune responses against the virus and allow for significant gene expression upon readministration in the lung. J Virol 75(10):4792–4801
16. Wonganan P, Croyle MA (2010) PEGylated adenoviruses: from mice to monkeys. Viruses 2(2):468–502
17. Kanerva A, Hemminki A (2004) Modified adenoviruses for cancer gene therapy. Int J Cancer 110(4):475–480
18. Dmitriev I et al (1998) An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent

cell entry mechanism. J Virol 72 (12):9706–9713

19. Nigatu AS et al (2015) Effects of cell-penetrating peptides on transduction efficiency of PEGylated adenovirus. Biomed Pharmacother 71:153–160
20. Nigatu AS et al (2013) Evaluation of cell-penetrating peptide/adenovirus particles for transduction of CAR-negative cells. J Pharm Sci 102(6):1981–1993
21. Gao J-Q et al (2007) Effective tumor targeted gene transfer using PEGylated adenovirus vector via systemic administration. J Control Release 122(1):102–110
22. Croyle MA, Yu Q-C, Wilson JM (2000) Development of a rapid method for the PEGylation of adenoviruses with enhanced transduction and improved stability under harsh storage conditions. Hum Gene Ther 11 (12):1713–1722
23. Stocks SJ et al (1986) A fluorometric assay of the degree of modification of protein primary amines with polyethylene glycol. Anal Biochem 154(1):232–234

Chapter 19

Production of Baculovirus and Stem Cells for Baculovirus-Mediated Gene Transfer into Human Mesenchymal Stem Cells

Friederike Eilts, Julie Harnischfeger, Daniel Loewe, Michael W. Wolff, Denise Salzig, and Peter Czermak

Abstract

The discovery of the genome-editing tool CRISPR-Cas9 is revolutionizing the world of gene therapy and will extend the gene therapy product pipeline. While applying gene therapy products, the main difficulty is an efficient and effective transfer of the nucleic acids carrying the relevant information to their target destination, the nucleus of the cells. Baculoviruses have shown to be very suitable transport vehicles for this task due to, inter alia, their ability to transduce mammalian/human cells without being pathogenic. This property allows the usage of baculovirus-transduced cells as cell therapy products, thus, combining the advantages of gene and cell therapy. To make such pharmaceuticals available for patients, a successful production and purification is necessary. In this chapter, we describe the generation of a pseudotyped baculovirus vector, followed by downstream processing using depth and tangential-flow filtration. This vector is used subsequently to transduce human mesenchymal stem cells. The production of the cells and the subsequent transduction process are illustrated.

Key words Gene therapy, Cell therapy, Tangential flow filtration (TFF), Baculovirus expression vector system (BEVS), Pseudotyped baculovirus, Human mesenchymal stem cell (hMSC)

1 Introduction

Gene therapy has evolved as a modern medicinal application [1], which offers a real cure for currently untreatable cancer types [2] or hereditary diseases [3]. The genome editing tool CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR) associated nuclease 9) is revolutionizing gene therapy and will extend the gene therapy product pipeline. In 2019, 2986 gene therapy products were in clinical studies, a growth by 12% compared to the preceding year (*see* **Note 1**).

The main difficulty in gene therapy is an efficient transfer of the nucleic acids carrying the relevant information into the nucleus of

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cells [4]. Therefore, effective transfer vectors need to fulfill the following criteria [5]:

1. Immune reactions to the nucleic acids must be prevented by the vector.
2. The cargo (in this case the nucleic acid) must be delivered to specific cells.
3. The vector should infect dividing and nondividing cells.
4. The vector must allow the unimpeded expression of the (recombinant) gene.
5. The transport of nucleic acids with varying sizes must be feasible.
6. The vector production must be economically profitable: simple, cost-effective, and in high concentrations.

Viral vectors were the first vectors to be used and still represent the major share of today's medicinal applications for gene therapy [2]. The most common candidates are the adenovirus [6], the adeno-associated virus [7], the gammaretrovirus [8], and lentivirus vectors [9]. Comprehensive descriptions of their assets and drawbacks can be found elsewhere [4, 10]. Here, the focus lies on the infrequently used, but very promising baculovirus vector (BV). Natural baculoviruses infect insect cells and are enveloped dsDNA viruses with rod-shaped nucleocapsids with a size of 30–60 nm × 250–300 nm and a circular DNA.

Different applications of baculoviruses have been used for decades. Since the discovery of the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV) (134 kb [11]) in 1971 [12], its use as a sustainable and safe pesticide was investigated. Next, the baculovirus expression vector system (BEVS) was developed for the production of recombinant proteins in the 1980s [13]. It is still extensively used for the production of vaccine candidates [14–16]. Furthermore, the BEVS was approved by EMA (*European Medicines Agency*) and FDA (*Food and Drug Administration*). Finally, the ability of the baculovirus to transduce eukaryotic cells was discovered in the mid-1990s [17], which opened the doors for gene therapy applications. The transduction of various types of cells and tissue is possible today [18–22].

In comparison to the prior mentioned viral vectors, the BV shows several advantages. First, the baculoviral genome allows a stable insertion of complex gene cassettes up to a length of 47 kb [23]. Secondly, it is nonpathogenic for humans and can be handled in biosafety level I laboratories. The baculovirus is inherently incapable of replicating in mammalian cells [24], that is, the DNA degrades inside the transduced cells [25] while its integration into the host genome is almost never found [26]. Thirdly, the baculovirus is not targeted by the human immune system as no specific

antibodies [27] or T-cells [28] are innate. Fourth, the baculovirus production is established on an industrial scale reaching high viral titers ($>10^9$ pfu/mL) [29]. Fifth, the production conditions are economically favorable as the virus is cultivated at 27 °C in serum-free medium without CO_2, and subsequently stored at 4 °C [30].

The aforementioned advantages make the BV very suitable for gene therapy, although further adaptations are possible to increase the efficiency of the gene transfer into human/mammalian cells. Genetic modifications of the BV may positively influence the transduction efficiency in mammalian cells. Modifications include (I) pseudotyping, (II) the integration of a corresponding promoter, and (III) the utilization of regulatory elements.

Pseudotyping (I) means, producing baculoviruses in combination with foreign viral envelope proteins. The glycoprotein G of the vesicular stomatitis virus (VSV), short VSV-G, is suitable to pseudotype the BV, and to enable the transduction of almost every mammalian cell type [31–34]. Apart from an efficient transduction, clearly the gene expression in the targeted cells or tissue is equally important. This requires the selection of a strong mammalian promoter (II) and/or tissue-specific promoters [35, 36]. Numerous studies have demonstrated the efficient transduction of different cell lines and their gene expression under the control of mammalian promoters, such as the cytomegalovirus (CMV) immediate early promoter/enhancer, or the Rous sarcoma virus promoter [17, 37, 38]. Moreover, regulatory elements (III) (e.g., the Woodchuck hepatitis virus posttranscriptional regulatory element) can be used to enhance gene expression [39].

Different preclinical studies have shown the potential of the BV as a viral vector targeting mammalian cells, for example for cancer therapy [40–43] or bone regeneration purposes [44–47]. Apart from these studies, future prospects of utilizing the baculovirus as a vaccine vector are evaluated [16, 48–50].

As the pseudotyped and further optimized BV is an efficient vector to deliver genetic information into human/mammalian cells, they can be used to transduce human stem cells. The generated genetically modified stem cells are then applied as cell therapy products. This combines the therapeutic effects of stem cells and transferred nucleic acids, which may encode for proteins (e.g., growth factors) or downregulate certain genes [51]. The choice of the target cell line strongly depends on the desired properties of the product. Mesenchymal stem cells (MSC) have been shown to be a favored cell type due to their immunomodulatory properties. Accordingly, native MSCs are logical candidates for the treatment of immune disorders, including the graft-versus-host disease, the inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, and diabetes [52]. Additionally, MSCs promote the regeneration of damaged tissue by stimulating cell proliferation and migration, promoting angiogenesis, and suppressing apoptosis

and fibrosis [53]. This regenerative capacity of MSCs has been used to treat the Alzheimer's disease, bone and cartilage diseases, diabetes, myocardial infarction, and osteoarthritis [54]. Clinical investigations with genetically modified MSCs, analyzing the cell's potential to treat graft-versus-host disease [55] or ischemia [56], repairing bone and cartilage, are still at the very beginning [57].

As already mentioned, the BV can be used to transduce MSCs. An optimization of this process has been conducted concerning the promoter. A recent promoter study, in which MSCs were transduced with a pseudotyped BV, recommends the human elongation factor 1 alpha promoter for regulating the gene expression [58]. In comparison, the CMV immediate early promoter/enhancer was shown to be considerably weaker due to promoter silencing [59, 60]. Others optimized the BV transduction of MSCs by constructing a hybrid vector of the baculovirus and the adeno-associated virus [61].

Due to the long history of applications using the baculovirus, several industrially attuned production systems are at hand [62]: They are the basis for the production of the BV in sufficient quantity and quality for gene therapy products, or for genetically modified cell therapeutics. As baculoviruses display a lytic life cycle, they are produced in a batch-mode in bioreactors (e.g., stirred tank reactor) [63, 64]. A production requires the consideration of the GMP guidlines. Usually, baculovirus/BV is amplified in serum-free medium [65] using a cell concentration at infection (CCI) of 1×10^6 cells/mL [66]. Subsequent infection is routinely completed with a multiplicity of infection (MOI) between 0.01 and 1 pfu/cell, taking into account that a low MOI reduces the amount of defective virus particles [67, 68]. Certain critical process parameters must be considered during a BV production, which include the time of harvest (TOH), time of infection (TOI), shear stress, dissolved oxygen, pH, and temperature [69]. In this chapter, methods of virus propagation are of a subordinate role, as they are intensively reviewed in Chap. 8.

After upstream processing has been conducted, the BV must undergo an extensive purification process to clear the culture supernatant from cell debris, residual protein and DNA. Generically, the downstream process (DSP) for viral vectors is split into the procedural steps of clarification, concentration, potential nuclease treatment, purification, polishing, and—for viruses of a smaller size—sterile filtration [70]. For the BV, a sterile filtration step is possible, but it can lead to high virus losses. As an alternative, a complete aseptic downstream process is suitable [71]. The performance of every DSP process step depends on the upstream process. Hence, it is inevitable, to reconcile up- and downstream processes in a holistic approach, while, at the same time, building robust and efficient methods that are in line with the approach of Quality by Design, requested by the FDA [72]. Additional regulatory obligations

come with the threshold values by EMA and FDA for residual contaminants. For gene therapy vectors, a batch release requires no "admissible levels" of host cell protein (HCP) or host cell DNA (hcDNA) according to Ph. Eur. 5.14. In literature, an hcDNA content of <5 pg per 10^{-11} virus particles is recommended [73]. For cell culture-derived vaccines (Ph. Eur. 6.3), the residual hcDNA (>200 bp) must not exceed 10 ng/dose, and the amount of residual bovine serum albumin should be <50 ng per dose. It is advised that the quantity of HCP is monitored and minimized throughout DSP, and reported using an appropriate assay. HCP ranges for biological products are typically between 1 and 100 ppm (<100 ng/mL) [74, 75]. A current draft guideline for gene therapy vectors of the FDA recommends to follow the limits applied to vaccines [76–78]. Useful guidelines for the quantification of the product and the contaminants can be found elsewhere [72, 79].

Clarification and concentration processes are usually performed via filtration methods [72] (in lab scale centrifugation [80]), whereas purification and polishing are relevant for manifold systems. Focusing on purity, contaminant removal, and high yields of a desired product [72, 79, 81], different strategies have been employed for the BV, relying on the key technologies of centrifugation, filtration, and chromatography [65]. Centrifugation techniques are based on ultracentrifugation methods which are solely used for lab-scale applications [30]. Chromatographic purification has been exploited by size exclusion chromatography [82], ion exchange chromatography [83–86], affinity chromatography [87–89] and affinity-binding to magnetic beads [90], and steric exclusion chromatography (*see* Chap. 12) [91]. Filtration processes for the BV purification usually involve tangential flow filtration (TFF), also called cross-flow filtration [71, 92, 93]. Membrane-based filtration is beneficial for handling a large-scale production whilst simultaneously yielding high virus concentrations of sustained infectivity and purity [84].

In the following methods chapter, we describe in detail the use of depth filtration for a primary clarification, and a subsequent TFF for the purification of the BV. Depth filtration is a dead-end method, using pore size ratings between 0.1 μm and 10 μm [94]. This procedure is very well suited for the filtration of high-cell-density cultivation broths, for an easy scaling of the parameters, and for employing single-use cartridges [95]. Recovery rates of virus particles and VLPs have been reported to lie above 85% using depth filtration [71, 96]. After cell separation, remaining contaminants like HCP and hcDNA are removed via a purification step. TFF is routinely applied in concentration mode or as diafiltration to further purify the BV. Due to the tangential flow (retentate), the buildup of a filter cake on the membrane surface is reduced, whilst a smaller fraction flows through the membrane (permeate).

For virus concentration/purification, the desired product should be found in the retentate. Virus recovery rates of 70% have been published [71, 92, 93].

2 Materials

In the following, we describe a protocol to generate pseudotyped baculoviruses, to produce the BV on a bioreactor scale, and to purify it via depth filtration and a subsequent TFF. Furthermore, we describe the transduction of human mesenchymal stem cells with purified BV. The whole process must be performed sterile; that is, a clean-bench system (Thermo Fisher Scientific, Waltham, MA, USA) and an autoclave (Systec, Linden, GER) are prerequisite. The origin of all materials mentioned is exemplary.

2.1 Generation of the Pseudotyped Baculovirus

The generation of the pseudotyped BV is mainly based on the BacMam-System using MAX Efficiency® DH10-Bac™ (Thermo Fisher Scientific, Waltham, MA, USA). We only describe the materials needed to generate the recombinant vector-encoding for the gene of interest. The materials for the generation of recombinant bacmid DNA and of the baculovirus stock can be found in Chap. 8.

- Transfer cloning vector (e.g., BacMam pCMV-Dest Vector, Thermo Fisher Scientific, Waltham, MA, USA).
- Sequence of the gene of interest (e.g., growth factor).
- Other sequences of genetic elements (e.g., a reporter gene like GFP, a secretion signal, etc.).

2.2 Production of the Baculovirus on a Bioreactor-Scale

The BV is produced in *Sf*-9 (immortalized ovary cells from *Spodoptera frugiperda*) insect cells in a stirred tank bioreactor. All materials needed for the bioreactor assembly, the process control, the cultivation of *Sf*-9 cells, the initiation of cultivation, the infection of *Sf*-9 cells with the baculovirus/BV, and the quantification of the BV, are described in Chap. 8.

2.3 Downstream Processing of the Baculovirus

When "water" is required, this always refers to ultrapure water (Milli-Q Academic, Merck Millipore, Burlington, MA, USA).

2.3.1 Quantification of Baculoviruses

Quantification methods for BV are described in detail in Chap. 8. This chapter covers the quantification of baculoviral DNA (vDNA), more specifically, DNA extraction and subsequent quantification by PCR, as well as the quantification of an infectious titer by an end-point dilution assay, that is, the 50% tissue culture infection dose ($TCID_{50}$), and a plaque assay. Although, in this chapter, these

assays are used for a different genotype of the virus, both show the same features necessary for assay application.

2.3.2 Quantification of Contaminants

The quantification of contaminants is performed by using assays in the 96-well microplate format. Reading is handled in a plate reader (Cytation3 [BioTek, Winooski, VT, USA]).

Residual Free DNA (dsDNA) (*See* **Note 2**)

- Quant-iT™ PicoGreen™ dsDNA Kit (Thermo Fisher Scientific, Waltham, MA, USA).
- Plate shaker (Thermomixer compact [Eppendorf, Hamburg, GER]).
- 96-Well plates, flat bottom, black (Greiner CELLSTAR® [Sigma-Aldrich, St. Louis, MO, USA]).
- Sterile, nuclease-free water (Thermo Fisher Scientific, Waltham, MA, USA).

Total Protein via Lowry (BCA) Assay (*See* **Note 3**)

- Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).
- Plate shaker (Thermomixer compact [Eppendorf, Hamburg, GER]).
- Incubator (HERAcell™ 240i [Thermo Fisher Scientific]), 37 °C.
- 96-Well plates, flat bottom, transparent (Greiner CELLSTAR® [Sigma-Aldrich, St. Louis, MO, USA]).
- Water.
- PBS for dilutions (Biochrom, Berlin, GER).

2.3.3 Clarification via Depth Filtration

The DSP process is performed using the benchtop filtration system SARTOFLOW® Slice 200 Benchtop System (Sartorius, Göttingen, GER). The system should be equipped with a peristaltic pump with a Tandem 1082-pump head (Sartorius, Göttingen, GER) (*see* **Note 4**). Pressures are measured with SciLog® SciPres® pressure sensors, connected to the pump.

- Sartopure® PP3 particle reduction filter element: size 8 (0.12 m^2 filtration area; 3 μm retention rate; filter material: polypropylene fleeces); single-use (Sartorius, Göttingen, GER).
- Sartopure® PP3 particle reduction filter element: size 4 (0.013 m^2 filtration area; 0.64 μm retention rate; filter material: polypropylene fleeces); single-use (Sartorius, Göttingen, GER).

- Sterile glass bottle topped with multiport connection system for bioreactors (Schott, Mainz, GER): one air filter (Merck Millipore, Burlington, MA, USA), one connector to probe vent of bioreactor (e.g., Luer lock [Sigma-Aldrich, St. Louis, MO, USA]), additional connectors should be sealed.
- PBS (Biochrom, Berlin, GER), adjusted to pH 6.4 with 1 M HCl (Carl Roth, Karlsruhe, GER), and filtered through 0.2 μm bottle-top filter (Fisher Scientific, Hampton, NH, USA). All buffers should be sterilized in an autoclave, or using a bottle-top filter.
- Water, 0.2 μm filtered, sterilized.
- 1 M NaOH (Carl Roth, Karlsruhe, GER), 0.2 μm filtered.
- Serological pipettes and holder (Sarstedt, Nümbrecht, GER).
- Sterile pipette tips and pipette (Eppendorf, Hamburg, GER).
- 1.5 mL tubes (Eppendorf, Hamburg, GER).
- 15 mL and/or 50 mL centrifuge tube (Sarstedt, Nümbrecht, GER).

2.3.4 BV Purification via TFF

- Sartocon® Slice 200 Polyethersulfone (PES) membrane (cassette), MWCO (molecular weight cut off) 300 kDa, filtration area 200 cm^2 (Sartorius, Göttingen, GER) (*see* **Note 5**).
- Sartocon® Slice 200 Holder (Sartorius, Göttingen, GER).
- PBS (Merck Millipore, Burlington, MA, USA), adjusted to pH 6.4 with 1 M HCl (Carl Roth, Karlsruhe, GER). 0.2 μm filtered, sterilized.
- Water, 0.2 μm filtered, sterilized.
- 1 M NaOH (Carl Roth, Karlsruhe, GER), 0.2 μm filtered.
- Serological pipettes (Sarstedt, Nümbrecht, GER) and holder.
- Sterile pipette tips and pipette (Eppendorf, Hamburg, GER).
- 1.5 mL tubes (Eppendorf, Hamburg, GER).
- 15 mL and/or 50 mL centrifuge tube (Sarstedt, Nümbrecht, GER).

Regeneration of Sartocon® Slice 200

- 1 M NaOH (Carl Roth, Karlsruhe, GER), 0.2 μm filtered.
- Water, 0.2 μm filtered, sterilized.
- Dry-heat oven (Memmert, Schwabach, GER), 50 °C.
- 20% ethanol (Carl Roth, Karlsruhe, GER), 0.2 μm filtered.

2.4 Transduction of MSC with BV

2.4.1 Thawing and Cultivating MSCs

- Cryovial with 1×10^6 hMSCs (e.g., from bone marrow, ATCC-PCS-500-012).
- Water bath (37 °C) (WNB 22, Memmert, Schwabach, GER).
- T25-flasks (Sarstedt, Nümbrecht, GER).
- Growth medium (Dulbecco's MEM supplemented with 10% fetal calf serum and 2 mM L-alanyl-L-glutamine) (Biochrom, Berlin, GER).
- Trypsin–EDTA solution (Biochrom, Berlin, GER).
- Phosphate-buffered saline (PBS), w/o Ca^{2+} and Mg^{2+} (#L1825 [Biochrom, Berlin, GER]).
- Serological pipettes and holder (Sarstedt, Nümbrecht, GER).
- Sterile pipette tips and pipette (Eppendorf, Hamburg, GER).
- 1.5 mL tubes (Eppendorf, Hamburg, GER).
- 15 mL and/or 50 mL centrifuge tube (Sarstedt, Nümbrecht, GER).
- Aspiration system (Vacusafe™, [Integra, Biebertal, GER]).
- Incubator (HERAcell™ 240i [Thermo Fisher Scientific]).
- Centrifuge (Heraeus™ Megafuge™ X1R [Thermo Fisher Scientific, Darmstadt, GER]).

2.4.2 Determination of MSC Concentration and Viability

- Hemocytometer/counting chamber, Neubauer improved (Marienfeld, Königshofen, GER).
- PBS (#L1825, Biochrom, Berlin, GER).
- Incident light microscope (DM1i, Leica, Wetzlar, GER).
- Sterile pipette and pipette tips (Sarstedt, Nümbrecht, GER).
- 0.4% trypan blue solution (Sigma-Aldrich, Hamburg, GER).

2.4.3 Transduction

- hMSCs cultured in growth medium (from Subheading 2.4.1).
- Psuedotyped BV (P2 viral stock).
- T-flasks (Sarstedt, Nümbrecht, GER).
- Growth medium (Dulbecco's MEM supplemented with 10% fetal calf serum and 2 mM L-alanyl-L-glutamine).
- Incubator (HERAcell™ 240i, Thermo Fisher Scientific).
- PBS with Ca^{2+} and Mg^{2+} (Merck Millipore, Burlington, MA, USA).
- Trypsin (Biochrom, Berlin, GER).
- Cytometer (Guava® easyCyte™ 6HT 2 L [Merck Millipore, Darmstadt, GER]).

3 Methods

3.1 Generating Pseudotyped BV

The sequence of the desired gene of interest must be known, and can either be synthesized de novo, or amplified by PCR. The desired target sequence must then be integrated into an acceptor vector. Depending on which BEVS is used, different acceptor vectors are available. To insert the desired gene sequence into the acceptor vector, Golden Gate cloning, Gateway cloning, or classical restriction ligation cloning can be used.

3.2 Production of the Baculovirus on a Bioreactor Scale

The BV is produced in *Sf*-9 insect cells in a stirred tank bioreactor. All methods, such as the bioreactor assembly, the process control, cultivation of *Sf*-9 cells, the initiation of cultivation, the infection of *Sf*-9 cells with baculoviruses/BV, and the quantification of the BV are described in Chap. 8 (*see* **Note 6**).

3.3 Downstream Processing

All experiments are carried out at room temperature (RT), unless stated otherwise.

3.3.1 Virus Harvesting

- Differing from the protocol described in Chap. 8, the BV is the desired product. The time of virus harvest strongly depends on the virus concentration and functionality. Thus, it is necessary to perform a study on time-dependent virus concentrations (vDNA and infectious titer). Refer to the cell viability as an indication for the time of harvest (*see* **Note 7**). Check the viability by methods using flow cytometry or simple counting with the trypan blue method. For reproducible results, counting should be performed in shorter intervals toward the end of cultivation (*see* **Note 8**).
- When not processed immediately, store the viruses under light exclusion at 4 °C, either by covering the container in foil, or by using opaque material. Keep in mind, that the BV tends to aggregate quickly [30]. A rapid purification reduces losses.

3.3.2 Clarification

All buffers must be sterile and filtered at 0.2 μm.

- For filtration, the system must be sterilized. Autoclave all bottles, tubing, and connectors, as well as the Sartopure® PP3 cartridges. Sterilization parameters for the cartridges can be obtained in the manual.
- Place the SARTOFLOW® Slice 200 Benchtop System in a sterile clean bench. Connect Sartopure® PP3 cartridges in series, first 3 μm retention rate, followed by 0.65 μm retention rate.
- Flush the system in total recycling mode (TRM) (*see* **Note 9**) with 500 mL water for 10 min to wet the filters, followed by 500 mL PBS, or other physiological saline, for 10 min for

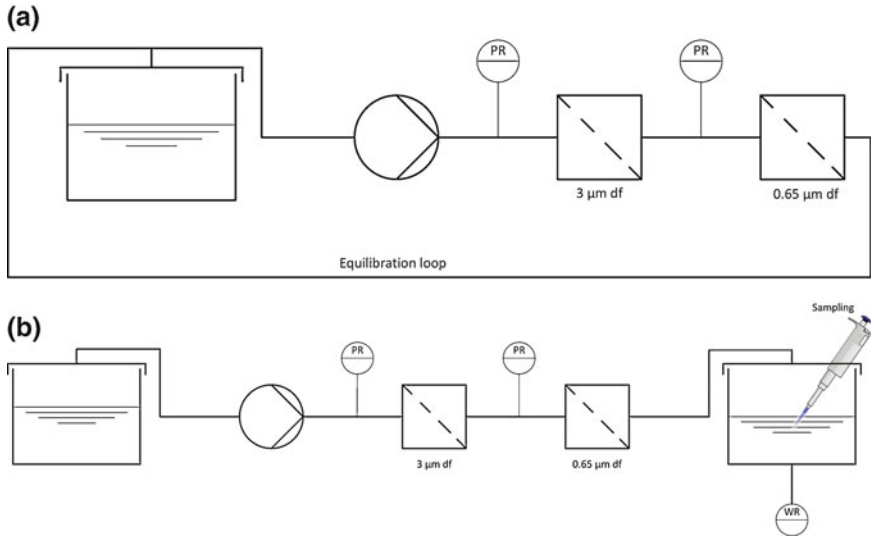

Fig. 1 Process flowchart of serial depth filtration for the BV clarification: (**a**) shows preparation of system in TRM; (**b**) the flow path for the clarification process. Abbreviations: *P* pressure, *R* reading, *W* weight, *df* depth filtration

equilibration purposes (*see* Fig. 1a). A sterility test should be performed afterward, for example in the cultivation medium or on agar plates. If it is not possible to sterilize all parts in contact with virus containing medium, use 1 M NaOH to sterilize the flow path (as described in the purification subheading 3.3.3).

- If the sterility test is positive, prepare the cell culture broth from the bioreactor. Volumes of up to 3 L were tested for this clarification setup. Under sterile conditions, convey the culture broth from the bioreactor into a sterile bottle, topped with a filter and tubing connector. One method to perform this, is to close the exhaust air vent: Stop the process control, apart from aeration and stirring. Connect the bottle to the probe vent of the bioreactor. Close the exhaust-air-vent, as the overpressure in the reactor, due to aeration, conveys the broth into the bottle. Stop the stirrer when the liquid level falls below the stirrer level and stop the aeration when the liquid level falls below probe inlet. Afterward, do not forget to disconnect the analytical sensors, and sterilize the reactor.
- Prepare the system for filtration by connecting the bottle containing the fresh bioreactor content, and change from TRM to the filtration mode (*see* Fig. 1b). Use a constant flow rate of 150 mL/min for filtration (*see* **Note 10**).
- Take samples (500 µL) from the permeate and the retentate fractions throughout the filtration process, for example following every 150 g of increased weight of permeate, plus one initial feed sample.

- Observe pressure measurements before and after first module (3 μm) (*see* **Note 11**).
- Clean the system by sterilization, either in the autoclave, or by liquids, as described for the regeneration of the Sartocon® Slice 200 cassette (Fig. 1).

3.3.3 Purification

All buffers must be sterile, and filtered at 0.2 μm.

- Place the system in a sterile clean bench.
- Install the Sartocon® Slice 200 cassette in the Sartocon® Slice 200 Holder as described by the manufacturer and, next, in the benchtop filtration system with a torque of 25 Nm. Flush the system with 10 L of water per 0.1 m^2 filter area before initial use (*see* **Note 10**). Afterward, perform an integrity test and determine the clean water flux as described in the manual.
- To prepare the membrane for the filtration runs, several washing steps in TRM are necessary (*see* Fig. 2a). The flow rate should be the same as in the following purification process. First, flush the membrane with 400 mL of sterilized and filtered water in TRM for 30 min. Secondly, sterilize the membrane with 400 mL of 1 M NaOH in TRM for 30 min. Thirdly, flush the membrane again with 400 mL of water in TRM for 10 min. Fourth, flush the membrane with 400 mL of PBS, or other physiological saline, in TRM for 10 min. Before starting the experiments, a sterility test should be done.
- Prepare the sample from the clarification step for filtration by measuring the volume. In the experiments conducted, up to 500 mL were used.
- Start filtration with a constant flow of 150 mL/min (*see* **Note 12**). Operate the system in the recycling mode for 5 min to equilibrate the membrane, followed by the filtration cross-flow mode (*see*Fig. 2b) until the entire sample has been filtered and no air is drawn into the system anymore.
- Take samples (500 μL) throughout the filtration run, e.g., after every 150 g of additional permeate weight, from the permeate and the retentate as well as from an initial feed sample.
- Monitor the differential pressure of filtration module (*see* **Note 13**).
- After filtration, clean the system, as described in the Subheading 3.3.4 of Sartocon® Slice 200 cassettes (Fig. 2).

3.3.4 Regeneration of Sartocon® Slice 200

- Use a flow rate of 500 mL/min. Flush cassette with 300 mL of water in TRM for 5 min in the cross-flow mode as well as back-flush. Flush cassette with 400 mL of 1 M NaOH, heated to

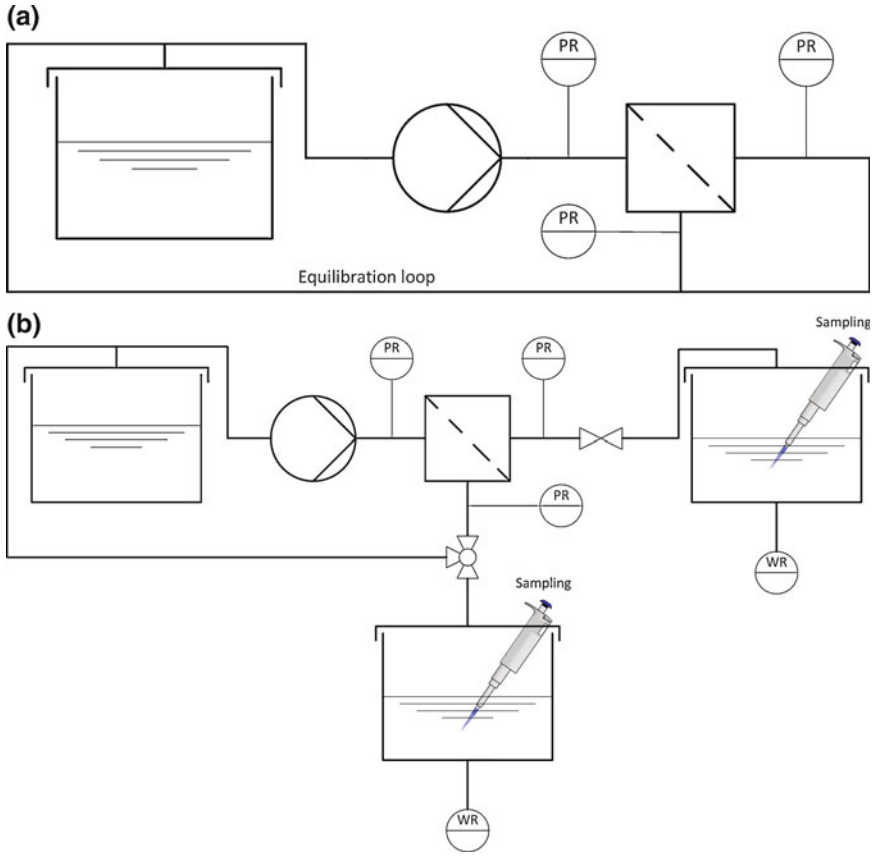

Fig. 2 Process flowchart of TFF for the BV purification: (**a**) shows preparation of system in TRM; (**b**) the flow path for the purification process. Abbreviations: *P* pressure, *R* reading, *W* weight

50 °C, in TRM for 60 min [97]. Afterward, flush with 300 mL of water in TRM for 5 min.

- For a full regeneration, and between every filtration run, store the cassette in 1 M NaOH at 50 °C for 40–48 h in a dry-heat oven [92]. Again, a clean water flux test should be performed, while a 95% regeneration of the initial water flux should be achieved [97].
- If no further runs are performed, store the cassette in 20% ethanol at 4 °C.

3.3.5 Quantification of Contaminants

dsDNA

- Let all reagents warm up to RT. Protect Quant-iT™ PicoGreen™ from light.
- Prepare the buffers as described by the manufacturer. Use the procedure for 96-well plates. Prepare the buffer for the number of samples plus 1–2 additional ones, to compensate losses throughout pipetting. When preparing the TE buffer, consider

the volume needed for dilutions. Usually, a dilution of 1:5 is needed.

- Prepare standards, as specified by the manufacturer (*see* **Note 14**). Prepare enough volume for every new 96-well plate. Standards can be stored at 2–6 °C for several weeks.
- Now, pipet the probes/standard, if necessary, TE buffer for dilution, and last, diluted Quant-iT™ PicoGreen™ in each well. Mix the plate thoroughly on the plate shaker for 30 s. Incubate for 2–5 min, excluded from light. Start reading with plate reader according to the manufacturer's instructions (*see* **Note 15**).

Total Protein

- Prepare standards as described in the manual with BGG or BSA. Standards can be stored at 2–6 °C for several weeks.
- Prepare working reagent (WR) following the manufacturer's instructions for the 96-well plate procedure.
- Pipet probes/standards and add PBS for a dilution into the wells containing the probes. A dilution of 1:10 is necessary for probes containing high loads of protein, for example the supernatant of lysed cells, or medium-containing serum.
- Add WR.
- Now, mix the plate thoroughly on the plate shaker for 30 s. Incubate for 30 min at 37 °C. After letting the plate cool down to RT, start the reading with the plate reader according to the manufacturer's instructions.

Mass Balance Study

- Determine the vDNA content via qPCR and infectious titer of the baculovirus via $TCID_{50}$ or a plaque assay of the feed, permeate for depth filtration, and retentate and permeate for TFF.
- By considering the volume/mass of individual fractions, the vDNA content of the feed is set to be 100%, and fractions should sum up to 100% or less (*see* **Note 16**). The loss of viruses in the membrane is the recovery loss, based on the following equation (*see* **Notes 17** and **18**):
- $$[\mathrm{vDNA}]_{\mathrm{Feed}} = [\mathrm{vDNA}]_{\mathrm{Retentate}} + [\mathrm{vDNA}]_{\mathrm{Permeate}} + [\mathrm{vDNA}]_{\mathrm{Loss}}$$
- Perform an equal balance for the infectious titer. Furthermore, the ratio of infectious viruses to defective virus particles in the feed is of particular interest (*see* **Note 19**). Preferably this value is high (*see* **Notes 20** and **21**).

- Perform a mass balance for the dsDNA content and the total protein content. Again, consider the mass/volume of each fraction. In this case, the reduction of both parameters in the retentate fraction is the value of interest. The higher the reduction, the better (*see* **Note 22**).

3.4 Transduction of hMSC with BV

All steps must be done under sterile conditions.

3.4.1 Thawing and Cultivation of hMSCs

- Add 0.12 mL of growth medium per cm^2 growth area into a T-flask (e.g., 3 mL growth medium in a T25-flask).
- Incubate the T-flasks at 37 °C and 5% CO_2 in an incubator to adjust pH and temperature.
- Thaw the cryovial with MSCs in a water bath (37 °C).
- When the cells have been thawed, pipet the complete cell suspension into the T-flask.
- Incubate the cells at 37 °C and 5% CO_2 in an incubator.
- Monitor the growth microscopically every day.
- If the cells are 80% confluent, remove the cell culture medium with a sterile pipette and an aspiration system.
- Wash the cells twice with 0.3 mL PBS per cm2 growth area (e.g., 7.5 mL of PBS in a T25-flask) (*see* **Note 23**).
- Add 0.012 mL/cm of 0.25% (w/v) trypsin–EDTA at 37 °C (e.g., 0.3 mL of trypsin solution in a T25-flask).
- Incubate for 5 min at 37 °C (*see* **Note 24**).
- Add 0.12 mL/cm of growth medium and mix it by pipetting to get a homogeneous cell suspension.
- Take a 100 µL cell sample in a 1.5 mL tube and determine the cell concentration.
- Centrifuge the cell suspension (300 × *g*, 5 min, RT, in a 15 or 50 mL centrifugation tube).
- Remove and discard the supernatant (to remove residual trypsin).
- Resuspend the pellet in the appropriate volume of a fresh prewarmed complete growth medium.

3.4.2 Determination of hMSC Concentration and Viability

- Use the manufacturer's instructions to prepare the Neubauer counting chamber.
- Take the 100 µL cell sample from your culture in a 1.5 mL tube.
- Dilute the cell suspension with PBS, if necessary (*see* **Note 25**).
- Mix 50 µL of cell suspension with 50 µL trypan blue solution (*see* **Note 26**).

- Rapidly transfer the stained cells to the prepared Neubauer counting chamber and count the cells using the inverse light microscope.
- Calculate the cell concentration and viability according to the manufacturer's instructions.

3.4.3 Transduction of hMSC with BV

- Plate 30,000 hMSCs/cm^2 in a T25 flask in the growth medium.
- Incubate cells overnight at 37 °C and 5% CO_2 in an incubator before proceeding to the transduction (*see* **Note 27**).
- Mix the pseudotyped BV (P2 viral stock) solution several times by inversion to ensure a homogeneous solution (*see* **Note 28**).
- Add the calculated volume of the pseudotyped BV (P2 viral stock) solution directly to the cells in the complete growth medium (*see* **Note 29**).
- Return the cells into the incubator and the incubate cells overnight at 37 °C and 5% CO_2.
- Detect your protein of interest or your reporter protein 16–48 h after transduction (e.g., GFP via fluorescence microscopy or flow cytometry).

4 Notes

1. www.clinicaltrial.gov; last checked 29. August 2019; Search: "*gene therapy*"; Recruitment status: "*recruiting; enrolling by invitation; active, not recruiting; completed*".
2. Quantification of residual DNA is performed using dyes that bind to double-stranded nucleic acids. As the baculovirus is a DNA virus, no discrimination between free viral DNA (vDNA) and host cell DNA (hcDNA) can be accomplished.
3. Instead of the BCA Assay, a Bradford Assay can be used. The BCA Assay is more sensitive, as it relies on a protein-copper chelation, measured by the detection of reduced copper. The Bradford Assay, on the contrary, is measured by a color shift due to protein-dye binding.
4. The baculovirus/BV is very shear-sensitive [98].
5. Different membranes may be used. PES membranes with a MWCO of 300 kDa have proven to be suitable for baculovirus purification [92]. Depending on alterations in the process (e.g., genetic modification or cell line), different materials may be more appropriate.
6. Store the BV stocks at 4 °C excluded from light, as the virus is very susceptible to photodegradation and aggregation due to

increased temperature [98]. Virus stocks can be used for 6 months before their expression is reduced.

7. Usually, a baculovirus/BV harvest is performed between 50% and 90% of cell viability. Influencing factors are proteases from the cells, an increase in the production of defective interfering particles, an increased protein load-impeding purification processes, as well as the viruses' concentration and functionality.
8. The harvesting procedure should be initiated before the culture enters the stationary phase. Insect cells produce strong viral proteases [99, 100], which lead to a degradation of the virus, and to defective viral particles. The processing of harvested baculoviruses should be performed quickly. Any further cell lysis needs to be evaluated with regard to an increased viral concentration and protein load.
9. This means, that all outgoing volume flows are reunited in the feed tank.
10. The ratio of retentate to permeate flow is approx. 1:1 for the Sartocon® Slice 200,300 kDa cassette.
11. A pressure increase, especially at the beginning, is expected for depth filtration. Keep in mind, that the maximum back pressure of 2 bar for the series connection should not be exceeded.
12. Flow rates may be varied to increase throughput. Desirable are high recovery rates at high filtration rates. The optimization of these two factors depends on the permeate flux, the capacity of the module, and the yield for the target particles. The permeate flux is limited by the retention of particles by the membrane, which build a polarization boundary layer. Additionally, the flux and the membrane capacity are impaired by membrane fouling [101]. Fouling is detectable as a rapid flux decline, followed by steady-state conditions [102]. Fouling is a major problem when filtering biological feeds, due to strongly heterogeneous components, which cause pore clogging and filter cakes. The amount of cell debris and proteins can be reduced by optimized cell harvesting and cell lysis. For this reason, the alignment of upstream and downstream procedures is very important.
13. A typical pressure gradient is described as follows [101]. At the beginning of the filtration process, the flux declines rapidly, due to fouling. Fouling pool mechanisms are pore clogging, pore constriction, and cake formation. After this initial decline, an extended period of a quasi-steady-state can be observed. Here, fouling and cleaning by crossflow are in balance. Due to the concentration of the virus in the feed, viscosity increases, which in turn leads to a persisting pressure increase.

14. Usually the standard curve for high concentrations is sufficient. In case of doubt, prepare both standards.
15. Depending on the plate reader, the fluorescence intensity should be adjusted to the highest standard available.
16. Usually, the standard error for a qPCR analysis makes mass balances difficult, 80–120% are still in a normal range.
17. In some cases, sums exceed 100%. This can have different reasons. (1) Check the interfering substances in the manual of the DNA extraction as well as the qPCR kit. Some chemicals, such as salts, may interfere with the assay, and result in higher or lower apparent concentrations. (2) Check the linearity of concentration-dependent qPCR measurements. Sometimes, very low or very high concentrations can lead to a big relative error.
18. In other cases, recovery rates are constantly low. In this case, the reasons are due to very complex interactions. (1) By modifying viruses, as done in pseudotyping, size and surface properties may change as well, which leads to altered adsorptive properties. An evaluation of membrane material and MWCO should be executed carefully in static and dynamic experiments. (2) Low recovery rates in the clarification process may indicate that cell lysis is not optimal. Hence, virus particles still adhere to cell debris, and are excluded in the filtration process. (3) Shear forces may be too high and the virus is lost.
19. Depending on the regeneration of the virus, values should be expressed in log-scale differences.
20. This value can indicate whether the point of harvesting and the cell lysis are optimal. Some viruses need to bud to fully assemble and become infectious, whereas infectious baculoviruses are formed in the cell and by budding. Furthermore, released viruses, by budding or lysis, may decay in the cultivation broth due to heat, UV radiation, or more complex processes.
21. If this ratio decreases due to an increased passaging, reasons could be sought in the presence of defective interfering particles.
22. If the reduction of dsDNA and total protein is not sufficient for the desired application, different approaches can be performed. Either a nuclease treatment, to reduce the size of DNA fragments and to facilitate DNA reduction, or a subsequent process step such as a chromatographic method can be used.
23. Wash cells very gently, so as to not detach them.
24. Verify the detachment of the cells with an incident light microscope. If the cells are not round, and still stick to the surface, increase the detachment time.

25. If you count more than 300 cells, then a dilution is recommended. If you count less than 100 cells, the cell suspension must be concentrated (e.g., by centrifugation and resuspension of the remaining pellet in a smaller volume).
26. Trypan blue is toxic. Wear protective gloves, clothing, eye- and face protection, when working with trypan blue.
27. Cells should be between 70% and 80% confluent to achieve an optimal transduction.
28. Vortexing is not recommended.
29. If the virus titer is not determined, as rule of thumb the required volume of the pseudotyped BV (P2 viral stock) solution is 5% (v/v) of the cell culture medium (e.g., 1 mL virus solution per 20 mL cell culture medium).

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References

1. Ginn SL, Amaya AK, Alexander IE et al (2018) Gene therapy clinical trials worldwide to 2017: an update. J Gene Med 20(5): e3015. https://doi.org/10.1002/jgm.3015
2. Wirth T, Parker N, Ylä-Herttuala S (2013) History of gene therapy. Gene 525 (2):162–169. https://doi.org/10.1016/j.gene.2013.03.137
3. Gardlík R, Pálffy R, Hodosy J et al (2005) Vectors and delivery systems in gene therapy. Med Sci Monit 11(4):RA110–RA121
4. Ibraheem D, Elaissari A, Fessi H (2014) Gene therapy and DNA delivery systems. Int J Pharm 459(1–2):70–83. https://doi.org/10.1016/j.ijpharm.2013.11.041
5. Somia N, Verma IM (2000) Gene therapy: trials and tribulations. Nat Rev Genet 1 (2):91–99. https://doi.org/10.1038/35038533
6. Wold WSM, Toth K (2013) Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. Curr Gene Ther 13 (6):421–433
7. Naso MF, Tomkowicz B, Perry WL et al (2017) Adeno-associated virus (AAV) as a vector for gene therapy. BioDrugs 31 (4):317–334. https://doi.org/10.1007/s40259-017-0234-5
8. Yi Y, Jong Noh M, Hee Lee K (2011) Current advances in retroviral gene therapy. Curr Gene Ther 11(3):218–228. https://doi.org/10.2174/156652311795684740
9. Milone MC, O'Doherty U (2018) Clinical use of lentiviral vectors. Leukemia 32 (7):1529–1541. https://doi.org/10.1038/s41375-018-0106-0
10. Merten O-W, Gaillet B (2016) Viral vectors for gene therapy and gene modification approaches. Biochem Eng J 108:98–115. https://doi.org/10.1016/j.bej.2015.09.005
11. Ayres MD, Howard SC, Kuzio J et al (1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202(2):586–605. https://doi.org/10.1006/viro.1994.1380

12. Vail PV, Sutter G, Jay DL et al (1971) Reciprocal infectivity of nuclear polyhedrosis viruses of the cabbage looper and alfalfa looper. J Invertebr Pathol 17(3):383–388. https://doi.org/10.1016/0022-2011(71)90013-9
13. Summers MD (2006) Milestones leading to the genetic engineering of baculoviruses as expression vector systems and viral pesticides. In: Bonning BC (ed) Insect viruses: biotechnological applications, vol 68. Elsevier Academic Press, Amsterdam, pp 3–73
14. Cox MMJ, Hashimoto Y (2011) A fast track influenza virus vaccine produced in insect cells. J Invertebr Pathol 107(Suppl):S31–S41. https://doi.org/10.1016/j.jip.2011.05.003
15. Mena JA, Kamen AA (2011) Insect cell technology is a versatile and robust vaccine manufacturing platform. Expert Rev Vaccines 10(7):1063–1081. https://doi.org/10.1586/erv.11.24
16. Lin S-Y, Chung Y-C, Hu Y-C (2014) Update on baculovirus as an expression and/or delivery vehicle for vaccine antigens. Expert Rev Vaccines 13(12):1501–1521. https://doi.org/10.1586/14760584.2014.951637
17. Hofmann C, Sandig V, Jennings G et al (1995) Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc Natl Acad Sci U S A 92(22):10099–10103. https://doi.org/10.1073/pnas.92.22.10099
18. Chen C-Y, Lin C-Y, Chen G-Y et al (2011) Baculovirus as a gene delivery vector: recent understandings of molecular alterations in transduced cells and latest applications. Biotechnol Adv 29(6):618–631. https://doi.org/10.1016/j.biotechadv.2011.04.004
19. Cheng T, Xu C-Y, Wang Y-B et al (2004) A rapid and efficient method to express target genes in mammalian cells by baculovirus. World J Gastroenterol 10(11):1612–1618. https://doi.org/10.3748/wjg.v10.i11.1612
20. Condreay JP, Witherspoon SM, Clay WC et al (1999) Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. Proc Natl Acad Sci U S A 96(1):127–132. https://doi.org/10.1073/pnas.96.1.127
21. Kost TA, Condreay JP (2002) Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol 20(4):173–180
22. Hu Y-C (2008) Baculoviral vectors for gene delivery: a review. Curr Gene Ther 8(1):54–65
23. Cheshenko N, Krougliak N, Eisensmith RC et al (2001) A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. Gene Ther 8(11):846–854. https://doi.org/10.1038/sj.gt.3301459
24. Kost TA, Condreay JP, Jarvis DL (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat Biotechnol 23(5):567–575. https://doi.org/10.1038/nbt1095
25. Wang K-C, Wu J-C, Chung Y-C et al (2005) Baculovirus as a highly efficient gene delivery vector for the expression of hepatitis delta virus antigens in mammalian cells. Biotechnol Bioeng 89(4):464–473. https://doi.org/10.1002/bit.20385
26. Merrihew RV, Clay WC, Condreay JP et al (2001) Chromosomal integration of transduced recombinant baculovirus DNA in mammalian cells. J Virol 75(2):903–909. https://doi.org/10.1128/JVI.75.2.903-909.2001
27. Luo W-Y, Lin S-Y, Lo K-W et al (2013) Adaptive immune responses elicited by baculovirus and impacts on subsequent transgene expression in vivo. J Virol 87(9):4965–4973. https://doi.org/10.1128/JVI.03510-12
28. Strauss R, Hüser A, Ni S et al (2007) Baculovirus-based vaccination vectors allow for efficient induction of immune responses against *Plasmodium falciparum* circumsporozoite protein. Mol Ther 15(1):193–202. https://doi.org/10.1038/sj.mt.6300008
29. Murhammer DW (ed) (2007) Baculovirus and insect cell expression protocols, Methods in molecular biology, vol 388, 2nd edn. Humana Press, Totowa, NJ
30. Jorio H, Tran R, Kamen A (2006) Stability of serum-free and purified baculovirus stocks under various storage conditions. Biotechnol Prog 22(1):319–325. https://doi.org/10.1021/bp050218v
31. Pan Y, Zhao Q, Fang L et al (2009) Efficient gene delivery into mammalian cells by recombinant baculovirus containing a hybrid cytomegalovirus promoter/Semliki Forest virus replicon. J Gene Med 11(11):1030–1038. https://doi.org/10.1002/jgm.1390
32. Tani H, Nishijima M, Ushijima H et al (2001) Characterization of cell-surface determinants important for baculovirus infection. Virology 279(1):343–353. https://doi.org/10.1006/viro.2000.0699
33. Mansouri M, Bellon-Echeverria I, Rizk A et al (2016) Highly efficient baculovirus-mediated multigene delivery in primary cells. Nat

Commun 7:11529. https://doi.org/10.1038/ncomms11529

34. Barsoum J, Brown R, McKee M et al (1997) Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Hum Gene Ther 8(17):2011–2018. https://doi.org/10.1089/hum.1997.8.17-2011
35. Li Y, Yang Y, Wang S (2005) Neuronal gene transfer by baculovirus-derived vectors accommodating a neurone-specific promoter. Exp Physiol 90(1):39–44. https://doi.org/10.1113/expphysiol.2004.028217
36. Wang CY, Wang S (2006) Astrocytic expression of transgene in the rat brain mediated by baculovirus vectors containing an astrocyte-specific promoter. Gene Ther 13(20):1447–1456. https://doi.org/10.1038/sj.gt.3302771
37. Boyce FM, Bucher NL (1996) Baculovirus-mediated gene transfer into mammalian cells. Proc Natl Acad Sci U S A 93(6):2348–2352. https://doi.org/10.1073/pnas.93.6.2348
38. Shoji I, Aizaki H, Tani H et al (1997) Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J Gen Virol 78(Pt 10):2657–2664. https://doi.org/10.1099/0022-1317-78-10-2657
39. Mähönen AJ, Airenne KJ, Purola S et al (2007) Post-transcriptional regulatory element boosts baculovirus-mediated gene expression in vertebrate cells. J Biotechnol 131(1):1–8. https://doi.org/10.1016/j.jbiotec.2007.05.022
40. Ang WX, Zhao Y, Kwang T et al (2016) Local immune stimulation by Intravesical instillation of baculovirus to enable bladder cancer therapy. Sci Rep 6:27455. https://doi.org/10.1038/srep27455
41. Lykhova AA, Kudryavets YI, Strokovska LI et al (2015) Suppression of proliferation, tumorigenicity and metastasis of lung cancer cells after their transduction by interferon-beta gene in baculovirus vector. Cytokine 71(2):318–326. https://doi.org/10.1016/j.cyto.2014.10.029
42. Swift SL, Rivera GC, Dussupt V et al (2013) Evaluating baculovirus as a vector for human prostate cancer gene therapy. PLoS One 8(6):e65557. https://doi.org/10.1371/journal.pone.0065557
43. Wang C-Y, Li F, Yang Y et al (2006) Recombinant baculovirus containing the diphtheria toxin A gene for malignant glioma therapy. Cancer Res 66(11):5798–5806. https://doi.org/10.1158/0008-5472.CAN-05-4514
44. Lin C-Y, Chang Y-H, Kao C-Y et al (2012) Augmented healing of critical-size calvarial defects by baculovirus-engineered MSCs that persistently express growth factors. Biomaterials 33(14):3682–3692. https://doi.org/10.1016/j.biomaterials.2012.02.007
45. Lin C-Y, Lin K-J, Kao C-Y et al (2011) The role of adipose-derived stem cells engineered with the persistently expressing hybrid baculovirus in the healing of massive bone defects. Biomaterials 32(27):6505–6514. https://doi.org/10.1016/j.biomaterials.2011.05.059
46. Lin C-Y, Lin K-J, Li K-C et al (2012) Immune responses during healing of massive segmental femoral bone defects mediated by hybrid baculovirus-engineered ASCs. Biomaterials 33(30):7422–7434. https://doi.org/10.1016/j.biomaterials.2012.06.083
47. Lu C-H, Yeh T-S, Yeh C-L et al (2014) Regenerating cartilages by engineered ASCs: prolonged TGF-β3/BMP-6 expression improved articular cartilage formation and restored zonal structure. Mol Ther 22(1):186–195. https://doi.org/10.1038/mt.2013.165
48. Hu Y-C, Yao K, Wu T-Y (2008) Baculovirus as an expression and/or delivery vehicle for vaccine antigens. Expert Rev Vaccines 7(3):363–371. https://doi.org/10.1586/14760584.7.3.363
49. Lu H-Y, Chen Y-H, Liu H-J (2012) Baculovirus as a vaccine vector. Bioengineered 3(5):271–274. https://doi.org/10.4161/bioe.20679
50. Madhan S, Prabakaran M, Kwang J (2010) Baculovirus as vaccine vectors. Curr Gene Ther 10(3):201–213
51. Yong S-B, Chung JY, Song Y et al (2018) Recent challenges and advances in genetically-engineered cell therapy. J Pharm Investig 48(2):199–208. https://doi.org/10.1007/s40005-017-0381-1
52. Castro-Manrreza ME, Montesinos JJ (2015) Immunoregulation by mesenchymal stem cells: biological aspects and clinical applications. J Immunol Res 2015:394917. https://doi.org/10.1155/2015/394917
53. Andrzejewska A, Lukomska B, Janowski M (2019) Concise review: mesenchymal stem cells: from roots to boost. Stem Cells 37(7):855–864. https://doi.org/10.1002/stem.3016
54. Brown C, McKee C, Bakshi S et al (2019) Mesenchymal stem cells: cell therapy and regeneration potential. J Tissue Eng Regen

Med 13(9):1738–1755. https://doi.org/10.1002/term.2914

55. Yu Y, Liao L, Shao B et al (2017) Knockdown of microRNA let-7a improves the functionality of bone marrow-derived mesenchymal stem cells in immunotherapy. Mol Ther 25 (2):480–493. https://doi.org/10.1016/j.ymthe.2016.11.015
56. Huang B, Jiang X-C, Zhang T-Y et al (2017) Peptide modified mesenchymal stem cells as targeting delivery system transfected with miR-133b for the treatment of cerebral ischemia. Int J Pharm 531(1):90–100. https://doi.org/10.1016/j.ijpharm.2017.08.073
57. Balmayor ER, Geiger JP, Koch C et al (2017) Modified mRNA for BMP-2 in combination with biomaterials serves as a transcript-activated matrix for effectively inducing osteogenic pathways in stem cells. Stem Cells Dev 26(1):25–34. https://doi.org/10.1089/scd.2016.0171
58. Sprick G, Weidner T, Salzig D et al (2017) Baculovirus-induced recombinant protein expression in human mesenchymal stromal stem cells: a promoter study. N Biotechnol 39(Pt B):161–166. https://doi.org/10.1016/j.nbt.2017.08.006
59. Brooks AR, Harkins RN, Wang P et al (2004) Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. J Gene Med 6(4):395–404. https://doi.org/10.1002/jgm.516
60. Meilinger D, Fellinger K, Bultmann S et al (2009) Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. EMBO Rep 10(11):1259–1264. https://doi.org/10.1038/embor.2009.201
61. Wang J, Zhu L, Chen X et al (2019) Human bone marrow mesenchymal stem cells functionalized by hybrid baculovirus-adeno-associated viral vectors for targeting hypopharyngeal carcinoma. Stem Cells Dev 28(8):543–553. https://doi.org/10.1089/scd.2018.0252
62. Contreras-Gómez A, Sánchez-Mirón A, García-Camacho F et al (2014) Protein production using the baculovirus-insect cell expression system. Biotechnol Prog 30 (1):1–18. https://doi.org/10.1002/btpr.1842
63. Maranga L, Cruz PE, Aunins JG et al (2002) Production of core and virus-like particles with baculovirus infected insect cells. In: Schügerl K, Aunins JG (eds) Tools and applications of biochemical engineering science, vol 74. Springer, Berlin, pp 183–206
64. Pijlman GP, van den Born E, Martens DE et al (2001) *Autographa californica* baculoviruses with large genomic deletions are rapidly generated in infected insect cells. Virology 283 (1):132–138. https://doi.org/10.1006/viro.2001.0854
65. Aucoin MG, Mena JA, Kamen AA (2010) Bioprocessing of baculovirus vectors: a review. Curr Gene Ther 10(3):174–186
66. Klöppinger M, Fertig G, Fraune E et al (1990) Multistage production of *Autographa californica* nuclear polyhedrosis virus in insect cell cultures. Cytotechnology 4(3):271–278. https://doi.org/10.1007/bf00563787
67. Zwart MP, Erro E, van Oers MM et al (2008) Low multiplicity of infection in vivo results in purifying selection against baculovirus deletion mutants. J Gen Virol 89 (Pt 5):1220–1224. https://doi.org/10.1099/vir.0.83645-0
68. Wickham TJ, Davis T, Granados RR et al (1991) Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. Biotechnol Lett 13 (7):483–488. https://doi.org/10.1007/BF01049204
69. Kwang TW, Zeng X, Wang S (2016) Manufacturing of AcMNPV baculovirus vectors to enable gene therapy trials. Mol Ther Methods Clin Dev 3:15050. https://doi.org/10.1038/mtm.2015.50
70. Wolff MW, Reichl U (2011) Downstream processing of cell culture-derived virus particles. Expert Rev Vaccines 10(10):1451–1475. https://doi.org/10.1586/erv.11.111
71. Vicente T, Peixoto C, Carrondo MJT et al (2009) Purification of recombinant baculoviruses for gene therapy using membrane processes. Gene Ther 16(6):766–775. https://doi.org/10.1038/gt.2009.33
72. Vicente T, Roldão A, Peixoto C et al (2011) Large-scale production and purification of VLP-based vaccines. J Invertebr Pathol 107 (Suppl):S42–S48. https://doi.org/10.1016/j.jip.2011.05.004
73. Ungerechts G, Bossow S, Leuchs B et al (2016) Moving oncolytic viruses into the clinic: clinical-grade production, purification, and characterization of diverse oncolytic viruses. Mol Ther Methods Clin Dev 3:16018. https://doi.org/10.1038/mtm.2016.18

74. EMA (1997) CPMP position statement on DNAa nd host cell proteins (HCP) impurities, routine testing versus validation studies
75. Champion K, Madden H, Dougherty J et al (2005) Defining your product profile and maintaining control over it, part 2: challenges of monitoring host cell protein impurities
76. Wright JF (2014) Product-related impurities in clinical-grade recombinant AAV vectors: characterization and risk assessment. Biomedicine 2(1):80–97. https://doi.org/10.3390/biomedicines2010080
77. Kramberger P, Urbas L, Štrancar A (2015) Downstream processing and chromatography based analytical methods for production of vaccines, gene therapy vectors, and bacteriophages. Hum Vaccin Immunother 11 (4):1010–1021. https://doi.org/10.1080/21645515.2015.1009817
78. FDA (2018) Chemistry, Manufacturing, and Control (CMC) information for human gene therapy Investigational New Drug Applications (INDs): Draft Guidance for Industry
79. Roldão A, Vicente T, Peixoto C et al (2011) Quality control and analytical methods for baculovirus-based products. J Invertebr Pathol 107(Suppl):S94–S105. https://doi.org/10.1016/j.jip.2011.05.009
80. Zitzmann J, Sprick G, Weidner T et al (2017) Process optimization for recombinant protein expression in insect cells. In: Gowder SJT (ed) New insights into cell culture technology. InTechOpen, Rijeka, Croatia
81. Hoffmann D, Leber J, Loewe D et al (2019) Purification of new biologicals using membrane-based processes. In: Basile A, Charcosset C (eds) Current trends and future developments on (bio-) membranes: membrane processes in the pharmaceutical and biotechnological field. Elsevier, Amsterdam, NL, Oxford/Cambridge, USA, pp 123–150
82. Transfiguracion J, Jorio H, Meghrous J et al (2007) High yield purification of functional baculovirus vectors by size exclusion chromatography. J Virol Methods 142(1–2):21–28. https://doi.org/10.1016/j.jviromet.2007.01.002
83. Barsoum J (1999) Concentration of recombinant baculovirus by cation-exchange chromatography. Biotechniques 26(5):834–836, 838, 840. https://doi.org/10.2144/99265bm07
84. Grein TA, Michalsky R, Vega López M et al (2012) Purification of a recombinant baculovirus of *Autographa californica* M nucleopolyhedrovirus by ion exchange membrane chromatography. J Virol Methods 183 (2):117–124. https://doi.org/10.1016/j.jviromet.2012.03.031
85. Wu C, Soh KY, Wang S (2007) Ion-exchange membrane chromatography method for rapid and efficient purification of recombinant baculovirus and baculovirus gp64 protein. Hum Gene Ther 18(7):665–672. https://doi.org/10.1089/hum.2007.020
86. Vicente T, Peixoto C, Alves PM et al (2010) Modeling electrostatic interactions of baculovirus vectors for ion-exchange process development. J Chromatogr A 1217 (24):3754–3764. https://doi.org/10.1016/j.chroma.2010.03.059
87. Chen G-Y, Chen C-Y, Chang MD-T et al (2009) Concanavalin A affinity chromatography for efficient baculovirus purification. Biotechnol Prog 25(6):1669–1677. https://doi.org/10.1002/btpr.253
88. Nasimuzzaman M, Lynn D, van der Loo JC et al (2016) Purification of baculovirus vectors using heparin affinity chromatography. Mol Ther Methods Clin Dev 3:16071. https://doi.org/10.1038/mtm.2016.71
89. Nasimuzzaman M, van der Loo JCM, Malik P (2018) Production and purification of baculovirus for gene therapy application. J Vis Exp (134):57019. https://doi.org/10.3791/57019
90. Kaikkonen MU, Viholainen JI, Närvänen A et al (2008) Targeting and purification of metabolically biotinylated baculovirus. Hum Gene Ther 19(6):589–600. https://doi.org/10.1089/hum.2007.177
91. Lothert K, Sprick G, Beyer F et al (2020) Membrane-based steric exclusion chromatography for the purification of a recombinant baculovirus and its application for cell therapy. J Virol Methods 275:113756. https://doi.org/10.1016/j.jviromet.2019.113756
92. Michalsky R, Passarelli AL, Pfromm PH et al (2009) Purification of the baculovirus *Autographa californica* M nucleopolyhedrovirus by tangential flow ultrafiltration. Desalination 245(1–3):694–700. https://doi.org/10.1016/j.desal.2009.02.039
93. Michalsky R, Passarelli AL, Pfromm PH et al (2010) Concentration of the baculovirus *Autographa californica* M nucleopolyhedrovirus (AcMNPV) by ultrafiltration. Desalination 250(3):1125–1127. https://doi.org/10.1016/j.desal.2009.09.123
94. Nestola P, Peixoto C, Silva RRJS et al (2015) Improved virus purification processes for vaccines and gene therapy. Biotechnol Bioeng 112(5):843–857. https://doi.org/10.1002/bit.25545

95. Prashad M, Tarrach K (2006) Depth filtration: cell clarification of bioreactor offloads. Filtr Separat 43(7):28–30. https://doi.org/10.1016/S0015-1882(06)70950-8
96. Kalbfuss B, Genzel Y, Wolff M et al (2007) Harvesting and concentration of human influenza A virus produced in serum-free mammalian cell culture for the production of vaccines. Biotechnol Bioeng 97(1):73–85. https://doi.org/10.1002/bit.21139
97. Grzenia DL, Carlson JO, Czermak P et al (2006) Purification of densonucleosis virus by tangential flow ultrafiltration. Biotechnol Prog 22(5):1346–1353. https://doi.org/10.1021/bp060077c
98. Michalsky R, Pfromm PH, Czermak P et al (2008) Effects of temperature and shear force on infectivity of the baculovirus *Autographa californica* M nucleopolyhedrovirus. J Virol Methods 153(2):90–96. https://doi.org/10.1016/j.jviromet.2008.07.030
99. Ikonomou L, Peeters-Joris C, Schneider Y-J et al (2002) Supernatant proteolytic activities of high-five insect cells grown in serum-free culture. Biotechnol Lett 24(12):965–969. https://doi.org/10.1023/A:1015692323167
100. Naggie S, Hu YC, Pulliam-Holoman TR et al (1997) Substrate (gelatin) gel electrophoretic method for analysis of protease activity in insect (Sf-9) cells. Biotechnol Tech 11(5):297–300. https://doi.org/10.1023/A:1018411210412
101. Czermak P, Nehring D, Wickramasinghe R (2007) Membrane filtration in animal cell culture. In: Walker JM, Pörtner R (eds) Animal cell biotechnology, vol 24. Humana Press, Totowa, NJ, pp 397–420
102. Belfort G, Davis RH, Zydney AL (1994) The behavior of suspensions and macromolecular solutions in crossflow microfiltration. J Membrane Sci 96(1–2):1–58. https://doi.org/10.1016/0376-7388(94)00119-7

Chapter 20

Lipofection-Based Delivery of DNA Vaccines

Monika Rak, Anna Góra-Sochacka, and Zbigniew Madeja

Abstract

The preventive and therapeutic potential of DNA vaccines combined with benefits of lipid-based delivery (lipofection) allow efficient nucleic acid transfer and immunization applicable in treatment of infections, cancer or autoimmune disorders. Lipofecting compositions consisting of cationic and neutral lipids can be used for both in vitro and in vivo applications and may also play the role of adjuvants. Here we describe a simple protocol of DNA vaccine carrier preparation based on cationic polyprenyl derivatives (PTAI—trimethylpolyprenylammonium iodides) and commonly used helper lipids with use of basic laboratory equipment. Such formulas have proven effective for immunization of animals as well as for cell transfection.

Key words Nonviral gene delivery, Lipofection, Lipid-based vaccine carriers, Polyprenyl-based vaccines, PTAI

1 Introduction

Wide preventive and therapeutic potential of DNA vaccines includes viral, bacterial, and parasitic diseases as well as cancer, autoimmune diseases and allergies. DNA biocompatibility, cost-efficient production, and good long-term storage properties are their most important advantages. Moreover, nucleic acids may also serve as vaccine adjuvants [1]. However, safety issues [2] and poor immunogenicity in humans [3] are major concerns. Lipid-based solutions have been employed to address these drawbacks for years [4–8] and still continue to be useful not only in DNA [9] but also in RNA [10] settings.

Lipofection can be easily modified to meet case-specific requirements via lipid composition and preparation [11, 12]. Lipofection strategy is based on cationic lipids that ensure spontaneous interactions with negatively charged nucleic acids to form complexes called lipoplexes (*see* Fig. 1). Additional cationic and neutral lipids (also designated as helper or co-lipids) modulate properties of lipoplexes. DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanola-

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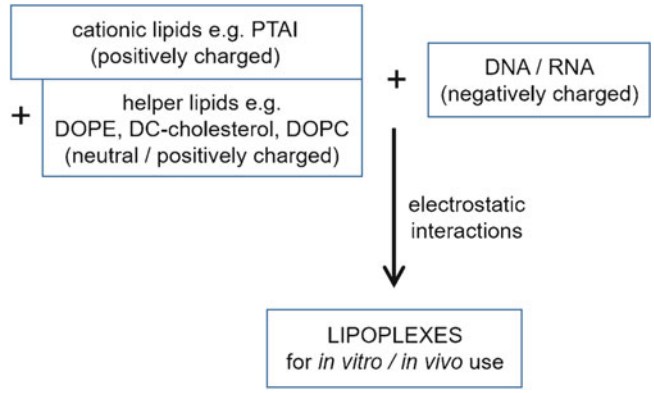

Fig. 1 Scheme of lipoplexes preparation. *PTAI* trimethylpolyprenylammonium iodides, *DOPE* 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, *DC-cholesterol* 3*ß*-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride, *DOPC* 1,2-dioleoyl-*sn*-glycero-3-phosphocholine

mine), as a helper lipid, promotes formation of more fusogenic inverted hexagonal structures, facilitating endosomal escape of nucleic acids [13, 14]. Additional implementation of DC-cholesterol (3*ß*-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride) or DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) may improve serum resistance and shelf life of compositions.

Here, we demonstrate a simple and convenient method of lipid-based vaccine carrier preparation and activity testing, utilizing only basic easily accessible laboratory equipment. The method is based on lipofecting activity of semisynthetic cationic forms of plant polyprenols (trimethylpolyprenylammonium iodides; PTAI; Fig. 2a) [15, 16] that were shown to be nontoxic [17, 18] and efficient in vaccine delivery [9]. These results also inspired synthesis of other cationic polyprenyl derivatives designed for practical applications in liposomology [19].

The protocol for preparation of PTAI-based vaccine carriers consists of dissolution of lipids in ethanol, mixing and vortexing of all lipid components in ethanol, and its subsequent mixing and vortexing with aqueous solution. This simple procedure results in preparation of stable reagents that can be used with DNA or RNA both in vitro and in vivo. This composition can be easily modified by users with different cationic and neutral lipids and other substances like poly-L-lysine or polyethylene glycol (PEG) for specific applications.

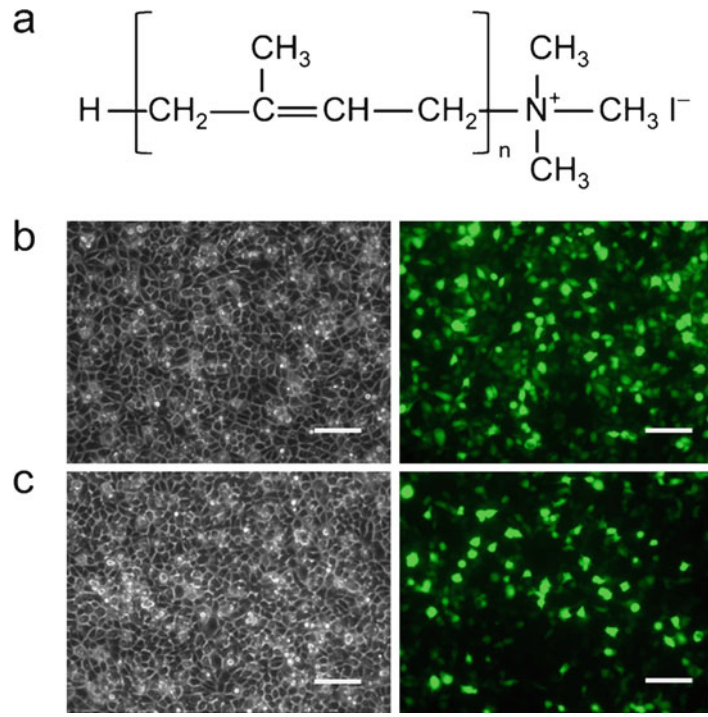

Fig. 2 Structure and efficiency of trimethylpolyprenylammonium iodides (PTAI) as lipofecting mixtures components. (**a**) Structure of PTAI, *n* corresponds to the number of isoprenoid units in the polyprenyl chain; in this protocol $n = 6–8$ or 10–14 or 7 or 11. (**b**, **c**) Efficiency of plasmid DNA (pEGFP-C1 encoding EGFP enhanced green fluorescent protein) transfer into DU145 cells in the absence (**b**) and in the presence of serum (**c**) in DMEM F-12 Ham medium with PTAI-11 + DOPE + DC-cholesterol (1:1:1 molar ratio) reagent. Phase contrast (left panel) and epifluorescence microscopy with FITC filter (right panel) images. Scale bar 100 μm

2 Materials

2.1 Preparation of Lipid-Based Vaccine Carriers

1. Trimethylpolyprenylammonium iodides with chain lengths from 6 to 8 or 10 to 14 isoprene units—PTAI-6-8 or PTAI-10-14, respectively or trimethylpolyprenylammonium iodides with chain lengths of 7 or 11 isoprene units—PTAI-7 or PTAI-11 (*see* **Note 1**). PTAI have been synthesized as described earlier [15]. PTAI and their use are subject of patents no. 230096 (*System of nucleic acid carriers, method for preparation of the system and their applications*), 211824 (*Trimethylamine derivatives of poly-cis and poly-trans line isoprene oligomers, method for their production and use*), 231158 (*Vaccine, pharmaceutical composition, carrier of nucleic acids and other biologically active substances, application of the composition in production of the vaccine and application of cationic derivatives*

of polyprenols PTAI for producing immunomodulating substances), and patent applications no. EP3185893, WO/2016/032348 (*Use of cationic derivatives of polyprenols PTAI in production of immunomodulating substances*), PCT/PL2015/000093 (*Use of cationic derivatives of polyprenols PTAI in production of immunomodulating substances*). All used PTAI were received from the Collection of Polyprenols, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland.

2. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE).
3. 3ß-[*N*-(*N′,N′*-Dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-cholesterol; DC-chol).
4. Ethanol (99%).
5. Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham With L-glutamine, 15 mM HEPES, and sodium bicarbonate, liquid, sterile-filtered, suitable for cell culture (DMEM F-12 Ham).
6. Glass bottles with Teflon seals.
7. Vortex.

2.2 Carriers Activity Testing In Vitro

1. DU145 human prostate cancer cells or XC rat Rous sarcoma cancer cells.
2. Cell culture flasks and dishes, tubes, 24-well cell culture plates.
3. Cell culture incubator.
4. Inverted phase contrast and fluorescence microscope.
5. Centrifuge.
6. Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham With L-glutamine, 15 mM HEPES, and sodium bicarbonate, liquid, sterile-filtered, suitable for cell culture (DMEM F-12 Ham) or Eagle's Minimum Essential Medium (EMEM).
7. Heat-inactivated fetal calf serum (FCS) or fetal bovine serum (FBS).
8. Antibiotics: penicillin, neomycin, streptomycin.
9. Plasmid DNA encoding EGFP enhanced green fluorescent protein (e.g., pEGFP-C1).
10. 0.25% trypsin with 0.02% EDTA.
11. Phosphate buffered saline (PBS).

2.3 Preparation of Vaccine Plasmid

1. *Escherichia coli* strain (e.g., DH5α or XL1-Blue) transformed with vaccine plasmid.
2. LB (Luria–Bertani) medium for bacterial culture propagation.

3. Antibiotics (e.g., ampicillin; working concentration: 20–50 μg/ml), depending on the antibiotic resistance carried by the used vector (*see* **Note 2**).
4. Endotoxin-free plasmid DNA purification Kit which ensures endotoxin level <0.1 EU/μg DNA.
5. PBS, pH 7.4.
6. Culture tubes, flasks, and dishes.
7. 37 °C shaking incubator.
8. Refrigerated centrifuge.
9. Pyrogen- or endotoxin-free plastic tubes and tips.
10. Glassware (*see* **Note 3**).

2.4 Carriers Activity Testing In Vivo

1. Purified vaccine plasmid—recombinant expression vector (e.g., pCI, Promega) carrying cDNA encoding appropriate antigen (e.g., hemagglutinin (HA) from influenza virus).
2. Animals to be vaccinated (e.g., mice, chickens).
3. Syringes with needles, and tubes for blood collection (e.g., 1.5 or 2 ml Eppendorf tubes).
4. 96-well flat-bottom plates: MediSorp Surface (Nunc) for detection of antibodies in chicken sera, MaxiSorp Surface (Nunc) for mouse sera.
5. Antigens for plates coating.
6. Enzyme-labeled anti-host secondary antibodies: goat anti-chicken IgY (Fc-specific)-horseradish peroxidase (HRP) (Pierce/Thermo Scientific), anti-mouse IgG-alkaline phosphatase (AP)-conjugated (Sigma-Aldrich).
7. 3,3′,5,5′-Tetramethylbenzidine (TMB) for HRP-based procedure, P-Nitrophenyl-phosphate (pNPP) for AP-based procedure.
8. PBS, pH 7.4.
9. PBST: 0.05% (v/v) Tween 20 in PBS.
10. 0.1% Tween 20 (v/v) in PBS.
11. Blocking buffer: 2% BSA (v/v) in PBS.
12. 0.5 M H_2SO_4, 3 M NaOH.
13. Sealing tape for 96-well plates (ThermoFisher).
14. Multichannel pipettes.
15. Automated microplate washer (optional).
16. Microplate reader.

3 Methods

3.1 Preparation of Lipid-Based Vaccine Carriers

1. Dissolve PTAI-6-8 (or PTAI-7), PTAI-10-14 (or PTAI-11), DOPE, and DC-cholesterol in ethanol (99%) to achieve concentrations: 20, 10, 25, and 33.3 mg/ml, respectively (*see* **Note 4**).
2. To prepare 1 ml of reagent in a 2 ml glass vial mix:
   (a) 85.80 μl PTAI-6-8 (or PTAI-7) and 51.32 μl DOPE (for reagent A consisting of PTAI-6-8/PTAI-7 + DOPE)—molar ratio of PTAI–DOPE 1.5:1.
   (b) 166.86 μl PTAI-10-14 (or PTAI-11) with 53.14 μl DOPE and 28.76 μl DC-cholesterol (for reagent B consisting of PTAI-10-14/PTAI-11 + DOPE + DC-chol)—molar ratio of PTAI–DOPE–DC-chol 1:1:1.
3. Vortex at the highest vortex settings for 1 min at RT (20–25 °C).
4. Add ethanolic solution of lipids to DMEM F-12 Ham medium (*see* **Note 5**).
   (a) 114.26 μl of lipids to 885.74 μl of medium for reagent A.
   (b) 207.30 μl of lipids to 792.70 μl of medium for reagent B.
5. Vortex at the highest vortex settings for 3 min at RT (20–25 °C).
6. Use immediately or store reagents at 4 °C (*see* **Note 6**).

3.2 Carriers Activity Testing In Vitro

1. Prepare culture media:
   (a) DMEM F-12 Ham (for DU145 cells) or/and EMEM (for XC cells) supplemented with 10% FCS or FBS.
   (b) DMEM F-12 Ham (for DU145 cells) or/and EMEM (for XC cells) supplemented with 20% FCS or FBS and 200 IU/ml penicillin, 20 μg/ml neomycin, and 20 μg/ml streptomycin.
2. Seed DU145 or XC cells into the wells of a 24-well plate at a density of 8×10^4 cell/well in 500 μl of DMEM F-12 Ham (for DU145 cells) or/and EMEM (for XC cells) supplemented with 10% FCS or FBS without antibiotics (*see* **Note 7**).
3. Cultivate cells in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h (to reach 70–80% confluence).
4. After 24 h, on the day of testing, prepare the PTAI-based reagents as described in Subheading 3.1 or take them out of the 4 °C storage conditions in advance. When they reach RT (20–25 °C), they are ready to be used. Vortex the reagents at the highest vortex settings for 3 min at RT (20–25 °C) immediately before use.

5. Prepare lipoplexes. To prepare 500 μl of lipoplexes:
   (a) For reagent A consisting of PTAI-6-8/PTAI-7 + DOPE.
   - Dilute 3 μl of PTAI-6-8/PTAI-7 based reagent prepared in Subheading 3.1 (**step 4(a)**) in 59.5 μl of DMEM F-12 Ham.
   - Add 62.5 μl of plasmid DNA solution at 0.08 μg/μl concentration (diluted in DMEM F-12 Ham).
   - Incubate 30 min at RT (20–25 °C) with gentle rocking.
   - Add 375 μl of DMEM F-12 Ham (for DU145 cells) or EMEM (for XC cells) and mix by gentle pipetting.

   (b) For reagent B consisting of PTAI-10-14/PTAI-11 + DOPE + DC-chol.
   - Dilute 7.5 μl of PTAI-10-14/PTAI-11 based reagent prepared in Subheading 3.1 (**step 4(b)**) in 55 μl of DMEM F-12 Ham.
   - Add 62.5 μl of plasmid DNA solution at 0.2 μg/μl concentration (diluted in DMEM F-12 Ham).
   - Incubate 30 min at RT (20–25 °C) with gentle rocking.
   - Add 375 μl of DMEM F-12 Ham (for DU145 cells) or EMEM (for XC cells) and mix by gentle pipetting.

6. Add lipoplexes to cells (*see* **Note 8**) in serum-free conditions or in the presence of serum:
   - Serum-free lipofection: aspirate medium from cells (optional: wash cells with medium without serum and antibiotics) and add the following:
     (a) For reagent A consisting of PTAI-6-8/PTAI-7 + DOPE.
     - 200 μl of DMEM F-12 Ham (for DU145 cells) or EMEM (for XC cells) and 200 μl of PTAI-6-8/PTAI-7 based lipoplexes prepared in **step 5(a)**.

     (b) For reagent B consisting of PTAI-10-14/PTAI-11 + DOPE + DC-chol.
     - 293.3 μl of DMEM F-12 Ham (for DU145 cells) or EMEM (for XC cells) and 106.7 μl of PTAI-10-14/PTAI-11 based lipoplexes prepared in **step 5(b)**.
   - Lipofection in the presence of serum: aspirate medium from cells partially—leave 200 μl of medium in each well of 24-well plate (*see* **Note 9**) and add the following:
     (c) For reagent A consisting of PTAI-6-8/PTAI-7 + DOPE.
     - 200 μl of PTAI-6-8/PTAI-7 based lipoplexes prepared in **step 5(a)**.

(d) For reagent B consisting of PTAI-10-14/PTAI-11 + DOPE + DC-chol.
- 93.3 μl of DMEM F-12 Ham (for DU145 cells) or EMEM (for XC cells) and 106.7 μl of PTAI-10-14/PTAI-11 based lipoplexes prepared in **step 5 (b)**.

7. After adding lipoplexes, gently slide the plate in a cross-like pattern (forward and backward and side-to-side) or in a figure-eight-like pattern three to four times.
8. Incubate cells at 37 °C with 5% CO_2 for 5 h.
9. Add 400 μl of DMEM F-12 Ham (for DU145 cells) or EMEM (for XC cells) supplemented with 20% FCS or FBS and 200 IU/ml penicillin, 20 μg/ml neomycin, and 20 μg/ml streptomycin.
10. Verify transfection efficiency 24 h after transfection (*see* **Note 10**) by fluorescence microscopy or flow cytometry. Examples of PTAI efficiency as components of lipofecting mixtures used in vitro are shown in Fig. 2b and c.

    Optional: for fluorescent microscopy stain cells with Hoechst 33342 (*see* **Note 11**):

    (a) Aspirate medium from wells of multiwell plate.

    (b) Add Hoechst 33342 solution at 1 μg/ml in PBS.

    (c) Incubate for 10 min at RT (20–25 °C).

    (d) Rinse three times with PBS.

3.3 Preparation of Vaccine Mixtures

1. Prepare plasmid DNA solution in PBS, pH 7.4 at required concentration, (e.g., 2.0 μg/μl).
2. Prepare vaccine mixtures with reagent A consisting of PTAI-6-8/PTAI-7 + DOPE with DMEM F-12 Ham prepared in Subheading 3.1 (**step 4(a)**).

    (a) Prepare one dose of vaccine containing 125 μg DNA in final volume of 160 μl (PTAI–DNA ratio 0.8:1 w/w) as follows:
    - Mix 75 μl of prepared PTAI composition with 85 μl of plasmid DNA solution containing 125 μg DNA (e.g., 62.5 μl plasmid solution at concentration 2 μg/μl + 22.5 μl PBS, pH 7.4).
    - Incubate for 30 min at RT (20–25 °C) with gentle rocking.
3. Prepare vaccine mixtures with reagent B consisting of PTAI-10-14/PTAI-11 + DOPE + DC-chol with DMEM F-12 Ham prepared in Subheading 3.1 (**step 4(b)**).

(a) Prepare one dose of vaccine containing 125 μg DNA in final volume of 160 μl (PTAI–DNA ratio 0.8:1 w/w) as follows:
- Mix 75 μl of prepared PTAI reagent B with 85 μl of plasmid DNA solution containing 125 μg DNA (*see* Subheading 3.3, **step 2(a)**).
- Incubate for 30 min at RT (20–25 °C) with gentle rocking.

(b) Prepare one dose of vaccine containing 62.5 μg DNA in final volume of 160 μl (PTAI–DNA ratio—1.6:1 w/w) as follows:
- Mix 75 μl of prepared PTAI reagent B with 85 μl of plasmid DNA containing 62.5 μg DNA.
- Incubate for 30 min at RT (20–25 °C) with gentle rocking.

(c) Prepare one dose of vaccine containing 62.5 μg DNA in final volume of 100 μl (PTAI–DNA ratio—0.8:1 w/w) as follows:
- Mix 37.5 μl of prepared PTAI reagent B with 62.5 μl of plasmid DNA solution containing 62.5 μg DNA.
- Incubate for 30 min at RT (20–25 °C) with gentle rocking.

(d) Prepare one dose of vaccine containing 10 μg DNA in final volume of 50 μl (PTAI–DNA ratio—0.8:1 w/w) as follows:
- Mix 6.0 μl of prepared PTAI reagent B with 44 μl of plasmid DNA solution containing 10 μg DNA.
- Incubate for 30 min at RT (20–25 °C) with gentle rocking.

3.4 Carriers Activity Testing In Vivo

1. Use vaccine mixtures prepared in Subheading 3.3 (**steps 2(a)** and **3(a)–(d)**) for intramuscular immunization (*see* **Note 12**).
2. Collect blood 1 or 2 weeks after each vaccination and use appropriate test (e.g., ELISA) to evaluate vaccination effectiveness.
3. Obtain blood sera as follows:
   (a) After collection, incubate blood at RT (20–25 °C) for 2 h then at 4 °C overnight.
   (b) Centrifuge for 10 min at 5000 × *g* at 4 °C.
   (c) Take out the serum and store at 4 °C.
4. Evaluate vaccination effectiveness by indirect ELISA assay (*see* **Note 13**).

(a) Dilute the antigen to an appropriate final concentration in PBS; for example, 2 μg/ml is a frequently used concentration for purified recombinant protein. Use the amount of the antigen per microplate well recommended by the supplier, or optimized by yourself.

(b) Coat plate wells by pipetting 50 μl of the prepared antigen solution, and incubate overnight at 4 °C (*see* **Note 14**).

(c) Remove the coating solution and wash plates four times by filling the wells with 300 μl PBST (*see* **Note 15**).

(d) Add 300 μl blocking buffer (2% BSA/PBS) to each well and incubate 90 min at 37 °C.

(e) Wash the plate twice with 300 μl PBST at RT (20–25 °C).

(f) Dilute sera samples (collected after animal immunization) in the blocking solution (2% BSA/PBS). Serum dilution depends on the antibody concentration in your samples and should be optimized; for example, you can begin with 50-, 100-, and 200-fold dilutions.

(g) Add 50 μl of the diluted tested sera to each well and incubate overnight at 4 °C.

(h) Wash plates five times with 300 μl 0.1% Tween/PBS (chicken sera samples) or 0.05% Tween/PBS (mouse sera samples) at RT (20–25 °C).

(i) Add 50 μl of HRP- or AP-conjugated (anti-host) secondary antibody, diluted in 2% BSA/PBS. Use dilution recommended by the manufacturer.

(j) Incubate for 1 h at 37 °C.

(k) Wash the plate five times with PBST at RT (20–25 °C).

(l) Add 50 μl/well of substrate solution: TMB for HRP or pNPP for AP. Incubate at RT (20–25 °C) in the dark for 30 min.

(m) Stop the HRP and AP reactions by adding 50 μl of 0.5 M H_2SO_4 and 3 M NaOH, respectively.

(n) Read the optical density (using a microplate reader) at 450 nm for HRP-based procedure and 405 for AP-based procedure (*see* **Note 16**).

4 Notes

1. PTAI-6-8 or PTAI-10-14 with chain lengths of 6–8 or 10–14 isoprene units, respectively, are derived from the natural mixtures of polyprenols with indicated chain length, whereas PTAI-7 and PTAI-11 are derived from single polyprenols

with a defined length of polyprenyl chain of 7 or 11 isoprene units, respectively. Natural mixtures of polyprenols are easier to obtain (they are isolated from plant material) than single polyprenols (have to be separated from the mixtures) and thus cheaper, while having the same lipofecting efficacy.

2. Bacterial cultures should be grown under antibiotic selection to prevent loss of plasmid.
3. In order to remove endotoxin from glassware, it should be sterilized at 180 °C overnight.
4. PTAI and DOPE should easily dissolve in ethanol at the indicated concentrations at room temperature (RT—20–25 °C). If DOPE is not dissolved completely (e.g., after storage in the freezer), place it in a shaker at 37 °C for 10 min. To dissolve DC-cholesterol, warm up the solution in a water bath to around 50 °C and vortex. Store dissolved lipids in glass bottles with Teflon seals at −20 °C.
5. Mixture of lipids in ethanol should be injected into medium very rapidly and mixed by intense pipetting.
6. Every time before use, reagents should be taken out of the 4 °C storage conditions in advance and brought to RT (20–25 °C). Immediately before use, reagents should be vortexed at the highest vortex settings for 3 min at RT (20–25 °C). When stored at 4 °C, reagents are stable for at least a week. Do not freeze the reagents.
7. To ensure proper confluency and even cell distribution, prepare a homogenous stock of cells in the medium and plate them to every well from this stock solution. After seeding cells, gently slide the plate in a cross-like pattern (forward and backward and side-to-side) or in a figure-eight-like pattern three to four times. If you are planning to perform your experiment in a plate with very small wells (e.g., 48- or 96-wells), you may not transfer the plate to the incubator immediately but leave them under the hood for up to an hour after seeding to let cells attach to the surface. A proper amount of medium (not too small, recommended by the manufacturer) for the dish/well should also be used; for example, for a 24-well plate, it should be not less than around 400 μl/well. This prevents meniscus effect/rings (increased cell density along the sides of the dishes), as the curve of the meniscus along the sides of the vessel is deeper and contains more medium and cells per unit surface area than the media-poor area in the center of the dish/well.
8. It is very important to add medium to cells first and then add lipoplexes. Add lipoplexes dropwise to the whole area of the well. Do not touch the sides of the well when adding lipoplexes.

9. Instead of leaving 200 μl of medium in the wells, you can also aspirate all medium and add 200 μl of fresh medium supplemented in FCS/FBS. Adding 200 μl of medium supplemented in 10% FCS/FBS and 200 μl of lipoplexes will result in 5% FCS/FBS in the final transfection mixture. At this step, by adding medium supplemented with higher content of FCS/FBS, you can control the % of FCS/FBS in the transfection mixture, if needed. Also note that transfection efficiency may vary depending on the procedure—it may be different when you add fresh medium instead of leaving the medium in wells.
10. Twenty-four hours is an optimal time to verify expression of most reporter genes used to test efficiency of transfection like those encoding EGFP or luciferase. This time may vary for different reporter genes, plasmids, and cells from 24 to 96 h after transfection.
11. For staining of live cells, dye that penetrate cell membrane like Hoechst 33342 should be used. For example propidium iodide is not cell permeable and requires cell permeabilization in the staining procedure, so it cannot be used for the staining of live cells.
12. Vaccine doses containing 62.5–125 μg DNA can be used for chicken immunization and those containing 10 μg for mice immunization. It is recommended to vaccinate animals twice—chickens at seventh and 21st day of life while mice at 35th and 49th day of life.
13. It is recommended that all tested serum samples, positive and negative controls should be analyzed in duplicate.
14. At all incubation steps, plates must be sealed with Parafilm to prevent evaporation of the solvent.
15. In all washing steps, use an automated 96-well plate washer or remove washes by flicking the plate over a sink followed by patting the plate on a paper towel.
16. The examples of ELISA results of chicken and mice immunization with DNA vaccines against influenza virus are presented in Stachyra et al. [9].

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References

1. Comberlato A, Paloja K, Bastings MMC (2019) Nucleic acids presenting polymer nanomaterials as vaccine adjuvants. J Mater Chem B 7:6321. https://doi.org/10.1039/c9tb01222b
2. Myhr AI (2017) DNA vaccines: regulatory considerations and safety aspects. Curr Issues Mol Biol 22:79–88. https://doi.org/10.21775/cimb.022.079
3. Hobernik D, Bros M (2018) DNA vaccines—how far from clinical use? Int J Mol Sci 19. https://doi.org/10.3390/ijms19113605
4. Un K, Kawakami S, Suzuki R et al (2010) Development of an ultrasound-responsive and mannose-modified gene carrier for DNA vaccine therapy. Biomaterials 31:7813–7826. https://doi.org/10.1016/j.biomaterials.2010.06.058
5. Christensen D, Korsholm KS, Rosenkrands I et al (2007) Cationic liposomes as vaccine adjuvants. Expert Rev Vaccines 6:785–796. https://doi.org/10.1586/14760584.6.5.785
6. Firouzmand H, Badiee A, Khamesipour A et al (2013) Induction of protection against leishmaniasis in susceptible BALB/c mice using simple DOTAP cationic nanoliposomes containing soluble Leishmania antigen (SLA). Acta Trop 128:528–535. https://doi.org/10.1016/j.actatropica.2013.07.021
7. Carroll TD, Matzinger SR, Barry PA et al (2014) Efficacy of influenza vaccination of elderly rhesus macaques is dramatically improved by addition of a cationic lipid/DNA adjuvant. J Infect Dis 209:24–33. https://doi.org/10.1093/infdis/jit540
8. Korsholm KS, Andersen PL, Christensen D (2012) Cationic liposomal vaccine adjuvants in animal challenge models: overview and current clinical status. Expert Rev Vaccines 11:561–577. https://doi.org/10.1586/erv.12.22
9. Stachyra A, Rak M, Redkiewicz P et al (2017) Effective usage of cationic derivatives of polyprenols as carriers of DNA vaccines against influenza virus. Virol J 14:168. https://doi.org/10.1186/s12985-017-0838-x
10. Erasmus JH, Khandhar AP, Guderian J et al (2018) A nanostructured lipid carrier for delivery of a replicating viral RNA provides single, low-dose protection against Zika. Mol Ther 26:2507–2522. https://doi.org/10.1016/j.ymthe.2018.07.010
11. Wang W, Li W, Ma N, Steinhoff G (2013) Non-viral gene delivery methods. Curr Pharm Biotechnol 14:46–60
12. Pozzi D, Marchini C, Cardarelli F et al (2014) Mechanistic evaluation of the transfection barriers involved in lipid-mediated gene delivery: interplay between nanostructure and composition. Biochim Biophys Acta 1838:957–967. https://doi.org/10.1016/j.bbamem.2013.11.014
13. Smisterová J, Wagenaar A, Stuart MC et al (2001) Molecular shape of the cationic lipid controls the structure of cationic lipid/dioleylphosphatidylethanolamine-DNA complexes and the efficiency of gene delivery. J Biol Chem 276:47615–47622. https://doi.org/10.1074/jbc.M106199200
14. Zuhorn IS, Oberle V, Visser WH et al (2002) Phase behavior of cationic amphiphiles and their mixtures with helper lipid influences lipoplex shape, DNA translocation, and transfection efficiency. Biophys J 83:2096–2108. https://doi.org/10.1016/S0006-3495(02)73970-2
15. Madeja Z, Rak M, Wybieralska E et al (2007) New cationic polyprenyl derivative proposed as a lipofecting agent. Acta Biochim Pol 54:873–876
16. Rak M, Ochałek A, Bielecka E et al (2016) Efficient and non-toxic gene delivery by anionic lipoplexes based on polyprenyl

ammonium salts and their effects on cell physiology. J Gene Med 18:331–342. https://doi.org/10.1002/jgm.2930

17. Gawrys O, Polkowska M, Roszkowska-Chojecka M et al (2014) Effects of liposomes with polyisoprenoids, potential drug carriers, on the cardiovascular and excretory system in rats. Pharmacol Rep 66:273–278. https://doi.org/10.1016/j.pharep.2013.09.009
18. Gawrys O, Olszyński KH, Gawarecka K et al (2014) Cationic derivative of polyprenol, a potential component of liposomal drug carriers, does not alter renal function in rats. Eur J Lipid Sci Technol 116:659–662. https://doi.org/10.1002/ejlt.201300489
19. Utkina NS, Danilov LL, Sizova OV et al (2010) Synthesis of quaternary ammonium salts containing a polyprenyl substituent. Russ Chem Bull Int Ed 59:1459–1462. https://doi.org/10.1007/s11172-010-0262-5

Chapter 21

Flavivirus DNA Vaccine Design and Adjuvant Selection

Lei Li, Yoshikazu Honda-Okubo, and Nikolai Petrovsky

Abstract

A DNA vaccine is a plasmid encoding a vaccine antigen together with an efficient eukaryotic promoter to drive protein expression. The chief problem of DNA vaccines has been their suboptimal immunogenicity in humans. Many different flaviviruses infect and cause serious illness and even death in humans, but human vaccines are not available against most of the relevant flaviviruses with the exception of Japanese encephalitis virus. DNA vaccines are easy and fast to produce at relatively low cost, do not require handling of dangerous pathogens, are stable at room temperature allowing for low-cost storage and transportation, and are highly versatile, allowing for rapid changes in coding sequence design and synthesis. This makes a DNA vaccine approach ideally suited for development as a broad-based flavivirus vaccine platform. However, to be useful as a flavivirus prophylactic vaccine platform in humans, a method would need to be found to enhance DNA vaccine immunogenicity without the need for the cumbersome and expensive equipment involved with electroporation. We describe here a protocol used to test different adjuvants with flavivirus DNA vaccines to determine an optimal formulation. An optimal regimen involving a DNA adjuvanted vaccine prime followed by an adjuvanted protein vaccine boost is described and can be applied by readers to solve barriers to the development of other DNA vaccines where immunogenicity is a problem.

Key words DNA vaccine, Flavivirus, Adjuvant, Codon optimization, Electroporation

1 Introduction

A DNA vaccine is usually a plasmid that encodes a vaccine antigen together with an efficient eukaryotic promoter for protein expression. DNA vaccines are usually administered intramuscularly, intradermally, or mucosally with the aim that the plasmid will gain access to the nuclei of cells and thereby induce expression of RNA encoding the relevant antigen, followed by protein translation and direct or cross-presentation on major histocompatibility molecules within the expressing cell. There is generally no risk of infection with DNA vaccines, they have minimal side effects and can induce both humoral and cellular responses. Hence DNA vaccines satisfy most points of the "ideal" vaccine profile recommended by the World Health Organization: they are easy and fast to produce at relatively low cost, do not require handling of dangerous pathogens, are

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stable at room temperature allowing for low-cost storage and transportation, can be rapidly produced, and are versatile, allowing for easy changes in coding sequence design and synthesis.

However, success of DNA vaccines in veterinary vaccines has not translated into successful human DNA vaccines. After more than 100 clinical trials, no DNA vaccine has yet been approved for humans, primarily due to their suboptimal immunogenicity in humans. To date, many strategies have been tried to improve their immunogenicity, including codon optimization, promoter selection, adjuvant inclusion, and optimization of delivery route but with electroporation (EP) remaining the most prominent method use [1]. The downside to electroporation remains that it requires bulky and expensive administration devices and is highly uncomfortable if not downright painful to the recipient.

In this protocol, we will describe examples of our design of several flavivirus DNA vaccines and the vaccination procedures we used, including optimization of coadministered adjuvants. Flaviviruses are a genus of viruses that include Dengue (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV), and Kunjin virus (KUNV) as well as Zika virus and tickborne encephalitis virus. They are positive-sense, single stranded RNA viruses with similar sized genomes (10.5–11 kb) that consist of three structural and seven nonstructural proteins. The structural prM/E proteins have shown to induce strong protection through neutralizing antibody responses. Within the genus, the JE serocomplex is a monophyletic group which includes WNV, JEV, MVEV and KUNV that are transmitted by the Culex genus of mosquitoes. MVEV is a clinically important virus which circulates in Australia and causes infrequent epidemics, the last being in 2012. Although symptomatic infections are rare, the mortality rate is ~20%. JEV has the highest incidence in the serocomplex, causing ~50,000 infections and ~10,000 deaths in Asia annually, with 30–50% of survivors suffering from irreversible neurological damage. JEV has been identified in Northern Australia and the presence of suitable vectors and hosts increases the risk that it will spread further, although stable transmission cycles have not yet established. KUNV is a strain of WNV which was the cause of a recent equine encephalitis outbreak in Australia, although reports of human infection have been rare. While outbreaks of Australian flaviviruses are relatively rare, changing climates favorable to transmitting mosquitos along with proposed plans for increasing the population in Northern Australia may lead to a higher incidence in the future.

Currently, there are no human vaccines for MVEV, KUNV, or WNV. For JEV, there are several available vaccines including an inactivated JEV vaccine formulated with alum adjuvant and a live-attenuated vaccine. MVEV and KUN infect a small number of people yearly, and so development of a traditional vaccine is unlikely

due to high costs. DNA vaccines present an alternative to traditional vaccines and have several advantages: lower cost of production, stable plasmids allowing for easier transportation and storage at room temperature, authentic protein translation and posttranslational modifications, and induction of both a cellular and humoral immune response [2]. Although they have many potential advantages, currently no DNA vaccines are approved for human use largely due to their poor overall immunogenicity in humans. To overcome this poor immunogenicity, traditional adjuvants such as alum have been tried but alum-adjuvanted DNA vaccines showed no major enhancement of antibody or T-cell responses. By contrast, Vaxfectin, a cationic lipid-based adjuvant, was shown to enhance the immunogenicity of DNA vaccines, including against JEV and DENV [3]. A prME JEV DNA vaccine formulated with Vaxfectin and administered to BALB/c mice via i.m. injection three times at a dose of 10 μg induced antibody titers of 1:80 compared to <1:10 for the unadjuvanted DNA vaccine [4]. Similarly, a polysaccharide adjuvant, zymosan, when combined with an HIV DNA vaccine and given i.m. to BALB/c mice significantly increased the IgG2a and CTL response with a twofold increase in IFN-γ [5]. Another polysaccharide adjuvant called Advax™, that is derived from delta inulin, has been extensively tested with protein, inactivated, and attenuated vaccines, including in phase I and II human clinical trials. A recent study of Advax formulated with a seasonal, inactivated influenza vaccine and injected i.m. into BALB/c mice showed a statistically significant increase in IgG1 and IgG2a antibody titers, as well as CD4+ and CD8+ T-cell proliferation and cytokine responses, that translated into enhanced protection against virus challenge [6]. Advax formulated with a cell culture grown, inactivated JEV vaccine given i.m. to C57BL/6 mice similarly induced significantly higher IgG1 and IgG2c responses and enhanced protection against a lethal JEV challenge [7]. This raises the question of whether Advax may similarly be used to enhance the immunogenicity of a DNA vaccine.

Another method of increasing DNA vaccine immunogenicity is to improve the uptake of the DNA into cells using electroporation (EP). This is a delivery method that involves standard i.m. injections of DNA followed by the application of brief electric pulses through electrodes into the site of injection. Studies have shown that the electric pulses induce transient pores to form in cell membranes [8], allowing extracellular macromolecules to gain access to the cell [9]. A study involving a DNA vaccine injected i.m. into C3H/HeN mice showed that EP led to a 1000-fold increase of expression of a luciferase marker [10]. Antibody titers were enhanced tenfold, with an increase in IgG2a compared to immunization without EP, indicating EP induced primarily a Th1 T-cell response. Other studies in cancer and infectious disease vaccine trials in various animal models have typically shown a

10- to 100-fold enhancement of vaccine efficacy using EP after i.m. DNA vaccine injection [11–13].

DNA-prime/protein-boost strategies have been successfully used to enhance antibody and T-cell responses to many viruses, although studies on the JEV serocomplex are limited. A study that used a WNV DNA vaccine targeting the E protein which was injected i.m. into BALB/c mice with EP, followed by a heterologous protein boost showed significantly enhanced neutralizing antibody titers [14]. Another study used a JEV DNA vaccine with multiple E-protein epitopes and immunomodulatory molecules, followed by two JEV protein boosts adjuvanted with complete Freund's adjuvant, and showed a twofold increase in antibodies, IL-4 and IFN-γ compared to either DNA or protein alone [15]. Hence, a DNA prime/protein boost strategy including relevant adjuvants may be an effective strategy for inducing strong antibody and T-cell protection against flaviviruses.

The following method describes the preparation and use of flavivirus DNA vaccines to be used as part of a DNA prime/protein boost strategy including various adjuvants to increase their immunogenicity (Fig. 1).

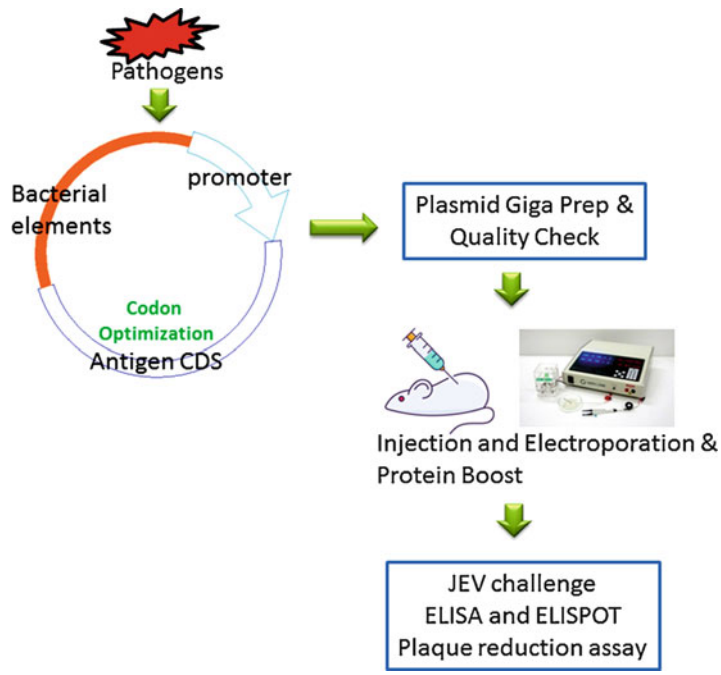

Fig. 1 Schematic of experimental design for testing of effectiveness of flavivirus DNA vaccines with or without added adjuvants

2 Materials

2.1 DNA Vaccine Design and Generation

1. Restriction enzymes from New England Biolab (NEB).
2. T4 DNA ligase (NEB).
3. TOP10 competent cells (Life Technologies).
4. 2YT medium (VWR).
5. Innova 40R shaker (Eppendorf).
6. Heraeus Megafuge 16R Refrigerated Benchtop Centrifuge (Thermo Scientific).
7. Qiagen Endotoxin Free Plasmid Purification kit (Giga prep size).
8. Nanodrop 2000 (Thermo Fisher).

2.2 Mouse Vaccination

1. Female C57BL/6 mice of 6–8 week old with ethics approval from Animal Welfare Committee.
2. Ketamine hydrochloride (100 mg/mL), Medetomidine hydrochloride (1 mg/mL), Atipamezole hydrochloride (5 mg/mL) and Saline for Injection.
3. 0.5 mL Insulin syringe with 29-G needle (BD).
4. 1 mL Tuberculin syringe and 29-G needle (BD).
5. Goldenrod animal lancet (4 mm) (Medipoint Inc.).
6. Heraeus Fresco17 Refrigerated Microcentrifuge (Thermo Scientific).
7. CpG2006 Phosphorothioate oligonucleotide (Oligo Factory).
8. Advax™ adjuvant manufactured by Vaxine Pty Ltd (GMP batch).
9. NEPA21 Electroporator (Nepa Gene).

2.3 JEV Challenge

1. Class II Biosafety cabinet.
2. Individually ventilated mouse cages and a rack.
3. 1 mL Insulin syringe with 29-G needle manufactured by BD.
4. Challenge virus (JEV Nakayama strain).
5. Animal Weighing Scale.

2.4 ELISA

1. Inactivated viruses or recombinant viral proteins of interest.
2. 96-Well ELISA plates (Greiner Bio-One).
3. 24-Well culture plates (Greiner Bio-One).
4. Sterile serological pipettes (Greiner Bio-One).
5. Multichannel pipette (20–200 μL).
6. Variable Adjustable Volume Pipettes.

7. Coating buffer: 0.1 M $NaHCO_3$, pH 9.6.
8. Biotinylated anti-mouse immunoglobulin of interest (Abcam).
9. HRP-conjugated streptavidin (BD Bioscience).
10. Blocking buffer: 1% bovine serum albumin (BSA) in Phosphate buffered saline (PBS).
11. Washing buffer: PBS + 0.05% Tween20 (Sigma-Aldrich).
12. TMB substrate kit (KPL, SeraCare).
13. Stop solution: 1 M phosphoric acid (Sigma-Aldrich).
14. Plate washer (NUNC).
15. Plate reader (OD_{450nm}).

2.5 Plaque Reduction Assay

1. Class II biosafety cabinet.
2. Water bath.
3. CO_2 incubator (Thermo Scientific).
4. Vero cells (ATCC CCL-81).
5. Assay virus (JEV Nakayama strain).
6. Sterile serological pipettes (Greiner Bio-One).
7. Dulbecco's Modified Eagle Medium (1× DMEM) (Gibco, ThermoFisher).
8. Double strength Minimum Essential Medium (2× MEM) (Gibco, ThermoFisher).
9. Fetal calf serum (FCS) (Gibco, ThermoFisher).
10. PBS (Sigma-Aldrich).
11. 0.25% trypsin–EDTA (Sigma-Aldrich).
12. 100× penicillin–streptomycin (10,000 U/mL) (Gibco, ThermoFisher).
13. 100× GlutaMAX Supplement (Gibco, ThermoFisher).
14. 0.22 μm Disk filter unit and 50 mL syringe.
15. Variable Adjustable Volume Pipettes.
16. Hemocytometer.
17. Inverted microscope (Olympus).
18. Avicel RC-591 (FMC BioPolymer).
19. Formalin (Sigma-Aldrich).
20. Methylene blue (Sigma-Aldrich).

2.6 ELISPOT Assay

1. Laminar flow hood.
2. 70 μm Cell strainer (Falcon, Corning).
3. 2 mL disposable syringe and 25-G needle (BD).
4. RPMI1640 medium (Gibco, ThermoFisher).

5. 100× penicillin–streptomycin (10,000 U/mL) (Gibco, ThermoFisher).
6. Sterile serological pipettes (Greiner Bio-One).
7. Multichannel pipette (20–200 μL).
8. Variable Adjustable Volume Pipettes.
9. MultiScreen HTS, 96-well filtration plate (Merck Millipore).
10. Anti-mouse IFN-ɣ, IL-2, IL-4 ELISPOT antibody pairs (BD Biosciences).
11. LEAF anti-mouse IL-17A and biotin-anti-mouse IL-17A antibody (BioLegend).
12. Phosphate buffered saline (PBS) (Sigma-Aldrich).
13. Fetal calf serum (FCS) (Gibco, ThermoFisher).
14. Red Blood Cell (RBC) Lysis Buffer (155 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA, pH 7.3).
15. Hemocytometer.
16. 70% ethanol.
17. Sterile forceps and scissors.
18. CO_2 incubator (Thermo Scientific).
19. ELISPOT plate reader.

3 Methods

3.1 DNA Vaccine Design and Generation

1. The coding DNA sequences of prME of different flaviviruses (e.g., MVEV, KUN, WNV, JEV, DENV, or Zika virus) are either synthesized or achieved by RT-PCR from isolated viruses. All sequences were codon-optimized by using JCat (http://www.jcat.de/) and double-checked by other similar tools (e.g., Genscript's codon optimization tool; https://www.genscript.com/gensmart-free-gene-codon-optimization.html) (*see* **Note 1**). In the meantime, common or conflicting restriction sites (*Eco*RI, *Bam*HI, *Xho*I, etc.) will be removed by silent mutation for easy cloning and flexibility of future subcloning into other DNA vaccine vectors (*see* **Note 2**).
2. Once the prME fragments (e.g., DENV2) are digested with *Kas*I/*Xho*I enzymes and gel purified, they are ligated into pcDNA3-JEss vector (Fig. 2) at *Kas*I/*Xho*I site to make pcDNA3-JEss-DENV2-prME DNA vaccine (Fig. 3). Because we need signal peptide for correct trafficking of expressed prME protein, we have included JEss signal coding sequence at the beginning of the cloning site (**CCATGG** GCAA-GAGGTCCGCCGGCTCAATCATGTGGCTCGC-GAGCTTGGCAGTTGTCATAGCTTGTGCAGGCGCC,

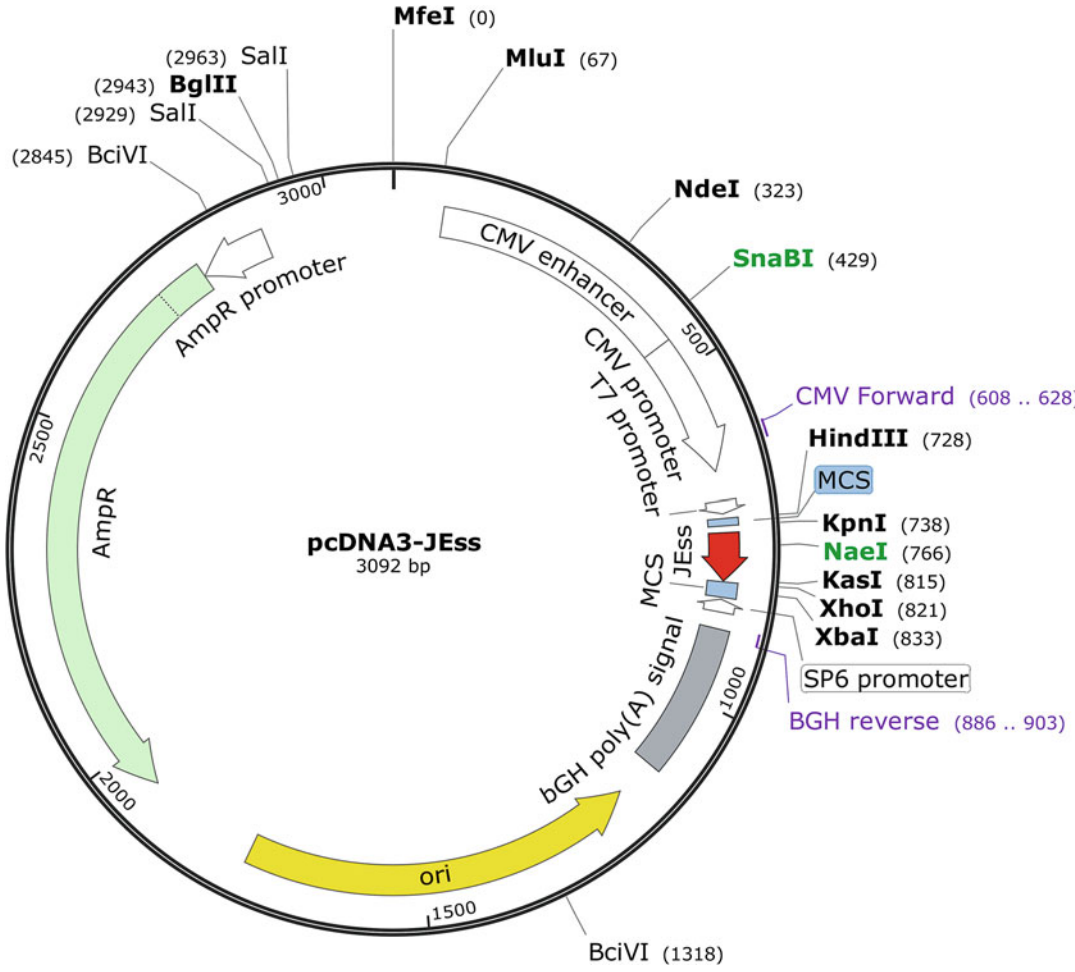

Fig. 2 Plasmid map of pcDNA3-JEss DNA vaccine vector

which codes the JEss MGKRSAGSIMWLASLAVVIACAGA, and the underlined is the Kozak sequence for efficient expression). Then the ligation is transformed into TOP10 competent cells and correct clones confirmed by restriction enzyme digestion after mini-prep (*see* **Note 3**). A glycerol stock is then generated for future use.

3. Every time when beginning the Mega or Giga prep, always start with a single colony from either a new transformation or by restreaking of glycerol stock. Pick a single colony and inoculate in 10 mL 2YT medium and shake at 220 rpm, 37 °C for 12–16 h.

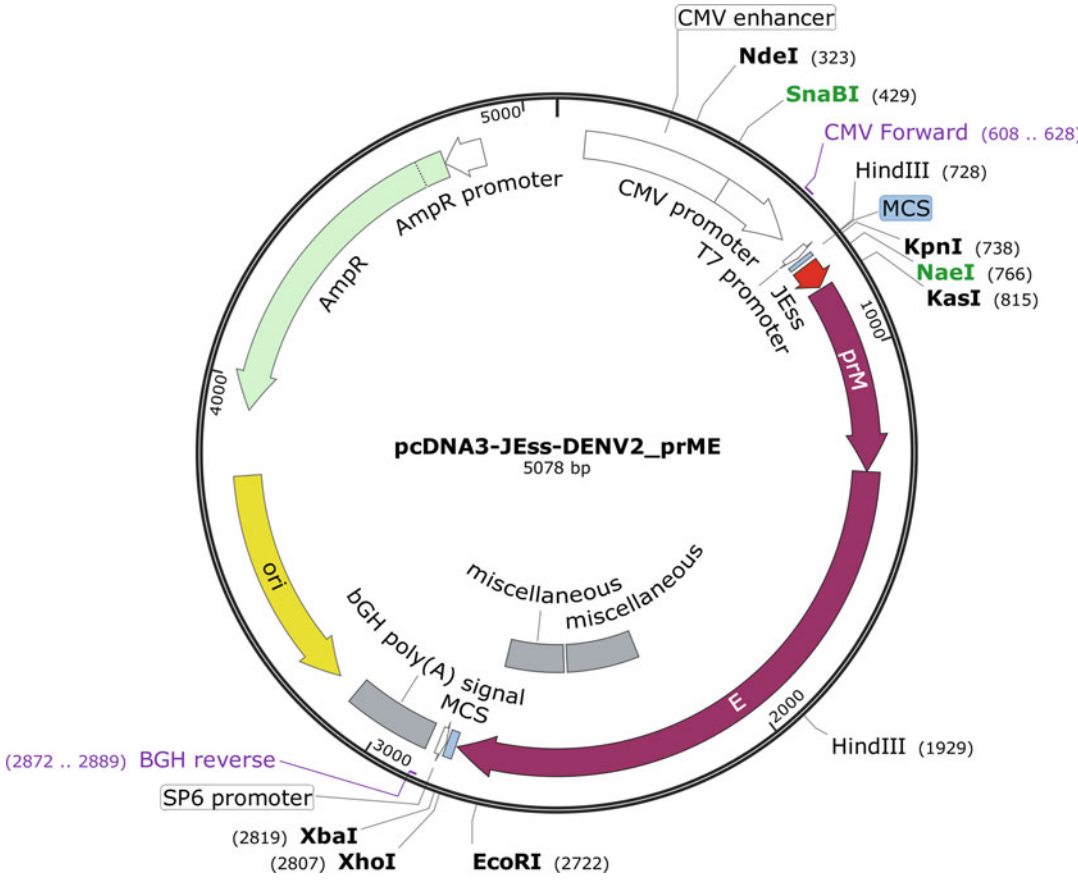

Fig. 3 Plasmid map of DENV2 DNA vaccine

4. Inoculate the small scale culture 1:500 into 650 mL 2YT medium in a 2 L shaker flask to make the total culture volume of 2.5 L for a Giga prep. Shake overnight as above.
5. Pellet the bacteria and perform Giga prep as per the manual of Qiagen Endotoxin Free Plasmid Purification kit. The expected yield is generally 7–10 mg (*see* **Note 4**).
6. Measure plasmid DNA concentration using NanoDrop or fluorescence-based method and also perform agarose gel electrophoresis to confirm the supercoiled plasmids (Fig. 4).

3.2 Mouse Vaccination

1. Prepare vaccine formulation according to Table 1 in sterile tubes. Select any desired adjuvant to assess its effects on DNA vaccine immunogenicity.
2. Inject 50 μL of vaccine intramuscularly for all mice that do not need electroporation.

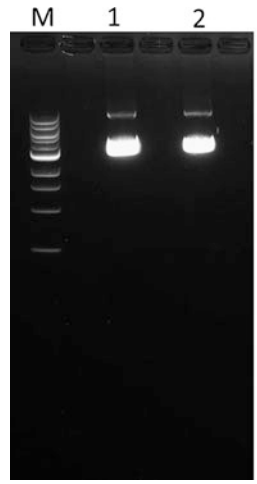

Fig. 4 Example of agarose gel electrophoresis of DENV2 DNA vaccine Giga prep

Table 1
Standard mouse experimental design to assess different DNA vaccine formulations and effect of adjuvants

Group number	No. of mice	Route	DNA vaccination	Protein boost
1	6	i.m.[a]	2× plasmid 1	2× protein + adjuvant
2	6	i.m.[a]	2× plasmid 1 + adjuvant	2× protein + adjuvant
3	6	i.m.[a] + EP[b]	2× plasmid 1	2× protein + adjuvant
4	6	i.m.[a] + EP[b]	2× plasmid 1 + adjuvant	2× protein + adjuvant
5	6	i.m.[a]	–	4× protein + adjuvant
6	6	i.m.[a] + EP[b]	4× plasmid 1 + adjuvant	–
7	6	i.m.[a] + EP[b]	2× saline + adjuvant	2× saline + adjuvant

[a]*i.m.* injection of DNA at 2 μg/μL
[b]Parameters: $V = 100$, time = 50 ms, interval = 100 ms, number of pulses = 3

3. For groups with electroporation, the mice need to be anesthetized by intraperitoneal injection of ketamine (75 mg/kg) mixed with medetomidine (1 mg/kg).
4. After anesthesia, the electrode needles are inserted into the tibialis posterior muscle at a depth of 3 mm and the impedance between electrodes is measured to make sure the electrodes are in the muscle. The electrodes are well positioned when the measured impedance is between 1 and 2 kΩ.
5. The syringe needle is then inserted at the same depth, between the two electrodes, and the injection of DNA vaccine is performed.

6. Then the electroporation is applied: three 50 ms long pulses at 100 V (200 V/cm) followed by three more pulses of the opposite polarity delivered to the injection site.
7. Then atipamezole (1 mg/kg) is injected to reverse the effect of anesthetics. Animals start to recover after 10 min (*see* **Note 5**).
8. At week 2, a second immunization is performed as above.
9. At week 4 and 6, two protein boosts alone or combined with adjuvant are given by intramuscular injection.
10. At week 3, 5 and 7, blood samples are collected by cheek bleeding, for collection of sera for the different immune response assays.
11. At week 8, half of the mice in each group will be anesthetized and cardiac bleeding is performed to get a maximum amount of sera for neutralization antibody assays.
12. Then ELISA and plaque-reduction neutralization test (PRNT) are performed to evaluate antibody responses.
13. Bones and spleens can be collected to measure cellular immunity by ELISPOT assay.
14. The remaining mice can be challenged by IP injection of virus (*see* **Note 6**).

3.3 Antibody Assay by ELISA

1. Dilute capture antigen in Coating Buffer to 1 μg/mL. 10 mL is required per ELISA plate (*see* **Note 7**).
2. Add 100 μL/well of the diluted antigen using a multichannel pipette.
3. Ensure the solution is distributed over entire surface of all wells.
4. Cover the plate with a plastic seal.
5. Incubate overnight at 2–8 °C.
6. Flick off contents of microplate into the sink and wash plates four times with Washing Buffer >250 μL/well (*see* **Note 8**).
7. Dry all surfaces of plates with a paper towel.
8. Add 200 μL/well of Blocking Buffer.
9. Cover the plate with a plastic seal.
10. Incubate for at least 30 min at RT.
11. Dilute samples serially in Blocking Buffer in a 96-well U-bottom plate. Always prepare enough volume for a duplicate set per sample as 100 μL/well of samples are required.

12. Flick off Blocking Buffer from plates and bang the plate onto absorbent pads to remove air bubbles and excess moisture.
13. Add 100 μL/well of samples.
14. In a 24-well plate, prepare Initial dilution of Standard Serum. Serially dilute serum in Blocking Buffer, Add 100 μL/well of Standard dilutions and blank to plate.
15. Cover the plate with a plastic seal.
16. Incubate for 2 h at RT.
17. Gently flick contents of microplate into the sink and wash plates six times with Washing buffer >250 μL/well each; bang the plate onto absorbent pads to remove air bubbles and excess moisture in between every two consecutive washes.
18. Dry all surfaces with a paper towel.
19. Dilute the biotinylated anti-mouse detection antibodies and streptavidin-HRP in Blocking buffer (total volume required is 10 mL per plate). Prepare this just before washing plates.
20. Gently add 100 μL/well of the diluted detection antibody using a multichannel pipette without forming air bubbles.
21. Cover the plate with a plastic seal.
22. Incubate for 1 h at RT.
23. Wash the plate six times.
24. Mix equal volume of Peroxidase Substrate and Peroxidase Substrate Solution B just before use. 10 mL are required per ELISA plate.
25. Add 100 μL/well of the TMB substrate solution using a multichannel pipette.
26. Incubate for 10 min at RT to allow color to develop.
27. Add 100 μL/well of stop solution using a multichannel pipette to stop the reaction.
28. Gently tap the plate to stop the reaction uniformly.
29. Measure OD at 450 nm with a VersaMax ELISA microplate reader (Molecular Devices, CA, USA) and analyzed using SoftMax Pro Software.
30. For determination of ELISA end-point titers, absorbance cut-off values are established as the mean absorbance of eight negative-control wells containing sera of naive mice plus 3 SD.
31. Absorbance values of test sera were considered positive if they were equal to or greater than the absorbance cut-off and end-point titers calculated as $\log_{10}$ of the reciprocal of the last dilution giving a positive absorbance value.

3.4 Antibody Assay by PRNT

3.4.1 Preparation of Growth Medium (DMEM + 5% FCS + Antibiotics)

1. Thaw required number of heat-inactivated FCS frozen aliquots at 4 °C and mix well.
2. Filter sterile 25 mL of FBS and 5 mL of 100× Penicillin/Streptomycin when adding them to 500 mL of DMEM.
3. Label container with appropriate information and record all information on the appropriate reagent formulation worksheet. Store at 2–8 °C.
4. Media should be verified that it is not contaminated before each use by visually inspecting for cloudiness or foreign particles. If contaminated, the media must be discarded.

3.4.2 Preparation of Maintenance Medium (DMEM + 3% FCS + Antibiotics)

1. Thaw required volume of heat-inactivated FCS at 4 °C and mix well.
2. Filter sterile 15 mL of FCS and 5 mL of 100× Penicillin/Streptomycin when adding them to 500 mL DMEM.
3. Label container with appropriate information and record all information on the appropriate reagent formulation worksheet. Store at 2–8 °C.
4. Media should be verified that it is not contaminated before each use by visually inspecting for cloudiness or foreign particles. If contaminated, the media must be discarded.

3.4.3 Preparation of Fixative Solution

1. Mix 100 mL of formalin with 900 mL of PBS.
2. Thoroughly mix.
3. Label container with appropriate information and record all information on the appropriate reagent formulation worksheet. Store at RT.

3.4.4 Preparation of Methylene Blue Stock Solution

1. Dissolve 2.25 g Methylene Blue in 200 mL water and add 0.375 mL 1 M NaOH.
2. Thoroughly mix.
3. Label dark container with appropriate information and record all information on the appropriate reagent formulation worksheet. Store at RT.

3.4.5 Preparation of Overlay Solution

1. Dissolve 9.6 g of Avicel Powder in 400 mL of Milli-Q Water.
2. Thoroughly mix and autoclave.
3. Label container with appropriate information and record all information on the appropriate reagent formulation worksheet. Store at RT.

3.4.6 Overlaying Media for Plaque Assay

1. Thaw heat-inactivated FCS, 100× Penicillin-Streptomycin and 100× GlutaMAX aliquots at 4 °C and mix well.

2. Mix 2.4% Avicel solution in water with equal volume of 2× MEM with 3% FCS, Pen/Strep (1/100), and GlutaMAX (1/100).

3.4.7 Performing the Plaque Reduction Assay

1. Warm maintenance medium and PBS to 37 °C in water bath.
2. Remove culture media from a T75 flask containing cultured Vero cells (*see* **Note 9**).
3. Wash cells with 5 mL of PBS and rinse with 2.5 mL of Trypsin-EDTA.
4. Incubate flask at 37 °C for 5–10 min until monolayer detaches.
5. Add 10 mL of maintenance medium to the flask.
6. Pipet cell suspension up and down at least five times against the bottom of the flask with a 10 mL pipette. This is to break up the cell clusters. Be careful not to create air bubbles.
7. Count cells using hemocytometer.
8. Dilute the cell suspension to 3×10^5 cells/mL using pre-warmed maintenance medium.
9. If a large number of cells are required for the assay, pool the cells from several flasks of the same passage number as required.
10. Add 3 mL/well of cell suspension of a 6-well plate and incubate at 37 °C in a 5% CO_2 incubator until cells make monolayer (overnight).

3.4.8 Neutralization

1. Heat-inactivate samples in water bath (56 °C for 30 min).
2. Prepare serial fourfold dilution in maintenance medium.
3. Dilute enough volume of virus to 200–240 PFU/mL in maintenance medium.
4. Mix diluted serum sample and virus and incubate at 37 °C for 90 min in a 5% CO_2 incubator.

3.4.9 Inoculation of Virus

1. Check plates under the microscope if cells at least 80% of bottom of the wells.
2. Aspirate cell medium from the wells.
3. Add 0.1 mL per well of neutralized samples per well in duplicate.
4. Incubate the plate at 37 °C for 90 min in a 5% CO_2 incubator (tilt the plate every 30 min to allow even distribution of the inoculum in the well).
5. Add 4 mL/well of overlaying media and incubate the plate at 37 °C for 6 days in a 5% CO_2 incubator.

3.4.10 Fixation, Staining, and Calculation of $PRNT_{50}$ Titers

1. After 6 days incubation, add 1.5 mL/well of fixative and mix well.
2. Leave plates for 1 h at RT.
3. Remove fixative and wash plates with tap water.
4. Add 1.5 mL/well of 1:30 diluted methylene blue dye solution in water and leave plates for 1 h at RT.
5. Remove dye solution and wash plates with tap water air-dry.
6. Count number of plaques in each well.
7. $PRNT_{50}$ titers are given as the reciprocal of serum dilutions that resulted in a 50% reduction in the number of plaques.

3.5 ELISPOT Assays

1. At the termination of immunogenicity studies, mice are euthanized by cervical dislocation and bones and spleens are collected to enable measurement of antigen-specific memory B and T cells.
2. Bone marrow (BM) are isolated from femurs by flushing with 3% FCS/PBS in a 2 mL syringe with 25-G needle after cutting both ends of the bone then pass through 70 μm cell strainer with a 2 mL syringe plunger.
3. Splenocytes are released by pressing against a 70 μm cell strainer with a 2 mL syringe plunger.
4. Red blood cells are removed by osmotic shock with RBC lysis buffer.
5. Cells are washed with 3% FCS/PBS then resuspended in RPMI complete medium with 10% heat-inactivated FCS.
6. The frequency of antigen-specific antibody- or cytokine-secreting cells is analyzed using biotinylated anti-mouse IgG, IgG1, IgG2c, or IgM antibodies (Abcam) or anti-mouse IFN-ɣ, IL-2, IL-4 antibody pairs (BD) or LEAF anti-mouse IL-17A and biotin-anti-mouse IL-17A antibody (BioLegend) with streptavidin-HRP (BD Biosciences), according to the manufacturer's instruction.
7. Briefly, single-cell suspensions are prepared from BM and spleens of mice are plated at 2×10^5 cells/well in 96-well filtration plates pre-coated with antigen of interest (for antibody detection) or anti-mouse cytokine antibodies (for cytokine detection) overnight at 4 °C then blocked with RPMI/10% FCS (*see* **Note 10**).
8. For cytokine assays, the cells are incubated with antigen protein (10 μg/ml) at 37 °C and 5% CO_2 for 2 days or 1 day for antibody producing cell assay (*see* **Note 11**).
9. Wells are washed and incubated with biotinylated anti-mouse cytokine detection antibodies or biotinylated anti-mouse Ig at RT for 2 h, washed, then streptavidin-HRP added for 1 h

before washing and addition AEC substrate solutions (BD Biosciences).

10. Carefully monitor spot formation and stop the reaction by gently washing the plate with tap water when development appears to slow.
11. Take the base off the plates and air-dry in dark.
12. Spots are counted by ImmunoSpot S6 ELISPOT analyzer (CTL, USA) and analyzed using ImmunoSpot Software.
13. Spots in negative control wells are subtracted from the number of spots in sample wells and the results are expressed as antibody secreting cells (ASC) per 10^6 BM cells or spots per 10^6 splenocytes.

4 Notes

1. Most of the freely available tools are suitable for this purpose, given that ideal codon optimization is not necessary for a DNA vaccine. Antigen sequence selection plays a more important role and has to be tried for each individual case either empirically or facilitated by computer modeling.
2. Because there are inconsistent data on effects of presence and frequency of CG motifs in DNA vaccine vectors, we do not routinely check or change this in our DNA vaccine constructs. For potential human trials, researchers can select vectors with the kanamycin resistance gene rather than ampicillin to avoid potential side effects.
3. In our experience, TOP10 is good for cloning and Giga-prep of most of our DNA vaccine constructs. If some constructs contain difficult sequences (e.g., secondary structure or high AT or GC content), other strains can be tried (e.g., DH5α or Stbl). Also, some constructs might show a degree of toxicity to bacteria (e.g., the JEV construct in our hands), which will yield lower amounts of plasmid DNA.
4. If using other brands kit for purification, follow the standard manual. Manual prep using the PEG6000 method is also possible, but endotoxin levels should be checked. Certain brands may have more RNA contamination in the final plasmid product, so agarose gel analysis is also recommended.
5. A careful consideration should be given to the mice legs to make sure the procedure was done properly without extreme pain and other side effects.
6. C57BL/6 mice are more susceptible to JEV virus infection than BALB/c mice if challenge studies are desired.

7. Either inactivated virus or recombinant viral proteins can be used but the concentration of antigen might need to be optimized for each antigen.
8. Bang the plate onto absorbent pads to remove air bubbles and excess moisture in between every two consecutive washes.
9. To maintain appropriate cell sensitivity, Vero cells should be in the log-growth phase and therefore cultured for a minimum of 48 h prior to being harvested for cell plate preparation.
10. Typically, cell numbers should usually range from 2×10^5 to 4×10^5 cells per well but might need to be optimized.
11. Do not stack the plates if you have more than one to prevent edge effects and do not move the plates while culturing.

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References

1. Li L, Petrovsky N (2017) Molecular adjuvants for DNA vaccines. Curr Issues Mol Biol 22:17–40
2. Li L, Saade F, Petrovsky N (2012) The future of human DNA vaccines. J Biotechnol 162 (2–3):171–182
3. Porter KR et al (2011) Immunogenicity and protective efficacy of a vaxfectin-adjuvanted tetravalent dengue DNA vaccine. Vaccine 30 (2):336–341
4. Nukuzuma C et al (2003) Enhancing effect of vaxfectin on the ability of a Japanese encephalitis DNA vaccine to induce neutralizing antibody in mice. Viral Immunol 16(2):183–189
5. Ara Y et al (2001) Zymosan enhances the immune response to DNA vaccine for human immunodeficiency virus type-1 through the activation of complement system. Immunology 103(1):98–105
6. Honda-Okubo Y, Saade F, Petrovsky N (2012) Advax, a polysaccharide adjuvant derived from delta inulin, provides improved influenza vaccine protection through broad-based enhancement of adaptive immune responses. Vaccine 30(36):5373–5381
7. Lobigs M et al (2010) An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel inulin-based adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses. J Gen Virol 91(Pt 6):1407–1417
8. Cukjati D et al (2007) Real time electroporation control for accurate and safe in vivo non-viral gene therapy. Bioelectrochemistry 70(2):501–507
9. Becker SM, Kuznetsov AV (2007) Local temperature rises influence in vivo electroporation pore development: a numerical stratum corneum lipid phase transition model. J Biomech Eng 129(5):712–721
10. Wu CJ et al (2004) In vivo electroporation of skeletal muscles increases the efficacy of Japanese encephalitis virus DNA vaccine. Vaccine 22(11–12):1457–1464
11. Livingston BD et al (2009) Comparative performance of a licensed anthrax vaccine versus electroporation based delivery of a PA encoding DNA vaccine in rhesus macaques. Vaccine 28(4):1056–1061
12. Luckay A et al (2007) Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. J Virol 81 (10):5257–5269

13. Simon AJ et al (2008) Enhanced in vivo transgene expression and immunogenicity from plasmid vectors following electrostimulation in rodents and primates. Vaccine 26 (40):5202–5209
14. Schneeweiss A et al (2011) A DNA vaccine encoding the E protein of West Nile virus is protective and can be boosted by recombinant domain DIII. Vaccine 29(37):6352–6357
15. Li P et al (2008) Enhancement of humoral and cellular immunity in mice against Japanese encephalitis virus using a DNA prime - protein boost vaccine strategy. Vet J 183(2):210–216

Chapter 22

Vaccine Delivery with a Detoxified Bacterial Toxin

Diana Diaz-Arévalo, Yanping Chen, and Mingtao Zeng

Abstract

It is still a challenge to develop needle-free mucosal vaccines. Despite progress in the development of the influenza vaccine, it must be reformulated annually because of antigenic changes in circulating influenza viral strains. Due to seasonal drift and shift of circulating strains, the influenza vaccine does not always match the circulating strains, and included adjuvants are not sufficient to induce a protective effect with long-lived memory cells. The adjuvants play a major role in the immune responses to a vaccine. Interestingly, the *Bacillus anthracis* detoxified anthrax edema toxin, which composes of protective antigen PA and N-fragment of edema factor (EFn), has shown improved effects for humoral and cellular immune responses. Here we describe the design of a universal influenza vaccine construct that consists of three tandem M2e repeats of the influenza antigen plus HA2 and detoxified toxin EFn, which is associated with the PA component, as well as the techniques used to corroborate protection. We present two major parts of description to demonstrate the vaccine strategy, using detoxified anthrax toxin for intranasal delivery of influenza antigen: (1) vaccine candidate design, production, and purification; (2) influenza virus microneutralization assay and cellular responses and lethal challenge with influenza viruses and *B. anthracis* Sterne spores. In the methods detailed here, we used different versions of the M2e–HA2 proteins.

Key words Vaccine, Adjuvant, *Bacillus anthracis*, Edema toxin, Edema factor, Influenza

1 Introduction

Bacillus anthracis escapes from host immune responses using two toxins: edema toxin (ET) and lethal toxin (LT). These toxins are formed after lethal factor (LF) or edema factor (EF) bind to protective antigen (PA), and individually they are nontoxic. The PA associated with EF or LF binds to ANTXR1 (tumor endothelial marker 8) and ANTXR2 (capillary morphogenesis protein 2). This protein heptamer complex forms a pore in the endosomal membrane facilitating the translocation of the toxins into the cytosol, where they exert their toxic activities [1]. LF is a zinc-dependent metalloprotease that cleaves six of the seven mitogen-activated protein kinase (MAPK) kinases, and EF is a calcium- and calmodulin-dependent adenylate cyclase that increases intracellular cAMP concentrations [2]. It is known that the EF binding region

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to PA is localized at the N-terminal (1–254 residues), and its toxicity is lost after removing the C-terminal [3, 4].

Influenza virus diseases are prevented with a seasonal vaccine; however, due to the frequent occurrence of mutations (drift and shift), some circulating virus strains may not match the seasonal vaccine. The best way to effectively protect people, especially young children and senior citizens, is to develop a universal vaccine with conserved viral regions. The ion channel protein M2 is found in all strains of the influenza A virus, and this structural protein is incorporated into the viral lipid envelope. The 23-amino acid extracellular N-terminal domain (M2e) is highly conserved, making it an attractive vaccine target for a broadly effective protein vaccine [5]. Hemagglutinin 0 is a precursor glycoprotein anchor at the viral membrane that its cleavage form by transmembrane anchor close to carboxyl terminal, it cleavage two disulfide-linked subunits, HA1 and HA2, eliminating an arginine residue, R329 [6]. HA2 is more highly conserved between influenza viruses, and anti-HA2-specific antibodies reduce the replication of influenza [7]. Also, vaccines based on conserved HA2 regions can have prophylactic efficacy [8, 9].

Taken together, anthrax and influenza induce cellular and humoral immune responses, whereas PA induces T cell immunity and low levels of anti-PA antibodies [10], indicating that the protection is associated with T cell responses. The same happens with lethal factor, which induces specific IFNγ-producing $CD4^+$ T cells in patients treated for cutaneous anthrax and generating long-lasting immunity to anthrax [11]. However, the immunization of BALB/c mice with PA and EF induces high antibody titers, which demonstrated an adjuvant effect. Mice vaccinated with PA + EF had higher titers than mice immunized with PA + LF [12]. The nasal delivery of ET coadministered with OVA resulted in high titers of OVA-specific IgA and IgG [13]. In addition, the nontoxic N-terminal fragment of EF was evaluated as a vaccine candidate against anthrax. This vaccine was replicated in an incompetent adenoviral vector (Ad/EFn) encoding the N-terminal region (1–254 amino acids) of the edema factor (EFn). This vaccine was administered to mice three times with 10^8 plaque-forming units (PFU)/dose and resulted in 37% and 57% of the protection after challenge with *B. anthracis* Sterne strain spores at a dosage of $200 \times LD_{50}$ and $100 \times LD_{50}$, respectively [14]. The N-terminal fragments of EF and LF bind to PA to further translocate to the cytosol. Taking advantage of this property, it is possible to use EF + PA to carry any type of vaccine into the antigen-presenting cells and increase the immune response, since EF has an adjuvant property [13].

In this chapter, we describe two methods to analyze the functionality of detoxified anthrax edema toxin EFn + PA as a carrier and adjuvant system. The first method describes the design,

production, and purification of a recombinant chimeric antigen containing the detoxified EFn (AA, 1–254), which binds to PA and allows the entrance of chimeric conserved M2e and HA2 amino acids from influenza. In this step it is necessary to use bioinformatics tools to find the regions or amino acids conserved between strains that are exposed to the immune system. The construct contains EFn (1–254)-linker (AAA) and the 3×M2e-linker (GSGGS)-HA2. The chimeric antigen is ligated to an expression vector and transfected into *E. coli*. The protein is purified using denaturing conditions and dialysis to remove contaminants [4, 15].

The second method described here measures vaccine-dependent T cell activation and antibody production and their correlation with protection. The chimeric vaccine without adjuvant allowed its own translocation into antigen-presenting cells to activate immune responses and induce dual protections against *B. anthracis*, Sterne spores, and influenza. Immune cells from vaccinated mice will be tested against the chimeric construct and negative controls. Antibodies are used to detect the different conserved antigens and their capacity to neutralize the entrance of influenza virus into cells by microneutralization assay. Finally, the protection of mice immunized with the chimeric vaccine and challenge with the three different strains of influenza and *B. anthracis* Sterne spores are described.

2 Materials

2.1 Production and Purification of the Recombinant Chimeric Antigen EFn-3×M2e-HA2

1. Synthetic EFn-3×M2e-HA2 gene: the anthrax EFn gene sequence (1–254 aa) was added upstream of three tandem M2e sequences from A/California/04/2009 (H1N1), A/Hong Kong/1/1968 (H3N2), and A/Vietnam/1204/2004 (H5N1) influenza viruses, and the conserved amino acids in each position were used to create an artificial, centralized, HA2 after alignment of H1N1, H3N2, H5N1, H7N1, H7N3, and H9N2. Ala-Ala-Ala and Gly-Ser linkers were designed for insertion between individual gene segments.
2. pET200/D-TOPO (Invitrogen, cat. #K10001).
3. BL21 StarTM (DE3) competent cells (Life Technologies).
4. Kanamycin.
5. IPTG.
6. D(+)-Glucose monohydrate (Fluka Sigma-Aldrich, St. Louis, MO, USA).
7. Cell lysis buffer (0.5 M NaCl, 40 mM imidazole, and 8 M urea).
8. Elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4).

9. LB medium.
10. LB agar plates.
11. Ni-NTA Superflow resin (Qiagen).
12. Sodium phosphate buffer.
13. 150 mM sodium chloride buffer.
14. Imidazole.
15. Complete EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics).
16. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), sample buffer, and running buffer (Invitrogen).
17. β-Mercaptoethanol.
18. SDS-PAGE electrophoresis chamber.

2.2 Vaccination, Immune Responses, and Dual Challenge

2.2.1 Immunization of Mice and Serum Collection

1. BALB/c mice (6–8 weeks) from Harlan Laboratories (Indianapolis, IN), A/J mice (6–8 weeks), and CD4-knockout (KO) and CD8-KO C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME).
2. Antigen EFn-3×M2e-HA2.
3. Recombinant anthrax protective antigen (BEI Resources, Manassas, VA).
4. 5-mm lancets to bleed mice using the submandibular method.
5. Ketamine and xylazine.

2.3 T Cell and Antibody Responses and Protection

2.3.1 ELISA

1. Mouse sera of immunized individuals.
2. Phosphate-buffered saline (PBS), pH 7.2.
3. Tween 20 detergent.
4. Immuno Clear standard module cx320.
5. Bovine serum albumin.
6. Anti-mouse IgG-Fc, IgG1, and IgG2a-phosphatase.
7. p-Nitrophenylphosphate phosphatase substrate system (KPL, Gaithersburg, MD).
8. ImmunoWash™ 1575 microplate washer (Bio-Rad, Hercules, CA).
9. PowereWave XS2 plate reader with GEN5 software (BioTek, Winooski, VT).

2.3.2 Influenza Virus Microneutralization Assay

1. H1N1 influenza virus A/PR/8/34 (PR8, from VR-95; ATCC, Manassas, VA).
2. Madin–Darby canine kidney (MDCK) cells.
3. 1% agarose in medium (minimum essential medium, 0.2% BSA, 1 μg/ml TPCK-treated trypsin).
4. Ethanol.

5. Coomassie blue.

2.3.3 T Cell Immune Responses by Detection of the Stimulated T Cell Population

1. Mouse immunized spleens.
2. Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (DMEM-10).
3. 100-μm nylon mesh cell strainers
4. ACK lysis buffer.
5. 10 ng of PMA/100 ng ionomycin.
6. M2, 3×M2e-HA2, PA.
7. M2e-region peptides from A/New York/348/03 (H1N1) virus.
8. HA2 region peptides from A/California/04/2009 (H1N1) virus.
9. Antibodies: anti-CD3–FITC, CD4–PerCP-Cy5.5, CD8–PE, CD8–V500, and FoxP3–PE (Table 1).
10. BD FACSCanto II flow cytometer (BD Biosciences).
11. FLOWJO v10 software (FlowJo, Ashland, OR).

2.3.4 Tetramers and Staining

1. I-A(d)/SLLTEVETPIRNEWGS–phycoerythrin (MHC Class II) and H-2K(d)/VETPIRNEW–allophycocyanin (MHC Class I), obtained through the NIH Tetramer Core Facility at Emory University (Atlanta, GA).
2. DMEM-10.
3. Spleen cells.
4. 100-μm nylon mesh cell strainers
5. Anti-CD3–Alexa Fluor 700, CD4–APC, CD8–phycoerythrin, and CD3–FITC (BD Biosciences, Table 1).

2.3.5 Lethal Challenge with Influenza Viruses or B. anthracis *Sterne Spores*

1. EFn-3×M2e-HA2-vaccinated mice.
2. 2×10^5 PFU of live A/PR/8/34 virus and 1×10^5 PFU of live A/CA/4/09 virus (ATCC, Manassas, VA).
3. *B. anthracis* Sterne spores.
4. Weight scale and thermometer.

3 Methods

3.1 Production and Purification of the Recombinant Chimeric Antigen EFn-3×M2e-HA2

A synthetic chimeric gene was generated by combining the EFn gene (1–254 residues); three tandem M2e sequences from influenza subtypes A/California/04/2009 (H1N1), A/Hong Kong/1/1968 (H3N2), and A/Vietnam/1204/2004 (H5N1, H3N2, and H5N1) (Fig. 1a) to increase the density and variation of M2e epitopes; and a highly centralized HA2 gene sequence.

Table 1
T cell antibody staining for FACS

Marker	Fluorochrome
CD25	FITC
CD3	Alexa Fluor 700
CD3	FITC
CD4	APC
CD4	PerCP-Cy5.5
CD8	PE
CD8	V500
FoxP3	PE

Antibodies against T cell membrane proteins and transcription factor. (Adapted from Arévalo et al. [4])

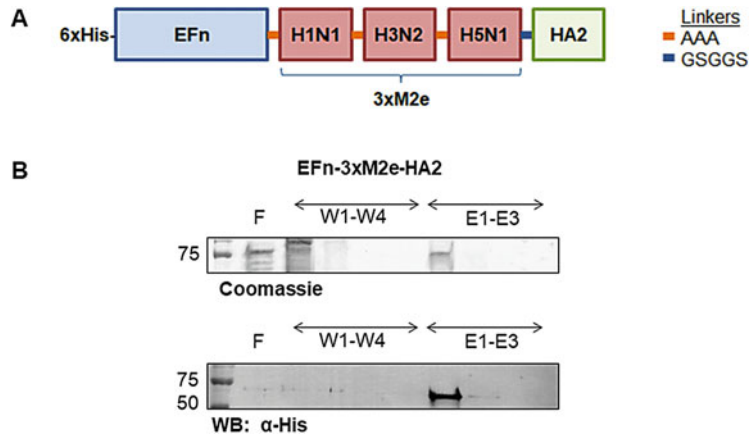

Fig. 1 Vaccine construct design and verification. (**a**) Schematic representation of the vaccine construct EFn-3×M2e-HA2. (**b**) Representative verifications by SDS-PAGE and western blot of EFn-3×M2e-HA2. *F* purification flow-through, *W* wash, *E* eluate. (Adapted from Arévalo et al. [4])

1. The synthetic gene are amplified using a forward primer with 4-base-pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer.
2. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce a blunt-end PCR product.
3. Check the size of the PCR product by agarose gel, using 0.8% agarose in TAE.

4. To fresh PCR product (0.5–4 μl) add 1 μl of salt solution, sterile water to a final volume of 5 μl, and 1 μl of the TOPO® vector.
5. Mix gently and incubate for 5 min at room temperature.
6. Place on ice and proceed to transform One Shot® TOP10 chemically competent *E. coli*.
7. Add 3 μl of the TOPO® cloning reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently.
8. Incubate on ice for 5–30 min.
9. Heat-shock the cells for 30 s at 42 °C without shaking.
10. Immediately transfer the tube to ice.
11. Add 250 μl of room-temperature S.O.C. medium.
12. Incubate at 37 °C for 1 h with shaking, then spread 100–200 μl of bacterial culture on a prewarmed selection plate and incubate overnight at 37 °C.
13. Select ten colonies to test the gene product by PCR.
14. Grow the TOP10 *E. coli* overnight in LB medium.
15. Purify the plasmid using a plasmid purification kit.
16. Transform BL21 Star™(DE3) with synthetic EFn-3×M2e-HA2-containing plasmid.
17. Check the gene product by PCR.
18. Select the best colony and grow in 20 ml of LB liquid medium overnight.
19. Transfer the 20 ml of bacteria culture to 4 L of LB, incubate for 2–4 h at 37 °C, and read the absorbance at 620 nm. The absorbance should be in the range 0.4–0.6 before adding IPTG (0.05 mM).
20. Grow the culture for 6 h at 30 °C.
21. Centrifuge the bacteria at 10,000 × *g* for 10 min at 4 °C. Place the bacterial pellet on ice.
22. Dilute the bacterial pellet by adding 5–10 ml of binding buffer (0.5 M NaCl, 40 mM imidazole, and 8 M urea) for each gram of cell paste.
23. Disrupt cells by sonication (in several short bursts) on ice for approximately 10 min.
24. Centrifuge the bacteria at 15,000 × *g* for 30 min at 4 °C.
25. Collect the supernatant and filtrate using a Minisart NY25 0.45-μm filter.
26. Equilibrate a Ni-NTA Superflow column using binding buffer for 30 min.
27. Load the lysed bacteria onto the column.

28. Collect the flow-through and keep at 4 °C.
29. Wash the column with 10 column volumes of binding buffer.
30. Elute the recombinant protein with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) and collect several 2-ml fractions.
31. Use an SDS-PAGE gel and western blotting to detect positive fractions (Fig. 1b).
32. Pool the positive fractions and dialyze four times with PBS.
33. Measure the recombinant protein concentration and store small aliquots at −80 °C.

3.2 Vaccination, Immune Responses, and Dual Challenge

3.2.1 Immunization of Mice and Serum Collection

1. BALB/c and A/J wild-type mice as well as C57BL/6 CD4-knockout (KO) and CD8-KO (6–8 week) mice are weighed and anaesthetized with 100 μl of 80 mg/kg ketamine and 6 mg/kg xylazine via intraperitoneal injection.
2. After 5 min of anesthetic injection, the mice receive 10 μl of a mixture containing 30 μg of chimeric vaccine candidate plus 60 μg of PA (*see* **Note 1**, Fig. 2) in each nostril. The control group receives only 60 μg of PA (*see* **Note 2**).
3. Mice are immunized at 0, 2, and 5 weeks and bled just before each immunization and on week 7 via the submandibular method with 5-mm lancets.
4. Centrifuge the blood sample at 13,000 × *g* for 10 min. at 4 °C, collect the sera, and keep at −20 °C until use.

3.2.2 ELISA

Antigen-specific antibody responses are measured in the sera of naive and immunized mice by ELISA.

1. Ninety-six-well Corning EIA/RIA plates are coated with 1 μg/ml of recombinant antigens (EFn-3×M2e-HA2, 3×M2e-HA2, and PR8) at 4 °C overnight.
2. Wash five times the plates with PBS-T (PBS with 0.05% Tween-20) using an ImmunoWash™ 1575 microplate washer (Bio-Rad, Hercules, CA).
3. Block the wells with 200 μl of 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature.
4. Dilute the serum samples 1:100, add them to the 96-well plate, and incubate for 2 h at room temperature with rocking.
5. Standard plates are coated with capture antibodies diluted 1:500 in PBS-T, including affinity-purified anti-mouse IgG-Fc, IgG1, IgG2a, or IgA (Bethyl Laboratories Inc., Montgomery, TX).
6. Repeat **step 2**.

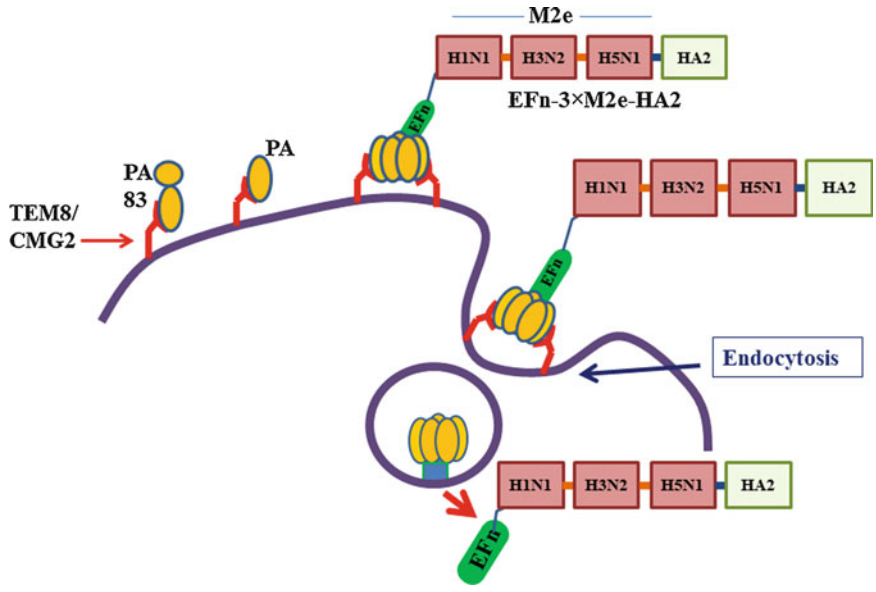

Fig. 2 Modified anthrax fusion proteins to deliver antigens. The chimeric vaccine without adjuvant allowed its own translocation into antigen-presenting cells. The PA could bind to either tumor endothelial marker 8 or capillary morphogenesis protein 2, present in antigen presenting cells. The binding between them allowed the self-associates to form a ring-shaped heptameric complex that results in the binding of the chimeric vaccine allowing the endocytosis and delivery into the cells

7. Add secondary anti-mouse IgG-Fc, IgG1, and IgG2a antibodies diluted 1:10,000 in PBS-T (100 μl/well), and incubate at RT for 1 h.
8. Repeat **step 2**.
9. Bound antibody is detected by using p-nitrophenylphosphate phosphatase as substrate.
10. Absorbances are measured at 405 nm using the PowereWave XS2 plate reader with GEN5 software (BioTek, Winooski, VT).

3.2.3 Influenza Virus Microneutralization Assay

1. Mouse sera are heat-inactivated at 56° for 30 min (*see* **Note 3**).
2. Dilute the mouse sera in serial dilutions.
3. Add 50 μl of each dilution and place in the wells of a microtiter plate and mix with 50 μl (2×10^4 PFU) of H1N1 influenza virus A/PR/8/34 (PR8, from VR-95; ATCC, Manassas, VA).
4. Incubate the plate for 1 h at 37°, and 50 μl of the suspension should be transferred onto Madin–Darby canine kidney (MDCK) cells.
5. After 1 h of incubation at 37° in 5% CO_2, the suspension containing virus and antibody is removed, and 1% agarose (*see* **Note 4**) in medium (minimum essential medium, 0.2% BSA, 1 μg/ml TPCK-treated trypsin) is added to each well.

Table 2
Microneutralization assay: neutralizing antibody titers against PR8 influenza virus

Groups	Titers (2 weeks after the third immunization)
EFn-3×M2e-HA2/PA	<10
EFn-3×M2e-HA2	<10
PR8	≥2560
PBS	<10

The antibodies produce after vaccination with EFn-3×M2e-HA2/PA or EFn-3×M2e-HA2 could not prevent the Influenza virus infection to Madin–Darby canine kidney (MDCK) cells. (Adapted from Arévalo et al. [4])

6. After 18–20 h, the agarose is removed, and the cells are fixed with ethanol and stained with Coomassie blue.
7. Count the number of plaques per well and determine the antibody titer (Table 2).

3.2.4 T Cell Immune Responses by Detection of the Stimulated T Cell Population

1. Humanely euthanize the vaccinated mice using cervical dislocation after anaesthetizing with 100 μl of 80 mg/kg ketamine and 6 mg/kg xylazine via intraperitoneal injection.
2. Under sterile conditions collect the splenocytes into a 15-ml centrifuge tube containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (DMEM-10).
3. Disrupt the cells by straining through a 100-μm nylon mesh cell strainer.
4. Remove the red cells with ACK lysis buffer, followed by a washing step, and transfer the cells to a 50-ml centrifuge tube.
5. Distribute 2×10^6 cells between samples for unstimulated and stimulated with EFn-3×M2e-HA2 plus PA or PA only, phytohemagluttinin, or 10 ng of PMA/100 ng ionomycin (*see* **Note 5**).
6. Analyze stimulation of the T cell population after 5 days in the presence of antigens. Cells are resuspended in BD FACS lysis buffer, diluted 1:10 in water, and incubated for 10 min at room temperature. Wash the cells and resuspend in 0.5 ml PBS, then add 0.5 ml BD Perm2 buffer diluted 1:10 in water, and incubate for 10 min at room temperature. Stain for 30 min with antibodies targeting T cell markers.
7. Read the samples in an FACSCanto II flow cytometer and analyze the data with FLOWJO v10 software (FlowJo, Ashland, OR; Table 1).

3.2.5 Tetramers and Staining

1. The following tetramers were obtained through the NIH Tetramer Core Facility at Emory University (Atlanta, GA): The MHC Class II tetramer I-A(d) / SLLTEVETPIRNEWGS and MHC Class II tetramer H-2K(d)/VETPIRNEW (influenza epitope) are labeled with phycoerythrin and allophycocyanin.
2. Stain 1×10^6 splenic mononuclear cells/ml with 1 μg MHC class II tetramer and incubate for 3 h at 37°. Wash the collected samples by centrifugation (using staining buffer at $800 \times g$ for 5 min).
3. Stain the cells with anti-mouse CD4–allophycocyanin and CD3–Alexa Fluor 700 for 30 min at room temperature in the dark.
4. For MHC class I experiments, label the cells with a 1:100 dilution of tetramer for 30 min at room temperature. After washing, stain with anti-mouse CD8–phycoerythrin and CD3–FITC (BD Biosciences) for 30 min at room temperature in the dark.
5. Wash the samples with staining buffer at $800 \times g$ for 5 min and resuspend in 300 μl of 2% formaldehyde in PBS.
6. Read the samples in an FACSCanto II flow cytometer and analyze the data with FLOWJO v10 software (FlowJo, Ashland, OR).

3.2.6 Lethal Challenge with Influenza Viruses

1. Challenge the vaccinated mice with H1N1 influenza virus PR8 2 weeks after administration of the third vaccine dose (*see* **Note 2**).
2. Weigh each mouse and then again after being anaesthetized with ketamine and xylazine.
3. Challenge the mice intranasally with 2×10^5 PFU of live A/PR/8/34 virus in 40 μl.
4. Challenge the mice intranasally with heterologous 1×10^5 PFU of live A/CA/4/09 virus in 40 μl.
5. Monitor the mice for signs of illness and weigh daily for 21 days postinoculation.
6. Death or 30% weight loss is considered an endpoint for survival analysis.
7. The results are showing in the Fig. 3. Survival of vaccinated mice following lethal challenge with influenza A. PR8 (H1N1) and B with CA09 virus.

3.2.7 Lethal Challenge with B. anthracis *Sterne Spores*

1. A/J (6–8 week) mice are sensitive to receiving an infection with *B. anthracis* Sterne spores (used to vaccinate military personal). These mice lack complement, making them susceptible to lethal challenge.

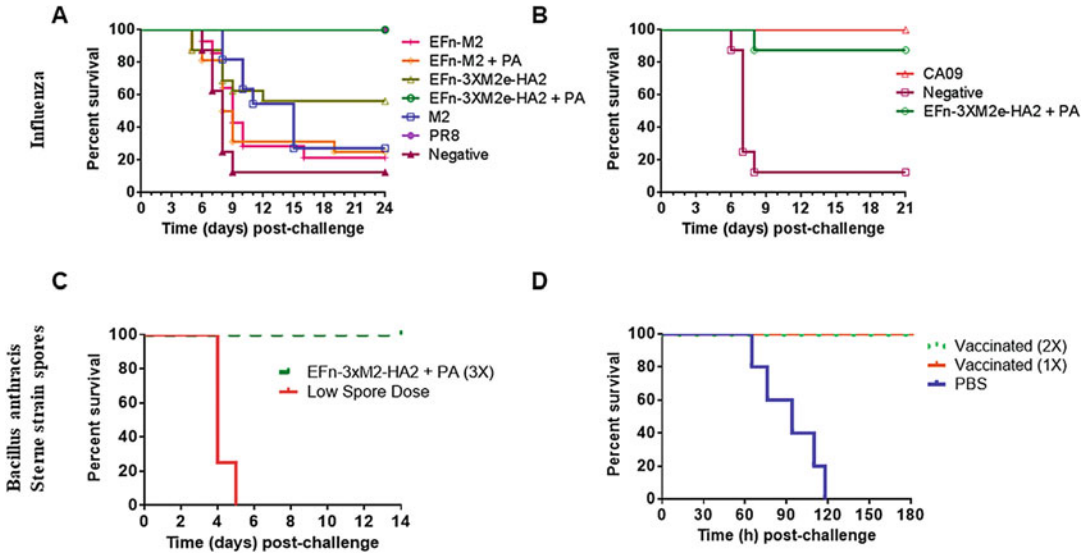

Fig. 3 Survival of vaccinated mice following lethal challenge with influenza virus. Immunized and naive (negative control) mice were challenged with 2×10^5 PFU of PR8 (H1N1) virus. Mice had been vaccinated with (**a**) EFn-M2 ($n = 14$), EFn-M2 + PA ($n = 16$), EFn-3×M2e-HA ($n = 16$), EFn-3×M2e-HA2 + PA ($n = 14$), or left unvaccinated ($n = 16$). For mice immunized with M2 or PR8, $n = 11$. Kaplan–Meier survival curves of vaccinated mice, which represents the results of two separate experiments. (**b**) Mice were immunized with EFn-3×M2e-HA2 + PA as before or with a low-dose of CA09 virus. Naive and immunized groups ($n = 8$ per group) were then challenged with 1×10^5 PFU of CA09 virus. (**c**) These mice were then challenged with 5×10^4 of *B. anthracis* Sterne spores. (**d**) The EFn-3×M2e-HA2 dose response against lethal challenge with 1.8×10^5 spores. Kaplan–Meier survival analysis. (Adapted from Arévalo et al. [4])

2. Immunize the mice three times with 30 μg EFn-3×M2e-HA2 plus 60 μg PA or inject subcutaneously once with 50 spores from the Colorado serum Company.
3. Two weeks after the final immunizations, mice are subcutaneously challenged with 5×10^4–1.8×10^5 spores. Monitor for signs of illness and weigh for up to 14 days postinoculation [7].
4. The results are showing in the Fig. 3. Survival of vaccinated mice following lethal challenge. These mice were then challenged with 5×10^4 of *B. anthracis* Sterne spores (Fig. 3c). The EFn-3×M2e-HA2 dose response against lethal challenge with 1.8×10^5 spores (Fig. 3d).

4 Notes

1. Previous immunization mix EFn-3×M2e-HA2 and PA and leave for 10 min to interact with and carried into antigen-presenting cells.
2. The intranasal immunization should be slow with small drops (3 μl) since the mice could die by drowning.

3. Mouse serum samples should be inactivated at 56 °C for 30 min to test with the influenza virus microneutralization assay, since the complement factors could affect the results.
4. Use low melting agarose (Gibco® 4% Agarose) with fluid at 37 °C to avoid damaging the cells and allowing the grow of the virus into the MDCK cells.
5. Include in the T cell responses assays other controls such as a group of mock-vaccinated (PBS) mice and PA to account for any artifacts from the culture.

References

1. Young JA, Collier RJ (2007) Anthrax toxin: receptor binding, internalization, pore formation, and translocation. Annu Rev Biochem 76:243–265
2. Tonello F, Montecucco CJ (2009) The anthrax lethal factor and its MAPK kinase-specific metalloprotease activity. Mol Aspects Med 30 (6):431–438
3. Xu Q, Zeng M (2008) Detoxified lethal toxin as a potential mucosal vaccine against anthrax. Clin Vaccine Immunol 15(4):612–616
4. Arévalo MT, Li J, Diaz-Arévalo D, Chen Y, Navarro A, Wu L et al (2017) A dual purpose universal influenza vaccine candidate confers protective immunity against anthrax. Immunology 150(3):276–289
5. Meng Q, Liu G, Liu Y, Deng X, Wang W, Xu K et al (2015) A broad protection provided by matrix protein 2 (M2) of avian influenza virus. Vaccine 33(31):3758–3765
6. Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC (1998) Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell 95 (3):409–417
7. Edwards M, Dimmock NJ (2000) Two influenza A virus-specific Fabs neutralize by inhibiting virus attachment to target cells, while neutralization by their IgGs is complex and occurs simultaneously through fusion inhibition and attachment inhibition. Virology 278 (2):423–435
8. Throsby M, van den Brink E, Jongeneelen M, Poon LL, Alard P, Cornelissen L et al (2008) Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One 3(12):e3942
9. Wang TT, Tan GS, Hai R, Pica N, Ngai L, Ekiert DC et al (2010) Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. Proc Natl Acad Sci U S A 107 (44):18979–18984
10. Turnbull P, Broster MG, Carman JA, Manchee RJ, Melling J (1986) Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Infect Immun 52(2):356–363
11. Ingram RJ, Metan G, Maillere B, Doganay M, Ozkul Y, Kim LU et al (2010) Natural exposure to cutaneous anthrax gives long-lasting T cell immunity encompassing infection-specific epitopes. J Immunol 184(7):3814–3821
12. Quesnel-Hellmann A, Cleret A, Vidal DR, Tournier JN (2006) Evidence for adjuvanticity of anthrax edema toxin. Vaccine 24 (6):699–702
13. Duverger A, Jackson RJ, Van Ginkel FW, Fischer R, Tafaro A, Leppla SH et al (2006) *Bacillus anthracis* edema toxin acts as an adjuvant for mucosal immune responses to nasally administered vaccine antigens. J Immunol 176 (3):1776–1783
14. Zeng M, Xu Q, Hesek ED, Pichichero ME (2006) N-fragment of edema factor as a candidate antigen for immunization against anthrax. Vaccine 24(5):662–670
15. Li J, Arévalo MT, Chen Y, Posadas O, Smith JA, Zeng M (2014) Intranasal immunization with influenza antigens conjugated with cholera toxin subunit B stimulates broad spectrum immunity against influenza viruses. Hum Vaccin Immunother 10(5):1211–1220

Chapter 23

Needleless or Noninvasive Delivery Technology

Akshay Datey, Jagadeesh Gopalan, and Dipshikha Chakravortty

Abstract

Injections of drugs or vaccines have become an indispensable part of living systems. Introduction to injections begins from the vaccination regimen at the neonatal stage and continues throughout the life span of an individual. Conventionally, injections are administered using hypodermic needles and syringes. These usually inject the liquid in the muscle, thus making intramuscular injections the most common form of administration. Although hypodermic syringes have been a clinician's tool in global vaccination efforts, they also have a set of undesirable characteristics. Pathogen transmission in case of HIV and HBV is one of the deadliest disadvantages of the needle-based injection system. Generation of plastic wastes in clinics, needlestick injury, and most importantly, pain associated with needle-based injections are a few more reasons of concern. In light of these issues, developing needle-free injection systems has excited researchers across the globe since the 1950s. Significant advancement has been reported in this field and various needle-free injection systems have been developed and are in clinical practice. This article briefly describes the history of needle-free injection systems and provides a detailed account of a few well-known methods of needle-less injections available.

Key words Needle-free, Injections, Vaccine, Nanoparticle, Microinjections

1 Introduction

Vaccination is most commonly administered using syringes and needles. Although it is an age-old practice which has proven its efficacy in clinics worldwide, needle-based methods are not fool-proof [1]. Several shortcomings of which include needle phobia, accidental needle-stick injury, and improper disposal and reuse of needles and syringes which leads to transmission of infections which include HIV and hepatitis B virus (HBV) [2]. Development of needle-free methods of injection has been of interest to researchers across the globe. Several methods have been reported to be efficient in delivering therapeutics and vaccines [3]. Needle-free liquid jet injections have the highest success rate and the longest history in delivering therapeutics to humans and animals so far [4]. Liquid jet injectors have been successfully demonstrated to be able to deliver several macromolecules, including vaccines, insulin, and growth

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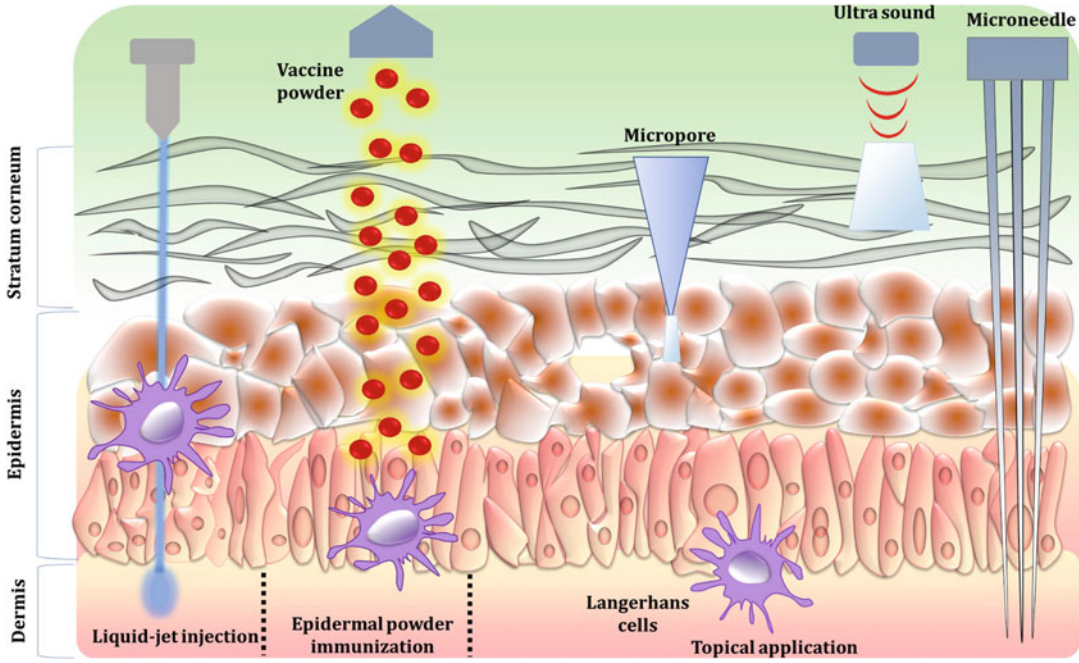

Fig. 1 A schematic of skin cross section depicting skin stratification. Different routes of immunization are highlighted. (**a**) Liquid-jet injection, (**b**) epidermal powder injection and (**c**) topical application for vaccine delivery [1]

hormones in many patients [5, 6] with erythropoietin [7] and interferon [8, 9] in a few cases. Anesthetic agents like lidocaine, midazolam, and ketamine have also been delivered using liquid jet injectors [10–12]. Apart from liquid jet injectors, there have been considerable developments in technologies which include epidermal powder injections, ultrasound-based needle-free injectors, and shock wave-based needle-free vaccine delivery systems [1, 2, 13]. Figure 1 depicts the cross section characteristics of human skin and various routes of needle-free vaccine delivery options.

1.1 Types of Needle-Free Delivery

The following are categories of needle-free vaccination/immunization: liquid-jet injection, epidermal powder immunization topical application, and mucosal immunization.

1.1.1 Liquid-Jet Injections

Jet injection is the oldest method of needle-free immunization, the origin of which can be traced back to the 1800s [14]. Aquapuncture was the first jet injection system reported in the literature [15]. A liquid-jet injector uses the kinetic energy of a high-velocity vaccine jet (typically more than 100 m/s) with a diameter that ranges from 76 to 360 μm, which is smaller than the outer diameter of a standard hypodermic needle (810 μm for a 21 G needle) [16]. Depending on the jet velocity, liquid jets penetrate different depths in the body ranging from skin and subcutaneous tissue to

muscle. Skin is a particularly attractive target for vaccine delivery as it is enriched with resident immune cells, the Langerhans cells [17]. These cells are efficient antigen presenting cells which help in mounting a robust immune response against any antigen [18].

1.1.2 Particle Bombardment of the Skin

Ballistic methods or particle-based methods accelerate powdered vaccines to high velocities that drive them into the skin. Usually, stratum corneum and layers of dermis are the sites of their deposition. Such a method of delivering vaccines into the epithelium is called epidermal powder immunization (EPI) [19]. This technique was developed in 1986, for the delivery of DNA-coated metal particles of ~1 μm in diameter into plants to genetically modify them, and it was known as the gene gun [20]. The ballistic method was further developed to deliver conventional and DNA vaccines to humans [21]. Ballistic methods typically deliver to the superficial layer of the skin thereby targeting Langerhans cells [22].

1.1.3 Topical Application to the Skin

Application of medicine to the skin is the easiest method of drug delivery. This method has been used for thousands of years. Systemic drug delivery through the skin developed with the use of transdermal scopolamine patches for treating motion sickness [23]. Administration of vaccines via the dermal route dates to more than 1000 years. Immunization against smallpox was practiced in India by scratching dry scabs from smallpox lesions onto the skin of healthy individuals [24]. Even today, the skin remains the site for immunization against smallpox using the bifurcated needle [25]. Although this method has a historical role, only recent times have drawn attention of the healthcare community to the use of topical vaccine application as a general mode of immunization [26].

1.1.4 Topical Adjuvants

Topical application of adjuvants such as cholera toxin (CT) together with the vaccine on the skin is known to generate a robust mucosal and systemic immune response [27]. This is an extensively studied method of all topical-immunization methods. Topical application of CT provides the required activation signal for Langerhans cells to mature and thereby transforming them into potent antigen-presenting cells [28]. These cells then prime the immune response to co-administered vaccines. However, the role of CT, a relatively large protein (86 kDa), which diffuses across the stratum corneum, remains unclear [29]. However, using emery paper (abrasive paper) to disrupt stratum corneum has been demonstrated to add an adjuvant effect to the application of vaccine and achieve efficient immune responses [30].

1.1.5 Colloidal Carriers

Colloidal carriers facilitate the encapsulation of vaccines for topical delivery. Studies have reported the use of colloidal carriers for topical delivery of vaccines, all in animals. Topical application of Tetanus Toxoid (TT) encapsulated in lipid vesicles, after booster immunization, has been shown to elicit a specific immune response (IgG) comparable to that produced by the conventional intramuscular injections of alum adsorbed TT [31]. DNA vaccines have also been delivered using a lipid-based system in animals. Topical application of this DNA–lipid vaccine can result in generating both humoral and cell-mediated immune responses. Several other combinations like cationic nanoparticles coated with DNA and microemulsion systems have been used for DNA immunization in animals [32]. The precise mechanisms by which colloidal carriers penetrate the stratum corneum and the feasibility of translating this concept to human use remains a topic of research.

1.1.6 Physical Methods

Microneedles, tape stripping, ultrasound, microporation or electroporation, and shock waves are the physical methods that have been used to deliver vaccines across the skin [1]. Most of these techniques have recently emerged as successful immunization techniques. Microporation involves focused deposition of thermal energy into the skin through an electrically heated element to remove small areas of the stratum corneum [33]. This leads to exposing the immunocompetent epidermis. Application of an adenoviral vector to microporated skin with hair removed resulted in 10- to 100-fold greater cellular and humoral immune responses as compared to unshaved skin. Microneedles are solid and hollow arrays of micrometer-scale silicon projections. They have also been used to carry out topical immunization with various vaccines [34]. Microprojection arrays have been demonstrated to deliver naked plasmid DNA, inducing stronger and less variable immune responses as compared to needle-based injections. Full seroconversion, which is the time required for eliciting a maximum response against an antigen, can be rapidly achieved with a smaller number of immunizations with these methods [35]. In another study, shock waves have been employed to deliver vaccines to the epidermis. Shock waves accelerate the vaccine/drug to form a high velocity liquid jet [2, 13]. This jet gets deposited into the skin making it accessible to the Langerhans cells for further immune processing (Fig. 1). Several of the protocols reported in the open literature have been described in the following section. Protocols have been written in a generalized manner for further adaptation by the reader.

2 Materials

2.1 Liquid Jet Injections

1. Spring powered liquid jet injectors.
2. DNA vaccine.
3. Anesthesia—Ketamine and Xylazine.
4. Microfine insulin syringe (29 G).
5. 70% ethanol.
6. 0.9% NaCl.
7. Fine capillary tubes for orbital puncture.

2.2 Particle Bombardment of Skin

1. Particles of polystyrene, gold of known radii.
2. Spermidine for DNA coating.
3. Commercial gene gun.

2.3 Physical Methods

1. Microprojection array.
2. 1%, 5%, and 20% solutions of OVA in phosphate buffered saline.
3. Polyisobutylene adhesive.
4. Isoflurane 3–3.5%.

2.4 Microporation

1. Microporation prototype.
2. Vaccine sample.

2.5 Vaccine Delivery Using Shock Waves

1. Shock wave device as described.
2. Vaccines to be injected.
3. 70% ethanol for skin surface sterilization
4. Hair removal cream/shaver to clear fur off animals.

2.6 Vaccine Delivery by Microneedles

1. Microneedle assemble and array.
2. Vaccine sample.
3. 70% ethanol.

3 Methods

3.1 Liquid Jet Injections

1. Spring powered jet injectors (Isajet™, Prolitec, Aouste/Sye, France) are used for the intradermal administration of DNA solutions [36].
2. A low force Isa10 injector is used in mice and the Isa40 injector, a jet-gun designed for human use, is used in monkeys.
3. Solutions to be injected are filled into the 5 ml glass chamber of the Isa10 or into a syringe which adapts onto the Isa40 injector.

Both injectors are equipped with a nozzle (rectangular for Isa10 and circular for Isa40) comprising five orifices.

4. These systems deliver fixed volumes of 100 µl per shot. The void volume corresponding to the space between the reservoirs and nozzles is of about 0.8 ml in Isa10 and 1.5 ml in Isa40.

3.1.1 Animal Experiments in Mice

1. Mice are anaesthetized by i.p. injection of a solution containing 8 mg/ml ketamine (Imalgène 500, Rhône-Mérieux) and 1.6 mg/ml xylazine (Rompun 2%, Bayer) in a total volume of 0.2 ml of saline.
2. Intramuscular injections: 50 µl of DNA are injected into the quadriceps muscle by using a Micro-Fine insulin syringe equipped with a 29 G1/2 needle.
3. Intradermal injections: 100 µl of DNA is injected into five spots of dorsal skin by using the Isa10 injector. The site of injection located all along the spinal cord is shaved and swabbed with ethanol prior to the injection. For serum antibodies titration, mice are bled by retroorbital sinus puncture, the bleedings could clot, centrifuged and the sera were stored at −20 °C until the assay (*see* **Note 1**).

3.1.2 Animal Experiments in Non-human Primates

1. Monkeys are anaesthetized by intramuscular administration in the thigh of 50 mg of ketamine (0.5 ml of Imalgene 1000, Rhône-Mérieux).
2. Intramuscular injections: 0.5 ml of DNA in 0.9% NaCl is injected into the quadriceps muscle by using a 3 ml syringe equipped with a 26 G3/8 needle (*see* **Note 2**).
3. Intradermal injections: 100 µl of DNA in 0.9% NaCl is injected into five spots of thigh skin by using the Isa40 injector. The injection site is shaved and swabbed with ethanol prior to the injection (*see* **Note 3**).

3.2 Particle Bombardment of Skin

1. For extracellular delivery, the following inert microparticles 8.5 ± 0.4, 11 ± 1.5, 20.5 ± 1.2, and 52.6 ± 2.0 µm polystyrene spheres (mean radius ± standard deviation); 2.6 ± 0.4, 10 ± 0.7, and 20 ± 1.4 µm glass spheres; and stainless-steel beads are manually sieved to bands of radii 10–19, 19–27, and 27–38 µm.
2. The micro-particles used for intracellular studies are gold with radii of 0.89 ± 0.58, 1.12 ± 0.56, and 1.52 ± 0.58 µm and a measured density of between 16.8 and 18.2 g/cm^3. Gold payloads were either 0.5, 0.75, or 1.5 mg.
3. DNA vaccines to be injected are coated on the particles by incubating with spermidine for 2 h prior to accelerating them using commercially available gene guns (*see* **Note 4**).

3.3 Physical Methods

3.3.1 Microprojection Array to Deliver OVA [37]

1. Microprojection arrays are produced using a controlled manufacturing process. The finished microprojection array is a titanium screen with a defined microprojection pattern, density, and length. Microprojection arrays used are generally 1 or 2 cm^2 in area.
2. Microprojection arrays are coated by immersion in a 1%, 5%, or 20% sterile aqueous solution of OVA (w/v, Grade V, Sigma Chemical Co., St. Louis, MO). Excess solution is removed by forced air and the arrays are air-dried for 1 h at ambient conditions.
3. The number of OVA coated on the microprojection arrays is determined using FITC–OVA against known standards by luminescence spectrometry (excitation 494 nm, emission 520 nm).
4. Microprojection arrays coated with FITC–OVA are also inspected visually by fluorescence microscopy before and after skin application.
5. After coating and drying, the microprojection array is affixed to an adhesive patch comprised of a low-density polyethylene backing with a polyisobutylene adhesive. The final systems should have a total patch area of 8 cm^2 containing either a 1 or 2 cm^2 area microprojection array.
6. Animals are anesthetized using a gas delivery system (isoflurane 3–3.5%, O_2 2–2.5 L/min) and skin treatment sites (lateral area of the thorax) are cleaned with isopropyl alcohol wipes (70%) and allowed to dry.
7. The skin sites should be lightly stretched manually during the system application with an impact applicator. After application, the system is left on the skin for specified times for efficient delivery.

3.3.2 Microporation

1. A prototype microporation system (Altea) [38] is used to create an array of microscopic pores in the stratum corneum of the mouse abdomen.
2. Each tiny spot of stratum corneum is removed by the application of focused thermal energy. Electrically heated wires of small diameter are used for skin removal.
3. The microporation tip comprises of a set of 80 μm diameter tungsten wires strapped over the edge of a ceramic substrate and electrically connected to the control circuitry via copper traces on each side of the substrate.
4. The temperature of the tip to be in contact with the skin is calibrated with the use of an optical calibrator device. The microporation parameters remain the same for various mice models tested.

5. The prototype microporation system parameters are set to create an array of micropores with the following details. Pore density of 75 micropores/cm^2, 80 μm wide, 300 μm long, and 40–50 μm in depth (*see* **Note 5**).

3.3.3 Vaccine Delivery Using Shock Waves

1. Shock wave-based needle-free device is used as described [2, 13].
2. Hair on the skin is removed in the abdomen region using a hair clipper or commercial hair removal cream.
3. The vaccine to be delivered is loaded in the sample holder.
4. The delivery device is placed in a perpendicular position against the shaved abdominal skin of the mice.
5. Shock wave formation is triggered, and liquid jet is accelerated to high velocity.
6. Multiple shots are administered to increase the volume of injection (*see* **Note 6**).

3.3.4 Vaccine Delivery Using Microneedles [39]

1. Mice are anesthetized and their abdominal hair is closely trimmed using an electric trimmer (no scraping of the skin surface).
2. After trimming, the skin is wiped clean with alcohol and lightly dried with a lint-free tissue. The mouse is then placed on the animal interface plate with the abdomen facing down and is positioned over an opening to allow access to the microporation tip assembly. A square array of micropores is then created and is loaded with the drug to be injected (*see* **Note 7**).

4 Notes

1. Serum should be free of hemoglobin. Hemolysis should be avoided for accurate results.
2. Fine gauge needle should be used to avoid collateral damage and hostile immune response.
3. Appropriate care should be taken to protect the integrity of DNA vaccine. It should not be vigorously pipetted/handled to prevent shearing.
4. Spermidine treatment is extremely crucial for DNA binding to gold nanoparticles. Improper incubation times might lead to infective DNA-particle binding.
5. The pore size of microinjector determines the velocity and depth of penetration of the vaccine. Pore size should be optimized according to the skin type and thickness for efficient vaccine delivery.

6. Volume of injection using this system is less. Care should be taken to increase the volume of injection by including multiple shots for delivering an effective dose of vaccine.
7. Needle-less drug/vaccine delivery involves novel methods for delivery. The major limitation and point of concern is the amount of vaccine delivered. The depth of penetration of the vaccine should not be greater than 300 μm from the skin surface. Using an array of injectors is a viable option to achieve maximum vaccine delivery at appropriate depths in the skin.

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References

1. Mitragotri S (2005) Immunization without needles. Nat Rev Immunol 5:905–916
2. Jagadeesh G, Prakash GD, Rakesh SG, Allam US, Krishna MG, Eswarappa SM, Chakravortty D (2011) Needleless vaccine delivery using micro-shock waves. Clin Vaccine Immunol 18:539–545
3. Chung SW, Hil-lal TA, Byun Y (2012) Strategies for non-invasive delivery of biologics. J Drug Target 20:481–501
4. Anselmo AC, Gokarn Y, Mitragotri S (2019) Non-invasive delivery strategies for biologics. Nat Rev Drug Discov 18:19–40
5. Bareille P, MacSwiney M, Albanese A, DeVile C, Stanhope R (1997) Growth hormone treatment without a needle using the Preci-Jet 50 transjector. Arch Dis Child 76:65–67
6. Bremseth DL, Pass F (2001) Delivery of insulin by jet injection: recent observations. Diabetes Technol Ther 3:225–232
7. Petersen J, Jani A (2001) Delivery of erythropoietin with a needleless injection system during hemodialysis maintains plasma levels. ASAIO J 47:608–610
8. Rogge MC, Charenkavanich S, DiBiase M, Jones W, Knox SJ, Alam JJ (1998) Impaired bioavailability of interferon beta-1a when administered intramuscularly by needle-free injection. Drug Deliv 5:275–280
9. Jaber A, Bozzato GB, Vedrine L, Prais WA, Berube J, Laurent PE (2008) A novel needle for subcutaneous injection of interferon beta-1a: effect on pain in volunteers and satisfaction in patients with multiple sclerosis. BMC Neurol 8:38
10. Lysakowski C, Dumont L, Tramer MR, Tassonyi E (2003) A needle-free jet-injection system with lidocaine for peripheral intravenous cannula insertion: a randomized controlled trial with cost-effectiveness analysis. Anesth Analg 96:215–219, table of contents
11. Bennett J, Nichols F, Rosenblum M, Condry J (1998) Subcutaneous administration of midazolam: a comparison of the Bioject jet injector with the conventional syringe and needle. J Oral Maxillofac Surg 56:1249–1254
12. Domino EF, Zsigmond EK, Kovacs V, Fekete G, Stetson P (1997) A new route, jet injection for anesthetic induction in children. 3. Ketamine pharmacokinetic studies. Int J Clin Pharmacol Ther 35:527–530
13. Subburaj J, Datey A, Gopalan J, Chakravortty D (2017) Insights into the mechanism of a novel shockwave-assisted needle-free drug delivery device driven by in situ-generated oxyhydrogen mixture which provides efficient protection against mycobacterial infections. J Biol Eng 11:48
14. Baxter J, Mitragotri S (2006) Needle-free liquid jet injections: mechanisms and applications. Expert Rev Med Devices 3:565–574
15. Koenig HM, Paisansathan C, Albrecht RF 2nd, Zsigmond EK (2004) Jet injection of local anesthetic decreases pain of arterial cannulation

in awake neurosurgical patients. J Neurosurg Anesthesiol 16:156–159

16. Seehanam W, Pianthong K, Sittiwong W, Milton B (2014) Injection pressure and velocity of impact-driven liquid jets. Eng Comput 31:1130–1150
17. Klareskog L, Tjernlund UM, Forsum U, Peterson PA (1977) Epidermal Langerhans cells express ia antigens. Nature 268:249–250
18. Stingl G, Wolffschreiner EC, Pichler WJ, Gschnait F, Knapp W, Wolff K (1977) Epidermal Langerhans cells bear Fc and C3 receptors. Nature 268:245–246
19. Weissmueller NT, Marsay L, Schiffter HA, Carlisle RC, Rollier CS, Prud'homme RK, Pollard AJ (2017) Alternative vaccine administration by powder injection: needle-free dermal delivery of the glycoconjugate meningococcal group Y vaccine. PLoS One 12:e0183427
20. Ismagul A, Yang N, Maltseva E, Iskakova G, Mazonka I, Skiba Y, Bi H, Eliby S, Jatayev S, Shavrukov Y, Borisjuk N, Langridge P (2018) A biolistic method for high-throughput production of transgenic wheat plants with single gene insertions. BMC Plant Biol 18:135
21. Aravindaram K, Yang NS (2009) Gene gun delivery systems for cancer vaccine approaches. Methods Mol Biol 542:167–178
22. Kendall M, Rishworth S, Carter F, Mitchell T (2004) Effects of relative humidity and ambient temperature on the ballistic delivery of micro-particles to excised porcine skin. J Invest Dermatol 122:739–746
23. Bailey PL, Streisand JB, Pace NL, Bubbers SJ, East KA, Mulder S, Stanley TH (1990) Transdermal scopolamine reduces nausea and vomiting after outpatient laparoscopy. Anesthesiology 72:977–980
24. Lofquist JM, Weimert NA, Hayney MS (2003) Smallpox: a review of clinical disease and vaccination. Am J Health Syst Pharm 60:749–756; quiz 757–758
25. Belongia EA, Naleway AL (2003) Smallpox vaccine: the good, the bad, and the ugly. Clin Med Res 1:87–92
26. Huang CM (2007) Topical vaccination: the skin as a unique portal to adaptive immune responses. Semin Immunopathol 29:71–80
27. Cope RB, Colditz IG (2000) Effect on systemic antibody concentrations of topical application of choleratoxin to skin of sheep. Aust Vet J 78:121–123
28. Price GA, McFann K, Holmes RK (2013) Immunization with cholera toxin B subunit induces high-level protection in the suckling mouse model of cholera. PLoS One 8:e57269
29. Eriksson K, Fredriksson M, Nordstrom I, Holmgren J (2003) Cholera toxin and its B subunit promote dendritic cell vaccination with different influences on Th1 and Th2 development. Infect Immun 71:1740–1747
30. Kim YC, Prausnitz MR (2011) Enabling skin vaccination using new delivery technologies. Drug Deliv Transl Res 1:7–12
31. Gupta PN, Mishra V, Singh P, Rawat A, Dubey P, Mahor S, Vyas SP (2005) Tetanus toxoid-loaded transfersomes for topical immunization. J Pharm Pharmacol 57:295–301
32. Zhao Z, Wakita T, Yasui K (2003) Inoculation of plasmids encoding Japanese encephalitis virus PrM-E proteins with colloidal gold elicits a protective immune response in BALB/c mice. J Virol 77:4248–4260
33. Lim JY, Park SH, Jeong CH, Oh JH, Kim SM, Ryu CH, Park SA, Ahn JG, Oh W, Jeun SS, Chang JW (2010) Microporation is a valuable transfection method for efficient gene delivery into human umbilical cord blood-derived mesenchymal stem cells. BMC Biotechnol 10:38
34. Bramson J, Dayball K, Evelegh C, Wan YH, Page D, Smith A (2003) Enabling topical immunization via microporation: a novel method for pain-free and needle-free delivery of adenovirus-based vaccines. Gene Ther 10:251–260
35. Raphael AP, Crichton ML, Falconer RJ, Meliga S, Chen XF, Fernando GJP, Huang H, Kendall MAF (2016) Formulations for microprojection/microneedle vaccine delivery: structure, strength and release profiles. J Control Release 225:40–52
36. Kwilas S, Kishimori JM, Josleyn M, Jerke K, Ballantyne J, Royals M, Hooper JW (2014) A hantavirus pulmonary syndrome (HPS) DNA vaccine delivered using a spring-powered jet injector elicits a potent neutralizing antibody response in rabbits and nonhuman primates. Curr Gene Ther 14:200–210
37. Matriano JA, Cormier M, Johnson J, Young WA, Buttery M, Nyam K, Daddona PE (2002) Macroflux (R) microprojection array patch technology: a new and efficient approach for intracutaneous immunization. Pharm Res 19:63–70
38. Arora A, Prausnitz MR, Mitragotri S (2008) Micro-scale devices for transdermal drug delivery. Int J Pharm 364:227–236
39. Mikszta JA, Dekker JP, Harvey NG, Dean CH, Brittingham JM, Huang J, Sullivan VJ, Dyas B, Roy CJ, Ulrich RG (2006) Microneedle-based intradermal delivery of the anthrax recombinant protective antigen vaccine. Infect Immun 74:6806–6810

Chapter 24

Lactic Acid Bacteria as Delivery Vehicle for Therapeutics Applications

Viviane Lima Batista, Tales Fernando da Silva, Luis Cláudio Lima de Jesus, Ana Paula Tapia-Costa, Mariana Martins Drumond, Vasco Azevedo, and Pamela Mancha-Agresti

Abstract

Lactic acid bacteria comprise a large group of Gram-positive organisms capable of converting sugar into lactic acid. They have been studied due to their therapeutic potential on the mucosal surface. Among the species, *Lactococcus lactis* is considered the model bacterium and it has been explored as an important vehicle for providing therapeutic molecules and antigens in the mucosa. They can be genetically engineered to produce a variety of molecules as well as deliver heterologous DNA and protein. DNA vaccines consist of the administration of a bacterial plasmid under the control of a eukaryotic promoter encoding the antigen of interest. The resulting proteins are capable of stimulating the immune system, becoming a promising technique for immunization against a variety of tumors and infection diseases and having several advantages compared to conventional nucleic acid delivery methods (such as bioballistic delivery, electroporation, and intramuscular administration).

Key words Lactic acid bacteria, DNA vaccine, DNA delivery, Mucosal administration, *Lactococcus lactis*

1 Introduction

Lactic acid bacteria (LAB) comprises a large group of gram-positive and nonsporulating microorganisms with fermentative properties, having the capacity to convert sugar into lactic acid. The majority of LAB have a "Generally Recognized as Safe" (GRAS) status according to the United States Food and Drug Administration (US FDA), meeting the criteria to be considered safe for human consumption [1].

LAB has been intensively studied due to their potential therapeutic effects on mucosal surfaces and can also be genetically engineered to efficiently produce a large variety of molecules either for the delivery of DNA or heterologous proteins [2–5]. In this context, among the various species of LAB, *Lactococcus lactis* is

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considered the model organism and has been explored as a vehicle for the delivery of therapeutic molecules and antigens in the mucosa [4, 6].

DNA vaccines consist of the administration of a bacterial plasmid under the control of a eukaryotic promoter encoding the antigen of interest, which is then synthesized by the vaccinated individuals themselves and presented to the immune system [7]. These proteins are capable of stimulating the immune system, becoming a promising technique of immunization against a variety of pathogens and tumors [7, 8]. DNA vaccines have several advantages over other vaccination strategies such as the ability to induce humoral and cellular immunity, longer shelf life, ease of manufacture, storage, transport and rapid production, besides the gene vaccines are highly stable at room temperature and do not require refrigeration [7, 8].

There are many forms for delivery of DNA vaccine: bioballistic delivery through the skin, which is a method of epidermal DNA delivery where Langerhans and keratinocyte cells are directly transfected by bombardment of gold particles coated with DNA plasmids [9, 10]; electroporation technique (EP) based on the application of high voltage pulses in a tissue that generates pores in the cell membrane, allowing the DNA vaccine to enter the cytoplasm of cells, leading to an inflammatory process [11]; and the bacterial delivery presented above.

Intramuscular administration is the most common form used; it consists of the optimized gene sequence of interest using naked DNA delivery. However, due to its poor distribution in the body and rapid degradation, it requires higher amounts of plasmid [12, 13]. As the mucosal surfaces are vulnerable to infection by pathogenic microorganisms, the local mucosal immune responses are important for protection against different diseases [14]. Thus, the mucosal route of administration has been the most widely explored. Among the mucosal administration routes, the oral, nasal, sublingual, anal, and vaginal routes are the most commonly used.

This chapter will describe protocols for the preparation and mucosal administration (nasal, intragastric, and sublingual) in mice of a DNA vaccine using *Lactococcus lactis* as a delivery vehicle.

2 Materials

Prepare all solutions using distilled or sterile water. All culture medium must be sterilized by autoclavation (121 °C/15 psi/15 min), and the glucose solution must be filtered (0.22 μm filter) (*see* **Note 1**).

1. M17 Broth: 0.05% ascorbic acid, 0.5% lactose, 0.025% magnesium sulfate, 0.5% meat extract, 0.25% meat peptone (peptic), 1.9% sodium glycerophosphate, 0.5% soya peptone (papainic), 0.25% tryptone, 0.25% yeast extract.
2. M17 agar: 0.05% ascorbic acid, 0.5% lactose, 0.025%magnesium sulfate, 0.5% meat extract, 0.25% meat peptone (peptic), 1.9% sodium glycerophosphate, 0.5% soya peptone (papainic), 0.25% tryptone, 0.25% yeast extract, 1.5% agar.
3. Glucose solution 50%.
4. PBS 0.01 M (1×): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.
5. Bacterial samples (*L. lactis*).
6. Xylazine hydrochloride.
7. Ketamine.
8. Forceps.
9. Micropipette and tips.
10. 0.22 μm syringe filter.
11. Petri dishes.
12. Syringe.
13. Gavage needle (20–22 G).
14. Autoclave.
15. Spectrophotometer.
16. pH meter.
17. 30 °C incubator.
18. Refrigerated centrifuge.
19. Laminar flow cabinet.
20. Magnetic bar.
21. Magnetic stirrer.

3 Methods

3.1 M17 Broth Preparation (1 L)

1. Suspend 42.25 g of the mixture in 900 mL distilled water (the quantity can vary according to the manufacturer).
2. Add water q.s. (*Quantum satis*, "quantity required").
3. Heat, if necessary, to dissolve the medium completely.
4. Sterilize by autoclaving.
5. Add glucose to 0.5% (i.e., for 1 L of broth add 10 mL of glucose 50% (stock solution)) (*see* **Note 2**).
6. Store in a tightened container at 2–8 °C (*see* **Note 3**).

3.2 M17 Agar Preparation (1 L)

1. Prepare the solution as detailed above.
2. Before sterilizing add 15 g of agar.
3. Mix it well.
4. Sterilize it by autoclavation.
5. After autoclavation, let cool to approximately 50 °C.
6. Add the glucose to 0.5%.
7. Pour the liquid in the petri dishes inside a laminar flow cabinet (20–25 mL of medium per 90-mm petri dish).
8. Let the agar solidify and then close the petri dishes, turn them upside-down and store at 2–8 °C.

3.3 Glucose Solution 50% Preparation (100 mL)

This solution (stock solution) will be used by being added to the medium to reach the concentration of 0.5%.

1. Dissolve 50 g of glucose in 100 mL of water (with the help of a magnetic stirring bar and magnetic mixer).
2. Sterilize by filtering (0.22 μm) inside a laminar flow cabinet (*see* **Note 1**).
3. Store at 2–8 °C.

3.4 Phosphate-Buffered Saline 0.01 M (PBS 1×) Preparation (1 L)

1. Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of distilled water.
2. Add water q.s. for 1 L.
3. Adjust the pH to 7.4 with HCl 1 M solution.
4. Sterilize by autoclaving.

3.5 Preparation of the Bacterial Strain: Lactococcus Lactis

1. Inoculate 50 μL of bacteria suspension in 30 mL of M17 broth supplemented with glucose to 0.5% (bacteria stored at −80 °C with 25% glycerol).
2. Incubate the culture at 30 °C, without shaking, for 20 h.

3.6 Quantitative Growth Evaluation of Bacterial Culture

1. Measure the OD_{600} of the bacterial culture previously grown.
2. To measure the OD_{600} adjust the spectrophotometer to a wavelength reading of 600 nm.
3. Calibrate the instrument using the noninoculated broth as a blank.
4. Measure the absorbance of the overnight cultured sample.
5. Dilute the bacterial culture in M17 + Glucose 0.5% broth for $OD_{600} = 0.04$ (bacterial lag phase).
6. Incubate the bacteria at 30 °C without shaking.
7. Measure the OD_{600} of the growing bacterial cultures hourly (until reaching the stationary phase).

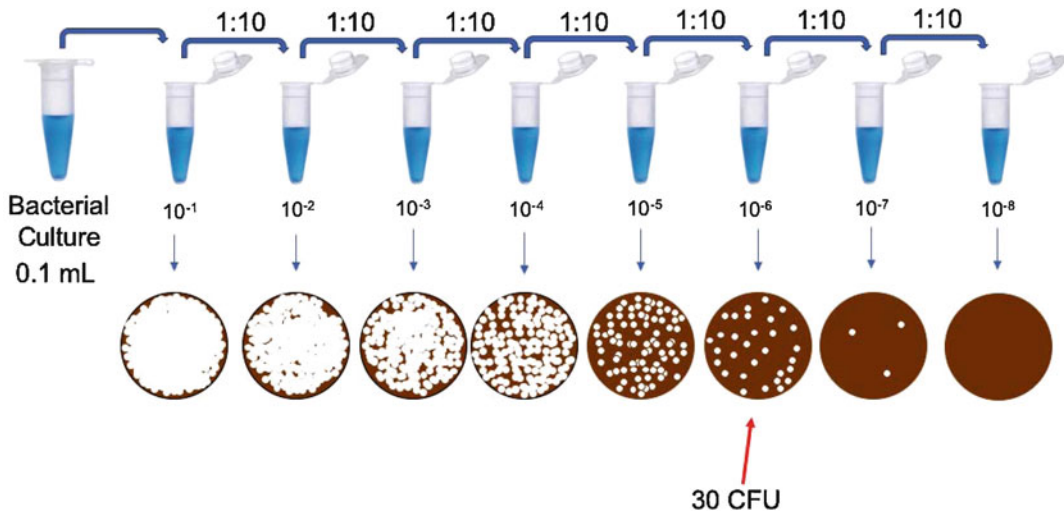

Fig. 1 Bacterial serial dilution. This scheme shows a logarithmic serial dilution, after each dilution, the tube contains ten times less bacteria than the previous. Pipette one part of the original solution and transfer it to nine parts of fresh medium, repeat this procedure for at least eight times. Collect 100 μL from each dilution and transfer it to a petri dish prepared with fresh agar medium. Spread the bacterial solution with Drigalski spatula and incubate it at 30 °C for 20 h. Count the number of colonies and calculate the bacterial concentration

8. From each measurement collect 100 μL of the bacterial culture to plate (after serial dilutions) and measure the number of colony-forming units (CFU) (Fig. 1) (*see* **Note 4**).

3.7 Serial Dilutions of Each Bacterial Sample Culture

1. Collect 100 μL of each sample from which the OD_{600} was measured.
2. Transfer this volume to 900 μL of fresh broth (dilution factor 10^{-1}).
3. Repeat the dilution process until reaching the dilution factor 10^{-8}.
4. Plate 100 μL of each dilution onto petri dishes with the help of a Drigalski spatula.
5. Incubate the petri dishes at 30 °C for 20 h.
6. Count the number of colonies to estimate the quantity of bacteria per mL in the original solution (Fig. 1) (Eq. 1) (*see* **Note 5**).

$$\text{CFU} = \frac{\text{number of colonies} \times \text{dilution factor}^{-1}}{\text{volume plated}} \quad (1)$$

7. After counting the number of bacterial colonies, the data will form a growth curve indicating in each OD_{600} the corresponding CFU (the result will be express in CFU/mL) (*see* **Note 6**).

3.8 Bacterial Doses Preparation for Mucosal Administration

Day 1

1. Add 50 μL of bacteria culture in 10 mL of M17 + Glucose 0.5% broth.
2. Incubate at 30 °C, without shaking, for 20 hours.

Day 2

1. Measure the OD_{600} of the bacterial culture previously grown.
2. Make a dilution for reaching the bacterial lag phase (based on the bacteria growth curve) (*see* **Notes 7** and **8**).
3. Incubate at 30 °C until the OD_{600} reaches the bacteria's exponential phase.
4. Centrifuge at 980 × *g* for 10 min at 4 °C.
5. Discard the supernatant.
6. Wash the pellet twice with PBS (0.01 M).
7. Repeat the **steps 4** and **5**.
8. Resuspend the pellet in 30 μL of cold sterile PBS (0.01 M).
9. Each microtube will contain one dose (*see* **Note 9**).
10. The dose can be used immediately after preparation or stored for future use at −80 °C for up to 5 days [5] (*see* **Note 10**).
11. Add PBS 0.01 M to the desired amount according to the chosen form of administration.
12. All procedures should be done in a sterile laminar flow.

3.9 Animal Restraints

One-hand restraint (Fig. 2).

1. Lift the mouse by holding the base of the tail and place it on the wire bar cage top or any similar rough surface (*see* **Note 11**).
2. With thumb and index finger hold the tip of the mouse's tail, tuck the tail between the third and fourth finger and pull back gently so the mouse grabs the surface.
3. With the second and third finger press gently the back of the mouse against the surface and with the thumb and index finger, grab the mouse scruff skin.
4. Be sure the grasp is firm and the mouse's head does not move [15].

Two-hand restraint (Fig. 3).

1. Lift the mouse by the base of the tail and place it on the wire bar cage top or any similar rough surface.
2. With the dominant hand hold the base of the tail (*see* **Note 11**).
3. With the thumb and index finger of the nondominant hand, grab the mouse scruff skin.

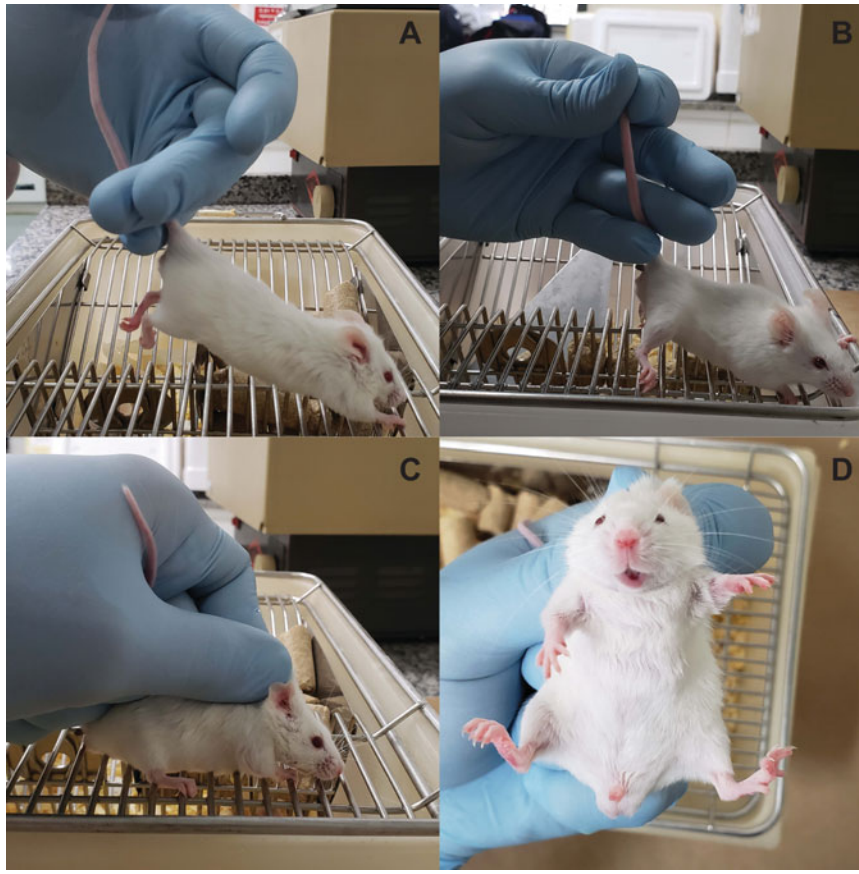

Fig. 2 One-hand mice restraint. Pick the mouse by the base of the tail allocating it in a rough surface (**a**), hold the tail with the index finger and thumb while tucking the base of the tail between third and fourth finger (**b**), press the mouse gently against the surface and, with the thumb and index finger, hold the skin of the back of its neck (**c**) and the restraining is completed (**d**)

4. Tuck the tail between the third and fourth finger of the non-dominant hand [15].

3.10 Sublingual Administration

Animal sedation is necessary for this procedure [16].

1. Sedate the animal.
2. To promote sedation of the animal, use a mixture of xylazine hydrochloride (10 mg/kg of body weight) and ketamine (100 mg/kg of body weight), or any other anesthetic authorized by the ethic committee.
3. With the animal restrained, inject the anesthetic mixture intraperitonially (volume calculated according to animal's weight) in the animal's right low quadrant of the abdomen (Fig. 4) (*see* **Note 12**).

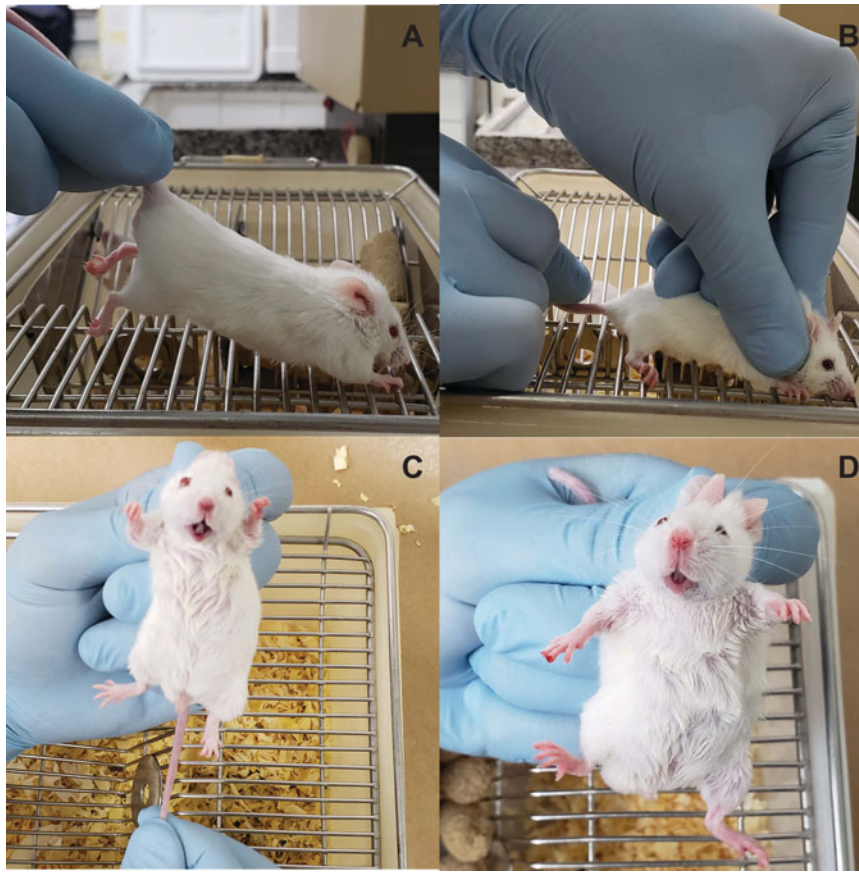

Fig. 3 Two-hand mice restraint. With the dominant hand pick the mouse by the base of the tail (**a**), with the nondominant hand press the mouse gently against the surface and, with the thumb and index finger, hold the skin of the back of its neck (**b**), raise the mouse and, with the dominant hand, tuck the tail between third and fourth finger of the nondominant hand (**c**) and the restraining is completed (**d**)

4. Guarantee the efficacy of the sedation by testing the mouse reflex to pain (i.e., by pressing the animal's feet and obtaining no reaction).
5. With the help of a forceps, hold the tongue maintaining the mouth open and keeping the sublingual region clear (Fig. 5).
6. With a micropipette, administer the dose (maximum of 10 μL per administration) under the tongue.
7. Wait until the dose is completely absorbed before repeating the administration.
8. To avoid swallowing, keep the mouse laying on its abdomen until the effects of the anesthesia has passed.
9. Repeat **steps 6** and **7** until the complete dose volume has been administered.

Fig. 4 Mice sedation for intraperitoneal anesthetic injection. With the mouse restrained, visually divide the animal's abdomen into four quadrants. Apply the injection in the lower right (animal's right side) quadrant in an approximately 45° angle

3.11 Intragastric Administration (Gavage)

1. Restrain the animal as described above.
2. Choose the right size of the gavage needle by measuring the distance between the mouth and the xyphoid cartilage (Fig. 6).
3. Insert the gavage needle on the animal's mouth beside the bottom teeth and above the tongue (Fig. 7).
4. Push the animal's head back with the needle so that the animal's nose is facing up and the path to the stomach is straighter.
5. Continue to pass the needle until the measured distance is reached.
6. Administrate the dose (maximum of 500 μL).

3.12 Intranasal Administration

1. Restrain the animal as described above.
2. With the help of a micropipette, place a small volume (i.e. 5 μL) of the dose on the nares of the animal to be inhaled (Fig. 8).
3. Wait until the dose disappears into the nares.

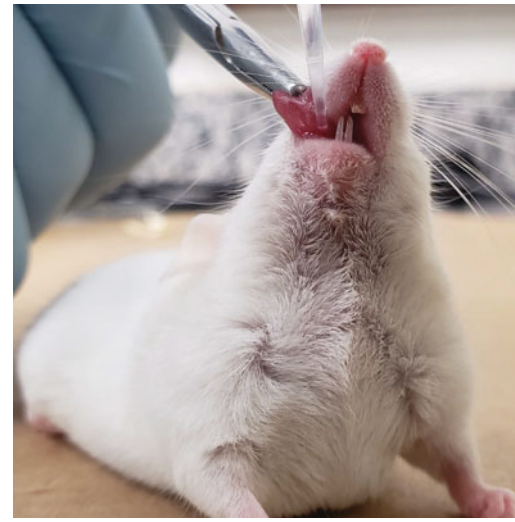

Fig. 5 Sublingual administration. Tongue positioning to achieve correctly sublingual administration. With the animal anesthetized, place it on a flat surface on its abdomen. Raise the animal's head and, with the help of a forceps, pick the tongue and lift it leaving the sublingual region exposed and ready for administration

Fig. 6 Gavage needle sizing. Procedure to measure and choose the correct gavage needle size. With the animal restrained, place the base of the gavage needle near the mouth of the animal with the tip of the needle pointing to its abdomen. The correct needle size must reach the xyphoid cartilage, located at the bottom of the animal's thorax

4. Repeat **steps 2** and **3** until the desired volume has been administered (up to 25 μL total), interchanging the nares between administrations.

Fig. 7 Intragastric administration (Gavage). With the animal restrained, place the tip of the gavage needle beside the animal's bottom teeth and above the tongue (**a**) then, with the help of the gavage needle, push the animal's head back to create a straighter path to the stomach (**b**). Insert the needle into the animal's esophagus (**c**) and administer the dose (**d**)

4 Notes

1. Alternatively, the glucose solution can be autoclaved for 10 min at 110 °C.
2. When the medium is removed from the autoclave, the fluid will be superheated. Allow the medium to cool to 50–60 °C at room temperature before adding thermolabile substances (e.g., glucose, sucrose, and antibiotics).

Fig. 8 Nasal administration. With the animal restrained, place the dose to be administered, with the help of a pipette, above the nares of the animal letting it be absorbed before administering the next dose

3. M17 broth can also be stored at room temperature if used in a short period of time. Keeping it at room temperature also helps identifying contamination of the medium.
4. Remove the petri dishes from the refrigerator and leave it on the bench until it reaches room temperature before inoculating the bacteria.
5. To count the number of CFU, choose the dilution factor in which between 30 and 300 CFU grow to make it easier to count and reduce mistakes.
6. The colony-forming unit (CFU) is a measure of viable bacterial cells, the results are given in CFU/mL (colony-forming units per milliliter). For the CFU measurement, perform serial dilution (10^{-6}, 10^{-7}, 10^{-8}, or more if necessary) or you will not be able to count the colonies.
7. The dilution is made to the lag phase so the dose will be prepared on the exponential phase.
8. For the bacterial growth curve, an initial of $OD_{600} = 0.04$ is used because this OD_{600} corresponds to the bacteria during the lag phase.
9. Each dose is equivalent to 2 mL of bacterial culture.
10. The prepared doses can be used up to 5 days after production if stored at −80 °C; after this time, the doses start to lose viability.

11. The animal restraint is usually done with the nondominant hand so that the dominant hand is free to do more precision-required procedures such as anesthetic injection and gavage.
12. The lower right quadrant of the abdomen is chosen as the intraperitoneal space because it has less organs and also to avoid cecum perforation.

References

1. Bermúdez-Humarán LG, Aubry C, Motta J-P et al (2013) Engineering lactococci and lactobacilli for human health. Curr Opin Microbiol 16:278–283. https://doi.org/10.1016/j.mib.2013.06.002
2. Bermúdez-Humarán LG (2009) Lactococcus lactis as a live vector for mucosal delivery of therapeutic proteins. Hum Vaccin 5 (4):264–267
3. Souza BM, Preisser TM, Pereira VB et al (2016) Lactococcus lactis carrying the pValac eukaryotic expression vector coding for IL-4 reduces chemically-induced intestinal inflammation by increasing the levels of IL-10-producing regulatory cells. Microb Cell Factories 15:150. https://doi.org/10.1186/s12934-016-0548-x
4. Mancha-Agresti P, Drumond MM, FLR do C et al (2017) A new broad range plasmid for DNA delivery in eukaryotic cells using lactic acid bacteria: in vitro and in vivo assays. Mol Ther Methods Clin Dev 4:83. https://doi.org/10.1016/j.omtm.2016.12.005
5. Coelho-Rocha ND, de Castro CP, de Jesus LCL et al (2018) Microencapsulation of lactic acid Bacteria improves the gastrointestinal delivery and in situ expression of recombinant fluorescent protein. Front Microbiol 9:2398. https://doi.org/10.3389/fmicb.2018.02398
6. Pontes DS, De Azevedo MSP, Chatel JM et al (2011) Lactococcus lactis as a live vector: heterologous protein production and DNA delivery systems. Protein Expr Purif 79:165
7. Fioretti D, Iurescia S, Fazio VM, Rinaldi M (2010) DNA vaccines: developing new strategies against cancer. J Biomed Biotechnol 2010:174378
8. Myhr AI (2017) DNA vaccines: regulatory considerations and safety aspects. Curr Issues Mol Biol 22:79–88. https://doi.org/10.21775/cimb.022.079
9. Babiuk LA, Pontarollo R, Babiuk S et al (2003) Induction of immune responses by DNA vaccines in large animals. Vaccine 21:649. https://doi.org/10.1016/S0264-410X(02)00574-1
10. Ferraro B, Morrow MP, Hutnick NA et al (2011) Clinical applications of DNA vaccines: current progress. Clin Infect Dis 53:296–302. https://doi.org/10.1093/cid/cir334
11. Roy MJ, Wu MS, Barr LJ et al (2000) Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 19:764. https://doi.org/10.1016/S0264-410X(00)00302-9
12. Wang B, Boyer J, Srikantan V et al (1993) DNA inoculation induces neutralizing immune responses against human immunodeficiency virus type 1 in mice and nonhuman primates. DNA Cell Biol 12:799. https://doi.org/10.1089/dna.1993.12.799
13. Kutzler MA, Weiner DB (2008) DNA vaccines: ready for prime time? Nat Rev Genet 9:776–788. https://doi.org/10.1038/nrg2432
14. Neutra MR, Kozlowski PA (2006) Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 6:148–158. https://doi.org/10.1038/nri1777
15. Machholz E, Mulder G, Ruiz C et al (2012) Manual restraint and common compound administration routes in mice and rats. J Vis Exp (67). https://doi.org/10.3791/2771
16. Song J-H, Nguyen HH, Cuburu N et al (2008) Sublingual vaccination with influenza virus protects mice against lethal viral infection. Proc Natl Acad Sci U S A 105:1644. https://doi.org/10.1073/pnas.0708684105

Chapter 25

A Hybrid Biological–Biomaterial Vector for Antigen Delivery

Ruiquan Qi, Andrew Hill, and Blaine A. Pfeifer

Abstract

A hybrid biological–biomaterial vector composed of a biocompatible polymeric biomaterial coating and an *Escherichia coli* core was designed and developed for antigen delivery. It provides a unique and efficient mechanism to transport antigens (protein or genetic) via different mechanisms of vector design that include antigen cellular localization (cytoplasm, periplasm, cellular surface) and nonnative functionalities that assist in antigen delivery. Based on a variety of *E. coli* strain development and polymer chemistry tools, the hybrid vector can be constructed into a number of formats for the purpose of optimized uptake and processing by antigen presenting cells, serving as the basis for a potent subsequent immune response. This chapter serves to outline a protocol for assembling a hybrid biological–biomaterial vector for use as a vaccine delivery system.

Key words Vaccine delivery, DNA vaccine, Biomaterial, Immunogenicity, *Escherichia coli*

1 Introduction

Vaccination strategies have proven effective in preventing or therapeutically treating diseases by generating potent and long-lasting immune reactivity spanning an array of infectious diseases and cancer [1]. However, there exist at least two major causes reducing vaccine effectiveness, namely, incomplete targeting of rogue cells and inadequate antigen delivery. To overcome these problems, vaccine development requires continual discovery efforts for ideal antigen candidates and methods that enable efficient antigen delivery to antigen presenting cells (APC) to subsequently activate immune responses.

With assistance from cationic biocompatible polymers which are capable of attaching to the surface of gram-negative bacteria via electrostatic interaction, a novel hybrid vector was designed to promote antigen production and delivery, aided by the innate delivery capabilities and engineering potential associated with the bacterial and biomaterial components of the final hybrid delivery device [2]. The hybrid vehicle, shown in Fig. 1 [3], features the

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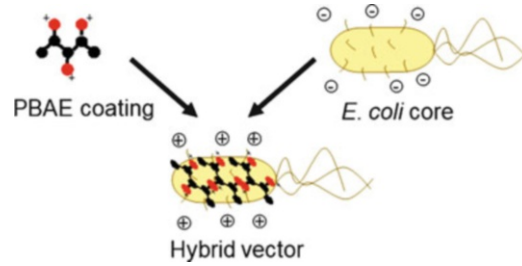

Fig. 1 The hybrid biological–biomaterial vector. Electrostatic interactions between a positively charged poly(beta-amino ester) (PBAE) and a negatively charged *E. coli* bacteria result in the hybrid vector composed of both components contributing to the delivery of antigenic cargo within the *E. coli* core of the vehicle

following: a positive charge [4] to decease cell membrane electronic repulsion and therefore enhance antigen uptake by APCs [5] in addition to the native and engineered features of both biomaterial and bacterial components for the dual purpose of adjuvancy and designed delivery of antigen content.

The bacterial component of the hybrid vector consists of a nonpathogenic *E. coli* strain with both natural adjuvant features and the capability to harbor antigens in simple plasmid-based formats. A genetic antigen will be designed for delivery to and expression within the target APC; whereas, a protein antigen will first be expressed within the *E. coli* cell prior to APC delivery. The surface cationic polymer component of the hybrid vector can feature either synthetic or natural polymers [6] and naturally associates with the negatively charged surface of *E. coli* [7]. By varying polymer content and structure [8] and the *E. coli* strains carrying antigen content, there is the potential to systematically optimize antigen delivery through this approach for the purpose of advanced vaccine response outcomes [9].

2 Materials

2.1 Cloning Materials

1. *dexB* forward cloning primer: TAAGCACATATGCAAG AAAAATGGTGGCATAATGCCGTAG.
2. *dexB* reverse cloning primer: TAAGCACTCGAGTTCCACA CAGAAAGCATCCCA.
3. *glpO* forward cloning primer: TAAGCAGAGCTCGAAT TTTCAAAAAAAACACGTGAATTGTC.
4. *glpO* reverse cloning primer: TAAGCACTCGAGATTTTTTA ATTCTGCTAAATCGTTGTTAG.
5. *stkP* forward cloning primer: TAAGCACATATGATCCAAAT CGGCAAGATTTT.

6. *stkP* reverse cloning primer: TAAGCAGCGGCCGCAGGA GTAGCTGAAGTTGTTTTA.
7. *dexB* forward pLF primer: GCGGGATCCCAAGAAAAAT GGTGGCATAATGCCGTAG.
8. *dexB* reverse pLF primer: ATAGGCGCGCCTTATAGTAA TTCCACACAG.
9. *glpO* forward pLF primer: GCGGTCGACAAGGAGATATAA TGGAATTTTCAAAAAAAAC.
10. *glpO* reverse pLF primer: GCGGCGGCCGCTTAATTTTTT AATTCTGC.
11. *stkP* forward pLF primer: GCGCATATGATCCAAATCGGC AAGATTTTTG.
12. *stkP* reverse pLF primer: GCGCAATTGTTAAGGAGTAGC TGAAGTTGTTTTAG.
13. *pspA* forward pLF primer: ATAGGCCGGCCAAGGAGATAT AATGGAAGAATCTCCCGTAGCCA.
14. *pspA* reverse pLF primer: ATACTCGAGTTATTCTGGGGCT GGAGTTTCTGGA.
15. pUAB055 containing *pspA*.
16. Phusion High-Fidelity DNA polymerase.
17. *Xba*I, *Age*I, *Xho*I, *Nde*I, *Sac*I, *Not*I, *Bam*HI, *Asc*I, *Sal*I, *Mun*I, and *Fse*I restriction enzymes.
18. Plasmid Miniprep kit.
19. GeneJET Gel Extraction Kit.
20. Agarose.
21. T4 DNA ligase.

2.2 Cell Culture Materials

1. Todd Hewitt blood agar plates: 10 g/L pancreatic digest of casein, 60 g/L yeast extract, 3.1 g/L beef heart infusion, 2.5 g/L sodium carbonate, 2 g/L dextrose, 2 g/L sodium chloride, 0.4 g/L sodium phosphate, and 5% v/v sheep blood.
2. Lysogeny broth (LB) medium: 10 g Bacto tryptone, 5 g yeast extract, 10 g/L NaCl, and antibiotic (100 mg/L ampicillin, 50 mg/L kanamycin, or 20 mg/L chloramphenicol, as needed).
3. LB agar plates: 10 g/L Bacto tryptone, 5 g/L yeast extract, 10 g /L NaCl, 15 g/L agar, and antibiotic (100 mg/L ampicillin, 50 mg/L kanamycin, or 20 mg/L chloramphenicol, as needed).
4. RPMI-1640 cell culture media: 10% FBS, 5 mM sodium pyruvate, 1% penicillin/streptavidin, 25 mM HEPES.
5. THY Media: 37 g/L Todd Hewitt Broth, 0.5% yeast extract.

6. Chemically Defined Medium (CDM): 10 g/L glucose, 4.5 g/L sodium acetate $3H_2O$, 3.195 g/L sodium phosphate monobasic H_2O, 7.350 sodium phosphate dibasic anhydrous, 1 g/L potassium phosphate monobasic, 0.2 g/L potassium phosphate dibasic, 0.7 g/L magnesium sulfate heptahydrate, 0.005 g/L manganese sulfate anhydrous, 0.005 g/L ferrous sulfate heptahydrate, 0.001 g/L ferric nitrate $9H_2O$, 0.0051 g/L calcium chloride anhydrous, 0.1 g/L DL-alanine, 0.1 g/L L-arginine free base, 0.1 g/L L-aspartic acid, 0.5 g/L L-cysteine free base, 0.05 g/L L-cysteine, 2HCl, 0.1 g/L L-glutamic acid, 0.2 g/L L-glutamine, 0.1 g/L glycine, 0.1 g/L L-histidine free base, 0.1 g/L trans-4-hydroxy L-proline, 0.1 g/L L-isoleucine, 0.1 g/L L-leucine, 0.1 g/L L-lysine, 0.1 g/L L-methionine, 0.1 g/L L-phenylalanine, 0.1 g/L L-proline, 0.1 g/L L-serine, 0.2 g/L L-threonine, 0.1 g/L L-tryptophan, 0.1 g/L L-tyrosine free base, 0.1 g/L L-valine, 0.02 g/L adenine free base, 0.02 g/L guanine hydrochloride, 0.02 g/L uracil, 0.0002 g/L PABA (4-aminobenzoic acid), 0.0002 g/L biotin, 0.0008 g/L folic acid, 0.001 g/L niacinamide, 0.0025 g/L B-NAD (β-nicotinamide adenine dinucleotide hydrate), 0.002 g/L D-Ca pantothenate (calcium-D-pantothenate), 0.001 g/L pyridoxal hydrochloride, 0.001 g/L pyridoxamine dihydrochloride, 0.002 g/L riboflavin, 0.001 g/L thiamine HCl, 0.0001 g/L cyanocobalamin, 5 g/L sodium bicarbonate, 1 g/L choline chloride, 0.75 g/L L-cysteine hydrochloride.
7. Isopropyl β-D-1-thiogalactopyranoside (IPTG): 100 mM stock.
8. 40% glycerol–water solution.

2.3 Protein Purification Materials

1. HisTrap HP column, GE Healthcare.
2. Buffer A: 50 mM Na_2HPO_4, 500 mM NaCl, and 10% glycerol.
3. Buffer B: 50 mM Na_2HPO_4, 500 mM NaCl, 10% glycerol, and 250 mM imidazole.
4. 100 mM $NiSO_4$.
5. 50 mM ethylenediaminetetraacetic acid (EDTA).
6. 1 M guanidinium hydrochloride.
7. 20% ethanol.
8. Sodium dodecyl sulfate.
9. 30% acrylamide–bis solution, 29:1.
10. Ammonium persulfate (APS): 10% solution.
11. 10× Tris–glycine–SDS (25 mM Tris, 192 mM glycine, and 0.1% SDS), pH 8.3.
12. Tris–HCl: 0.5 M, pH 6.8.

13. *N*,*N*,*N*,*N*-Tetramethylethylene-diamine (TEMED).
14. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH of 7.4.
15. Paraformaldehyde (PFA): 4% aqueous solution.

2.4 Materials for Hybrid Vector Assembly

1. D(+)-Mannose (3 g).
2. p-Toluenesulfonyl chloride: at 900 mg/L, dissolved in allyl alcohol.
3. Ethylene diamine: 5 M in DMSO.
4. Neopentyl glycol diacrylate.
5. 2-Aminopropane-1,3-diol.
6. NaOAc: 25 mM.

3 Methods

3.1 Isolation of Streptococcus pneumoniae Antigens

1. Streak a cryogenic stock of the D39 *Streptococcus pneumoniae* strain onto a Todd Hewitt blood agar plate using a sterile cue-tip.
2. Incubate the plate at 37 °C for 16–20 h.
3. Collect with a sterile toothpick a small portion of a colony into 5 μL of deionized water and incubate at 98 °C for 10 min to serve as the template for colony PCR.
4. Amplify antigen genes (*dexB*, *glpO*, and *stkP*) by PCR with the cloning primers (Primers 1–6) using the Phusion HF DNA polymerase (New England Biolabs) under the following conditions: 30 s at 98 °C for initial denaturation, 30 amplification cycles (10 s at 98 °C, 15 s for annealing, and 72 °C for extension at the rate of 30 s per kb), 4 min at 72 °C for final extension and holding at 4 °C when finished.
5. Confirm PCR products using agarose gel (1% w/v) with 0.1% w/v SYBR Safe DNA Gel Stain electrophoresis at a constant voltage (100 V).
6. Visualize the PCR products with UV light to confirm bands are the correct size, as compared to a 1 kb DNA ladder standard.
7. Extract desired bands from the gel using a razor and purify the DNA using a Thermo Scientific Gel Extraction kit following the manufacturer's instructions.

3.2 Assembly of the Individual Pneumococcal Antigen Plasmids

1. Prepare the PCR products for cloning into the pET21c plasmid (Amp^R) via digestion of 2 μg of DNA with the flanking restriction sites: *Nde*I and *Xho*I (*dexB*), *Sac*I and *Xho*I (*glp*O), and *Nde*I and *Not*I (*stkP*). Digestions should be carried out for 1 h at 37 °C.

2. Following digestion, purify the reaction products using agarose gel extraction as described above.
3. Inoculate *E. coli* containing the pET21c plasmid backbone into 5 mL of LB broth containing ampicillin and incubate overnight at 37 °C and 225 rpm (*see* **Note 1**).
4. Purify pET21c DNA from *E. coli* cultures using a Qiagen Miniprep Kit following the manufacturer's instructions.
5. Following purification, digest 3 μg of the plasmid backbone with the same restriction enzymes for 1 h at 37 °C.
6. After completion of the digestion reaction, purify the insert-free plasmid backbone from a 1% agarose gel using a Thermo Scientific Gel Extraction kit following the manufacturer's instructions.
7. Measure the DNA concentrations of the pET21c and antigen PCR product digestions using a NanoDrop spectrophotometer via absorbance at 260 nm.
8. Ligate the digested insert into the digested pET21c constructs utilizing T4 DNA Ligase. Incubate the ligation reaction at room temperature overnight (*see* **Note 2**). Include a negative control for each plasmid digestion in which no insert is added to confirm complete digestion of the backbone plasmid.
9. Following the overnight incubation, heat-inactivate the ligation reactions by incubating in a 65 °C heat bath for 10 min.
10. After heat inactivation, transform the ligation products into competent GeneHogs (Thermofisher Scientific) *E. coli* (*see* **Note 3**) to form the pCJ05 (*dexB*), pCJ06 (*glpO*), and pCJ07 (*stkP*) plasmids. Include a negative control in which sterile water is added to the competent cells to evaluate contamination.
11. Verify that the resulting transformants contain the desired plasmids by colony PCR and restriction digest analysis using the appropriate primers and enzymes, respectively.

3.3 Assembly of the Consolidated Pneumococcal Antigen Plasmid

1. Using the confirmed plasmids, as well as the pUAB055 plasmid for *pspA*, as templates, amplify the *dexB*, *glpO*, *stkP*, and *pspA* genes using the listed pLF primers (Primers 7–14) via PCR with the Phusion HF DNA polymerase as described above.
2. Prepare the PCR products for cloning into the pLF plasmid (based upon the pDuet plasmids from Novagen) via digestion of 2 μg of DNA with the flanking restriction sites: *Bam*HI and *Asc*I (*dexB*), *Sal*I and *Not*I (*glpO*), and *Nde*I and *Mun*I (*stkP*), and *Fse*I and *Xho*I (*pspA*). Digestions should be carried out for 1 h at 37 °C.

3. Following digestion, purify the reaction products using agarose gel extraction as described above.
4. Inoculate *E. coli* containing the pET21c plasmid backbone into 5 mL of LB broth containing ampicillin and incubate overnight at 37 °C and 225 rpm (*see* **Note 1**).
5. Purify pACYCDuet-1 DNA from *E. coli* cultures using a Qiagen Miniprep Kit following the manufacturer's instructions.
6. Following purification, digest 3 μg of the plasmid backbone with one set of restriction enzymes listed above for 1 h at 37 °C.
7. After completion of the digestion reaction, purify the insert-free backbone from a 1% agarose gel using a Thermo Scientific Gel Extraction kit following the manufacturer's instructions.
8. Measure the DNA concentrations of the pACYCDuet-1 and antigen PCR product digestions using a NanoDrop spectrophotometer via absorbance at 260 nm.
9. Ligate the appropriate the digested inserts into the digested pACYCDuet-1 constructs utilizing T4 DNA Ligase. Incubate the ligation reaction at room temperature overnight (*see* **Note 2**). Include a negative control in which no insert is added to confirm complete digestion of the backbone plasmid.
10. Following the overnight incubation, heat-inactivate the ligation reactions by incubating in a 65 °C heat bath for 10 min.
11. After heat inactivation, transform the ligation products into competent GeneHogs (Thermofisher Scientific) *E. coli* (*see* **Note 3**). Include a negative control in which sterile water is added to the competent cells to evaluate contamination.
12. Verify that the resulting transformant contains the desired plasmids by colony PCR and restriction digest analysis using the appropriate primers and enzymes, respectively. Repeat **steps 6–13** until all four antigens are transformed into a single plasmid to form the pLF plasmid.
13. Transform the pCJ05, pCJ06, pCJ07, pUAB055, and pLF plasmids into *E. coli* YWT7-hly using the chemical transformation method (*see* **Note 3**).
14. Select a single colony for each plasmid and culture with shaking at 250 rpm under 37 °C before mixing with 40% glycerol at a 1:1 ratio prior to strain cryostorage at −80 °C.

3.4 Determination of Hybrid Vector Protein Expression

1. To ensure proper level of protein consistent with that of the hybrid vector in future studies, the quantity of antigen present in a given dose of hybrid vector should be determined. Inoculate the bacterial cores into 5 mL of LB media containing ampicillin and incubate overnight at 37 °C.

2. After 16 h of growth, transfer 1 mL of the bacterial culture into 1 L of LB media containing ampicillin and incubate at 37 °C until an OD_{600nm} of 0.4–0.6 is reached (approximately 3 h).
3. Once an OD_{600nm} of 0.4–0.6 has been reached, induce gene expression using 100 μM IPTG.
4. Transfer the culture to a 22 °C shaker and incubate for an additional 20 h.
5. Following the incubation, measure the OD_{600nm} of the culture and harvest 500 mL of the cell culture via centrifugation for 20 min at 4000 rpm (Eppendorf 5415D centrifuge) and 4 °C.
6. After removing the supernatant, resuspend the cell pellet in 20 mL of buffer A and place on ice.
7. Lyse the cells using sonication (Model CL-18, Fisher Scientific) at an amplitude of 40–50. Cells should be sonicated 10 s on and 30 s off for 10 cycles.
8. Following sonication, centrifuge the cells for 20 min at 4000 rpm and 4 °C and transfer the supernatant to microcentrifuge tubes.
9. Centrifuge the tubes at 13,000 rpm for 10 min to remove any remaining debris and collect the supernatant into a single fresh tube.
10. Purify the overexpressed protein(s) using a prepared HisTrap HP column (GE Healthcare) using a syringe protocol (*see* **Note 4**).
11. The fractions collected should then be evaluated for protein content using a plate reader to measure absorbance at 280 nM. Analyze fractions containing a spike in absorbance, typically observed at the ~100 mM imidazole wash step, with SDS PAGE using a 10% acrylamide gel.
12. After SDS PAGE is completed, stain the gel with Coomassie Blue for 1 h then wash with deionized water to visualize protein bands. Fractions containing the proteins of interest can be identified using a protein ladder.
13. Combine fractions containing substantial quantities of the proteins of interest with little contamination into a single fresh tube.
14. Dialyze the combined protein fractions overnight into PBS at 4 °C.
15. Measure the protein concentration within the solution using the Bradford Assay with a clear polystyrene plate. Mix 5 μL of the extract with 250 μL of the Bradford reagent and incubate at room temperature for 20 min. Afterward, measure the absorbance at 595 nm and compare to a standard curve containing 0–1 mg/mL of BSA.

16. Utilize the protein concentration as determined by the Bradford assay in conjunction with the prior OD_{600nm} measurement to either determine the dose of protein per 10^7 cells or to determine the quantity of cells needed for a particular protein antigen dose.

3.5 Assembly of the Polymer Component

1. Add amine (2-aminopropane-1,3-diol, 400 mg) and diacrylate (neopentyl glycol diacrylate) into 2 mL DMSO at 1:1.2 molar ratio.
2. Conduct the reaction for 5 days at 60 °C with continuous stirring at 1000 rpm.
3. Polymers are purified by dialysis which is conducted against acetone using molecular porous membrane tubing, with an approximate molecular weight cutoff of 3500 Da.
4. Remove acetone by evaporation under vacuum.
5. Allyl-a-D-mannopyranoside (ADM) is synthesized by dissolving 3 g of D(+)-mannose and 18 mg of p-toluenesulfonyl chloride in allyl alcohol (20 mL) at 90 °C under reflux for 24 h.
6. Purify resulting ADM product via dialysis.
7. Concentrate the resulting reaction solution by vacuum distillation at 35 °C (*see* **Note 5**).
8. Dissolve the synthesized acrylate-terminated polymer in DMSO (167 mg/mL) and react with 5 M ethylene diamine (in DMSO) at room temperature for 24 h to form amine-capped polymer.
9. Purify amine-capped polymer by dialysis followed by evaporation under vacuum.
10. React the amine-capped polymer with ADM at a 1:2 molar ratio in DMSO at 90 °C for 24 h.
11. Purify the final polymer with dialysis followed by evaporation under vacuum.

3.6 Hybrid Vector Preparation

1. Start an overnight culture by inoculating a hybrid vector strain (i.e., YWT7-*hly* with pCJ05, pCJ06, pCJ07, pUAB055, or pLF) into LB media containing ampicillin and grow for 16 h at 37 °C.
2. Utilize the overnight culture to seed 10 mL (2% v/v) of fresh LB media containing ampicillin and incubate at 37 °C while periodically measuring the optical density at 600 nm (OD_{600nm}).
3. Once the optical density reaches 0.4–0.5 (*see* **Note 6**), induce samples with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 22 °C for 20 h (*see* **Note 7**).

4. Wash bacterial vectors once and standardize to 0.5 OD_{600nm} in PBS, whereas bacterial strains to be used in hybrid vector formation should be washed once and standardized to 1.0 OD_{600nm} in 25 mM NaOAc (pH 5.15) (*see* **Note 8**).
5. Desiccate the polymer doses that are dissolved in chloroform and resuspend in 25 mM NaOAc (pH 5.15) prior to equal volume addition to 1.0 OD_{600nm} bacterial strains.
6. Incubate hybrid vectors (final 0.5 OD_{600nm}) and bacterial vectors in PBS at 22 °C for 15 min (*see* **Note 9**).
7. Utilize additional PBS to perform further dilutions of both bacterial and hybrid vectors as desired.

3.7 Hybrid Vector Immunization

1. Following the preparation described above, the hybrid vector can then be prepared for animal immunizations. Normalize dose levels for the hybrid vector to 10^7 by measuring the OD_{600nm} of the solution via spectrophotometer and diluting with PBS, as needed.
2. Prior to the first immunization, collect serum from the mice via retro-orbital bleeding followed by centrifugation to establish baseline antibody and cytokine levels.
3. For animal immunizations, utilize CD-1 mice. Immunize mice in groups of 6–12 using either subcutaneous (SC), intraperitoneal (IP), or intranasal (IN) administration. Administer the quantity of hybrid vector desired in 200 μL PBS for SC and IP injections or a 40 μL aspiration for IN delivery. A sham vaccination with PBS should be included as a negative control for each administration route. In addition, hybrid vectors containing the background plasmids (i.e., the parent plasmid backbones for CJ05, CJ06, CJ07, pUAB055, and pLF) should be tested as controls to ensure resulting immune response is derived from the encoded antigens.
4. After 14 days, collect serum from the mice as described above to measure antibody and cytokine values after primary immunization.
5. After collecting the after primary immunization serum, vaccinate the mice with a secondary immunization using the same conditions.
6. After another 14 days (28 days total), collect serum from the mice as described above to measure antibody and cytokine values post-secondary immunization.

3.8 Immunological Characterization

1. To characterize antibody titers within serum samples, an enzyme-linked immunosorbent assay (ELISA) is performed by coating a 96-well Costar high-binding polystyrene plate with the pneumococcal antigens GlpO, DexB, StkP, or PspA (10 μg/mL) in tris-buffered saline (TBS) at 4 °C overnight.

2. Block the plate with 3% bovine serum albumin in TBS–Tween 20 (TBS-T) for 1 h at 22 °C.
3. Dilute the isolated sera into TBS-T in ratios of 1:1000, 1:5000, 1:7500, and 1:10,000 and add to the plate.
4. Incubate the plates at 37 °C with mild agitation for 3 h.
5. Add the secondary antibody (anti-mouse IgG, IgA, IgM (H + L), IgE, highly X-adsorbed (biotin)) to the wells in a 1:1000 ratio and agitate for 2 h.
6. Add streptavidin to each well in a 1:1000 ratio and shake for 30 min.
7. Develop the signal with *p*-nitrophenylphosphate and the reaction using 0.75 M NaOH.
8. Quantify the signal using a plate reader spectrophotometer at an absorbance of 405 nm.
9. Quantify the production of cytokines using IFN-γ and IL-17A ELISA kits (R&D Systems).

3.9 Preparation of Biofilm-Dispersed Pneumococci

1. Propagate NCI-H292 epithelial cell line in T75 cell culture flasks in RPMI-1640 medium at 37 °C and 5% CO_2. Approximately 5–10 flasks will be needed to generate a sufficient quantity of biofilms.
2. Incubate cultures at 37 °C renewing the cell culture media every 2 days.
3. Once the cells have reached 100% confluency, remove and discard culture medium.
4. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
5. Add 2.0–3.0 mL of trypsin–EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5–15 min).
6. Add 6.0–8.0 mL of complete growth medium and aspirate cells by gently pipetting.
7. Add 0.5 mL of cell suspension to each well of a 24-well cell culture plate and incubate at 37 °C and 5% CO_2. As with the T75 cell culture flasks, continue renewing cell culture media every 2 days.
8. Once the cell layer has reached 100% confluency, remove and discard the culture medium from the culture vessel.
9. Wash cell substratum three times with 1 mL PBS per well to remove medium residues.

10. Fix the confluent epithelial substratum by adding 0.5 mL of 4% paraformaldehyde (PFA) solution per well for 1 h at room temperature.
11. Aspirate the 4% PFA solution and wash the cell substratum three times with 1 mL PBS per well to remove any residual PFA solution. Use immediately or briefly store in refrigerator after adding 0.5 mL PBS per well.
12. Streak a *S. pneumoniae* strain from glycerol stock onto a blood agar plate and grow overnight.
13. Inoculate the streak from above step into 3–5 mL of THY media in a 10-mL glass tube or a 15-mL conical tube and grow statically at 37 °C to an OD_{600nm} of approximately 0.6. Bacteria from this step can be harvested for challenge models as the planktonic phenotype.
14. Dilute *S. pneumoniae* 1:10 in CDM to an OD_{600nm} of approximately 0.06 and seed 0.5 mL *S. pneumoniae* suspension in each well containing fixed epithelial cells.
15. Incubate at 34 °C for bacterial biofilm formation for 48 h, carefully exchanging the supernatant with 0.5 mL fresh CDM media approximately every 12 h. Avoid disturbing the biofilm as much as possible.
16. To disperse biofilms with heat, wash the biofilms with fresh CDM medium and expose the *S. pneumoniae* to 38.5 °C for 4 h.
17. Collect and pellet biofilm-released bacteria by centrifugation at 9000 × *g* for 2 min at 4 °C in a microcentrifuge.
18. Resuspend pellets in 0.5 mL 0.9% NaCl and incubate for 5 min at room temperature.
19. Pellet the biofilm-dispersed pneumococci by centrifugation at 9000 × *g* for 2 min in a microcentrifuge. Remove and discard supernatant by pipetting without disturbing the pellet. The pellet can be stored at −80 °C at this time (Fig. 2).

3.10 Pneumococcal Challenge Model

1. After allowing for 14 days to pass following the secondary immunization described above, challenge mice with 1×10^4 (sepsis model) or 1×10^6 (pneumonia model) CFU of pneumococci strains through intraperitoneal or intranasal (with isoflurane) administration, respectively. Bacteria utilized in these studies can be prepared using either planktonic pneumococci (*see* **step 13** in Subheading 3.9) or using biofilm-dispersed pneumococci (step 19 in Subheading 3.9; *see* Fig. 3).
2. Monitor mice monitored every 4 h for signs of morbidity (huddling, ruffled fur, lethargy, and abdominal surface temperature). Mice found to be moribund should be euthanized via CO_2 asphyxiation and cervical dislocation (*see* **Note 10**).

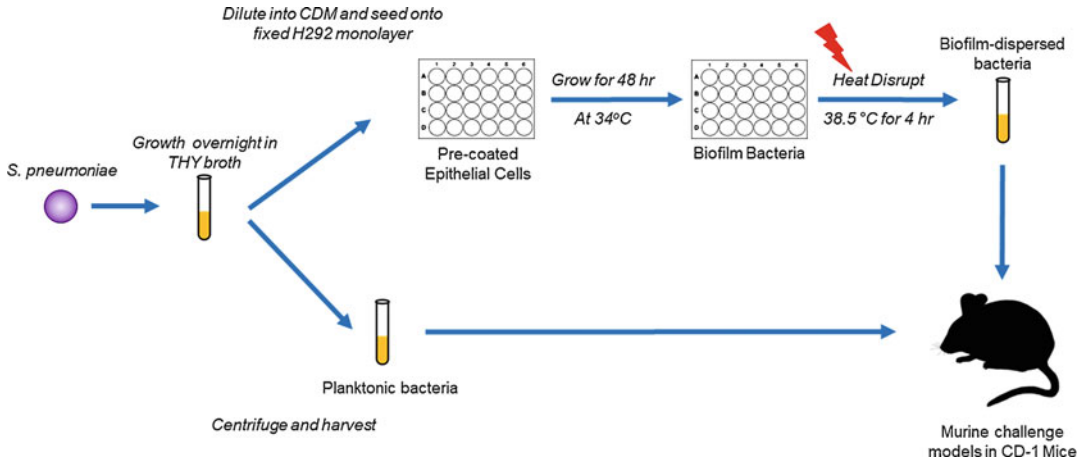

Fig. 2 Preparation of *S. pneumoniae* for murine challenge models of vaccination effectiveness

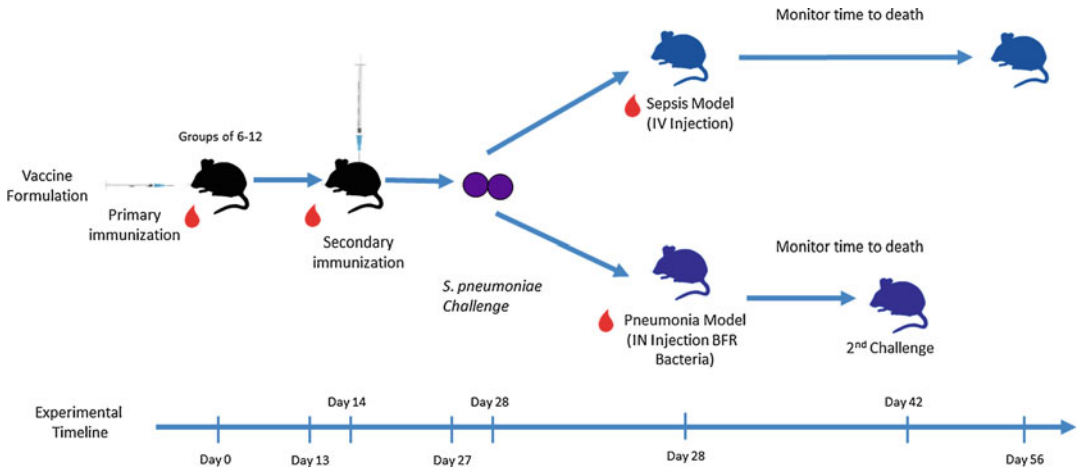

Fig. 3 Murine immunization and disease challenge timeline

3. Once 14 days have passed for mice challenged with the pneumonia challenge model or 21 days have passed for the sepsis challenge model, mice should be considered to have demonstrated complete survival.

4 Notes

1. It is more time efficient if preparation of the plasmid backbone is conducted in parallel with preparation of the insert. Ideally, the *E. coli* culture used to prepare the backbone should be inoculated the night before the antigen is PCR amplified.

2. Ligation reactions were carried out using 50 ng of vector DNA and an insert to vector ratio of 5:1. The temperature at which the reactions were carried out was generally between 20–25 °C.
3. A conventional chemical transformation procedure was performed to transfer these constructs during this process, but larger single plasmids or multiple plasmids might require an electrotransformation process. The plasmids presented in this method either contain one specific antigen (the pCJ or pUAB) or the combined antigens (pLF).
4. An array of metal-chelation affinity chromatography options exist for purifying 6× histidine-tagged recombinant protein products. In this case, we used a HisTrap column system that relied on NTA-Nickel chelation.
5. It is common to concentrate or evaporate the solution to the densest level possible. In doing so, we observed no component breakdown at 35 °C.
6. At OD_{600nm} 0.4–0.5, *E. coli i*s at a period of exponential growth when the proliferation rate is at the highest level, likely resulting in cellular health supporting optimal gene expression.
7. IPTG is used to induce the expression of genes in the plasmid or genome in *E. coli*. Normally, the induction temperature should be less than 30 °C and induction time depends on the extent of gene expression desired (i.e., how much protein is required for a given dose).
8. The NaOAc solution provides an environment that facilitates polymer coating onto the *E. coli* surface.
9. Shaking at 22 °C helps the polymer coating process. PBS is applied during dilution to maintain cellular health and pH.
10. Sufficient monitoring and timely humane euthanasia when mice are observed to be experiencing distress is critical for ethics in conducting studies with vertebrate animals.

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References

1. Harris CC (1991) Chemical and physical carcinogenesis: advances and perspectives for the 1990s. Cancer Res 51(18, Suppl):5023s–5044s
2. Jones CH, Chen M, Ravikrishnan A, Reddinger R, Zhang G, Hakansson AP, Pfeifer BA (2015) Mannosylated poly(beta-amino esters) for targeted antigen presenting cell

immune modulation. Biomaterials 37:333–344. https://doi.org/10.1016/j.biomaterials.2014.10.037

3. Li Y, Beitelshees M, Fang L, Ahmadi MK, Chen M, Hill A, Davidson BA, Knight P 3rd, Smith RJ Jr, Andreadis ST, Hakansson AP, Jones CH, Pfeifer BA (2016) In situ pneumococcal vaccine production and delivery through a hybrid biological-biomaterial vector. Sci Adv 2 (7):e1600264
4. Samal SK, Dash M, Van Vlierberghe S, Kaplan DL, Chiellini E, van Blitterswijk C, Moroni L, Dubruel P (2012) Cationic polymers and their therapeutic potential. Chem Soc Rev 41 (21):7147–7194. https://doi.org/10.1039/c2cs35094g
5. Qiao Y, Huang Y, Qiu C, Yue X, Deng L, Wan Y, Xing J, Zhang C, Yuan S, Dong A, Xu J (2009) The use of PEGylated poly [2-(N,N-dimethylamino) ethyl methacrylate] as a mucosal DNA delivery vector and the activation of innate immunity and improvement of HIV-1-specific immune responses. Biomaterials 31 (1):115–123. https://doi.org/10.1016/j.biomaterials.2009.09.032
6. Jones CH, Chen M, Gollakota A, Ravikrishnan A, Zhang G, Lin S, Tan M, Cheng C, Lin H, Pfeifer BA (2015) Structure-function assessment of mannosylated poly (β-amino esters) upon targeted antigen presenting cell gene delivery. Biomacromolecules 16 (5):1534–1541. https://doi.org/10.1021/acs.biomac.5b00062
7. Jones CH, Gollakota A, Chen M, Chung T-C, Ravikrishnan A, Zhang G, Pfeifer BA (2015) Influence of molecular weight upon mannosylated bio-synthetic hybrids for targeted antigen presenting cell gene delivery. Biomaterials 58:103–111. https://doi.org/10.1016/j.biomaterials.2015.04.033
8. Jones CH, Chen C-K, Ravikrishnan A, Rane S, Pfeifer BA (2013) Overcoming nonviral gene delivery barriers: perspective and future. Mol Pharm 10(11):4082–4098. https://doi.org/10.1021/mp400467x
9. Beitelshees M, Li Y, Pfeifer BA (2016) Enhancing vaccine effectiveness with delivery technology. Curr Opin Biotechnol 42:24–29. https://doi.org/10.1016/j.copbio.2016.02.022

Chapter 26

Liposomal Dual Delivery of Both Polysaccharide and Protein Antigens

Roozbeh Nayerhoda, Andrew Hill, and Blaine A. Pfeifer

Abstract

Pneumococcal disease is caused by *Streptococcus pneumoniae*, a colonizing microorganism characterized by transitioning from a benign commensal to a virulent pathogen in the presence of suitable circumstances, which then poses a serious infectious disease threat afflicting millions of people. Especially affected are the young and elderly through outcomes that include pneumonia, bacteremia, meningitis, and otitis media. Current prevention vaccine options on the market contain capsular polysaccharides conjugated to the Diphtheria CRM197 protein (Pfizer) or are composed of only pneumococcal polysaccharides (Merck), and in both cases, limitations prevent the generation of comprehensive disease protection. Through the use of a liposomal carrier, we present an alternative methodology for producing a vaccine product via noncovalent colocalization of both polysaccharide and protein classes of complementary pneumococcal disease immunogens.

Key words Liposome, Encapsulation, Polysaccharide, Noncovalent, Vaccine, Pneumococcal disease

1 Introduction

Commensal bacterial pathogens cause disease outcomes including pneumonia, bacteremia, meningitis, and otitis media [1–4]. As an example, *Streptococcus pneumoniae*, a commensal bacterial microorganism capable of shifting to a virulent state, is considered a priority pathogen posing great risk to human health, as recognized by the World Health Organization [5–8].

The current standard of care for preventing pneumococcal infections includes routine vaccination of infants with the pneumococcal conjugate vaccine (PCV), Prevnar 13. PCVs such as Prevnar 13 prevent pneumococcal colonization by introducing the surface capsular polysaccharides (CPSs) associated with *S. pneumoniae* as the vaccine antigen. However, unlike the pneumococcal polysaccharide vaccine, Pneumovax 23, the polysaccharides in Prevnar 13 are covalently conjugated to the Diphtheria CRM197 immune-stimulating protein. By conjugating the CPSs to the

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carrier protein, Prevnar 13 is able to generate a T cell-dependent response to the CPSs, which are traditionally T cell-independent antigens. This enables PCVs to generate a potent and long-lasting immune response (demonstrated in part by IgM to IgG class switching) in infant populations that serve as reservoirs for pneumococcal colonization.

Despite their utility, current vaccine formulations for pneumococcal disease face challenges due to the diversity of pneumococcal CPSs (resulting in over 90+ unique *S. pneumoniae* strains or serotypes). Compounded by the difficulty of scaling the glycoconjugation process, which needs to be developed and performed separately for each individual polysaccharide, broad coverage of pneumococcal serotypes is limited due to the narrow subset within current vaccine options (i.e., the 13 and 23 designations of Prevnar and Pneumovax, respectively, represent the number of *S. pneumoniae* CPS serotypes included in the vaccine products). To overcome this limitation, we have developed a liposome-based system for generating a glycoconjugate-like immune response to pneumococcal polysaccharides that is more readily scalable regarding broader disease coverage.

To do so, we developed a liposome vaccine against *S. pneumoniae* that provides a two-pronged protection strategy against bacterial infection [5, 9]. In this strategy, we encapsulated 20+ pneumococcal capsular polysaccharides within a liposome that was surface-decorated with pneumococcal protein antigens that are upregulated in the biofilm-dispersed phenotype of the bacteria [5, 9, 10]. Through the physical colocalization of the CPSs and immunogenic protein antigens to the liposome, we were able to achieve a glycoconjugate-like immune response against the CPSs, without requiring direct covalent attachment [5, 9]. Consequently, we demonstrated the ability to provide potent immunization (i.e., a T cell-dependent immune response) against pneumococcal CPS using a mechanism that is more amenable to expanding to additional serotypes than conventional conjugation chemistry.

Here, we describe our approach to formulating a liposomal carrier that can harbor both *S. pneumoniae* polysaccharide content and noncovalently colocalize surface proteins (such as CRM197). Efforts to date have developed a process for encapsulating up to 24 pneumococcal capsular polysaccharides and attaching up to two proteins to the liposome surface [5, 9]. The methods outlined within this chapter illustrate the liposome encapsulation of polysaccharides (LEPS) featuring the pneumococcal 19F polysaccharide and the surface-attachment of the green fluorescent protein (GFP, as a surrogate protein useful in characterizing protein surface attachment) using an Ni-NTA system.

2 Materials

2.1 LEPS Components

1. Lipids for assembly of LEPS include:
   (a) 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC).
   (b) 1,2-Dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG).
   (c) 1,2-Dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA-Ni).
   (d) Cholesterol.
   (e) 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000).
2. Pneumococcal Polysaccharide: Isolated 19F serotype.
3. GFP is obtained through production in recombinant *E. coli* and purified chromatographically using Ni-NTA resin. The protocol for doing so is outlined within the method presented here.

2.2 Components Used for Assembly and Purification of LEPS

1. Fisher Scientific CL-18 Sonicator.
2. Buchi Rotavapor (R-200) rotary evaporator.
3. Buchi Heating Bath (B-490).
4. Handheld Extruder with 200 nm polycarbonate filter membrane.
5. Centrifugal Tubes with 300 kDa filters.

2.3 Protein Production and Purification Materials

1. BL21(DE3) *E. coli* containing pET21b with GFP inserted into the multiple cloning site is use to enable production of GFP.
2. Lysogeny broth (LB) medium: 10 g Bacto tryptone, 5 g yeast extract, and 10 g/L NaCl.
3. LB agar plates: 10 g/L Bacto tryptone, 5 g/L yeast extract, 10 g /L NaCl, and 15 g/L agar. When bacteria are transformed with the plasmids outlined here, resulting cultures are plated on solid agar supplemented with antibiotics and incubated overnight at 37 °C.
4. Bacterial growth medium is supplemented with ampicillin at 100 mg/L.
5. IPTG: isopropyl β-D-1-thiogalactopyranoside (100 μM) is added to bacterial media to induce gene expression.
6. HisTrap HP column, GE Healthcare.
7. Buffer A: 50 mM Na_2HPO_4, 500 mM NaCl, and 10% glycerol.
8. Buffer B: 50 mM Na_2HPO_4, 500 mM NaCl, 10% glycerol, and 250 mM imidazole.

9. 100 mM $NiSO_4$.
10. 50 mM ethylenediaminetetraacetic acid (EDTA).
11. 1 M guanidinium hydrochloride.
12. 20% ethanol.
13. Sodium dodecyl sulfate.
14. 30% acrylamide–bis solution, 29:1.
15. Ammonium persulfate (APS).
16. 10× Tris–glycine–SDS (25 mM Tris, 192 mM glycine, 0.1% SDS) at pH 8.3 for the resolving gel.
17. Tris–HCl (0.5 M, pH 6.8) for the stacking gel.
18. *N*,*N*,*N*,*N*-Tetramethylethylene-diamine (TEMED).
19. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 at pH of 7.4.

2.4 Assay Materials

1. Flat Bottom 96-well plates.
2. Synergy 4 Multi-Mode Microplate Reader (BioTek Instruments Inc.).
3. Concentrated sulfuric acid.
4. Autoclave bags.
5. Phenol.

3 Methods

3.1 Production of Recombinant GFP

1. BL21(DE3) *E. coli* containing the GFP plasmid is inoculated into 5 mL of LB media containing ampicillin and grown for 16–20 h at 37 °C in a shaker set to 225 rpm.
2. One milliliter of the bacterial culture is then transferred into 1 L of LB media containing fresh ampicillin and shaken at 37 °C until an OD_{600nm} of 0.4–0.5 is reached (approximately 3 h).
3. Protein expression is induced by supplementing the media with 100 μM IPTG. Afterward, the culture is incubated in a shaker set to 225 rpm and 22 °C for 20 h.
4. The cell culture is then harvested via centrifugation for 20 min at 4000 rpm and 4 °C (Sorvall Biofuge Primo R Centrifuge).
5. After removing the supernatant, the cell pellet is resuspended in 20 mL of buffer A and placed on ice.
6. The cells are then lysed using sonication (Model CL-18, Fisher Scientific) at an amplitude of 40–50. Cells are sonicated 10 s on and 30 s off for 10 cycles.

7. Following sonication, the cells are centrifuged for 20 min at 4000 rpm and 4 °C (Sorvall Biofuge Primo R Centrifuge) and the supernatant is transferred to microcentrifuge tubes.
8. The tubes are then centrifuged at 13,000 rpm (Eppendorf Centrifuge 5415 D) for 10 min to remove any remaining debris and the supernatant is collected into a single fresh tube.
9. Protein purification is then carried out with a prepared HisTrap HP column (GE Healthcare).
10. To prepare the HisTrap column, with a column volume (CV) of 1 mL, the following steps are performed:
    (a) Pass 2 CV of DI water over the column.
    (b) Pass 2 CV of 100 mM $NiSO_4$ over the column.
    (c) Pass 2 CV of DI H_2O over the column.
    (d) Pass 2 CV of buffer B over the column.
    (e) Pass 2 CV of DI H_2O over the column.
    Add the crude protein extract to the column and collect 1 mL fractions in a 96-well plate.
    (f) Pass 20 mL of buffer A containing 5 mM imidazole through the column followed by successive 5 mL of buffer A containing the following concentrations of imidazole: 10, 15, 20, 30, 50, 100, and 250 mM (i.e., buffer B), followed by an additional 10 mL of buffer B passed over the column during which fractions were no longer collected.
11. The fractions collected are then evaluated for protein content using a plate reader to measure absorbance at 280 nM. Fractions which demonstrate an increase in absorbance, typically fractions containing ~100 mM imidazole, are then evaluated for the presence of GFP using SDS PAGE using a 10% acrylamide gel.
12. After the SDS PAGE is completed, the gel is stained using Coomassie Blue, then washed with water to visualize protein bands. Fractions containing the protein of interest with little contaminants are identified using a protein ladder and then combined.
13. The combined protein fractions are dialyzed overnight into PBS at 4 °C.
14. The concentration of the protein solution is determined using the Bradford Assay with a clear polystyrene plate. Five microliters of the extract is mixed with 250 μL of the Bradford reagent and incubated at room temperature for 20 min. Afterward, the absorbance at 595 nm is measured and compared to a standard curve containing 0–1 mg/mL of BSA (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL).

3.2 Assembly of the LEPS Construct

1. Dissolve (DOPC), (DOPG), (DGS-NTA-Ni), cholesterol, (DSPE-PEG2000) at a molar ratio of 3:3:1:4:0.1 to a total lipid mass of 500 μg in chloroform.
2. Add 1 mL of warm solution of Pneumococcal polysaccharide 19F serotype with the concentration of 0.6 mg/mL in PBS (*see* **Note 1**) to the lipid solution and sonicate at an amplitude of 50% for 1 min.
3. Using rotatory evaporator, evaporate the solvents at 50 °C until a thin white film comprised of the lipids and Pneumococcal polysaccharide 19F serotype.
4. Rehydrate the film by rotating it with 1 mL of double-filtered PBS at the same temperature until the thin film is fully dissolved (Fig. 1).
5. Pass the dissolved lipids 10–12 times through a polycarbonate membrane (200 nm pore size) at 60 °C using a handheld extruder (*see* **Note 2**). This process should yield unilamellar liposomes of a defined size (200 nm) (Fig. 2).
6. To purify postextrusion liposomes from free/unencapsulated polysaccharides and small vesicles, Transfer 500 μL of postextrusion sample to the 300 kDa microcentrifuge tubes and centrifuge for 5 min at 12,000 rcf at 4 °C.

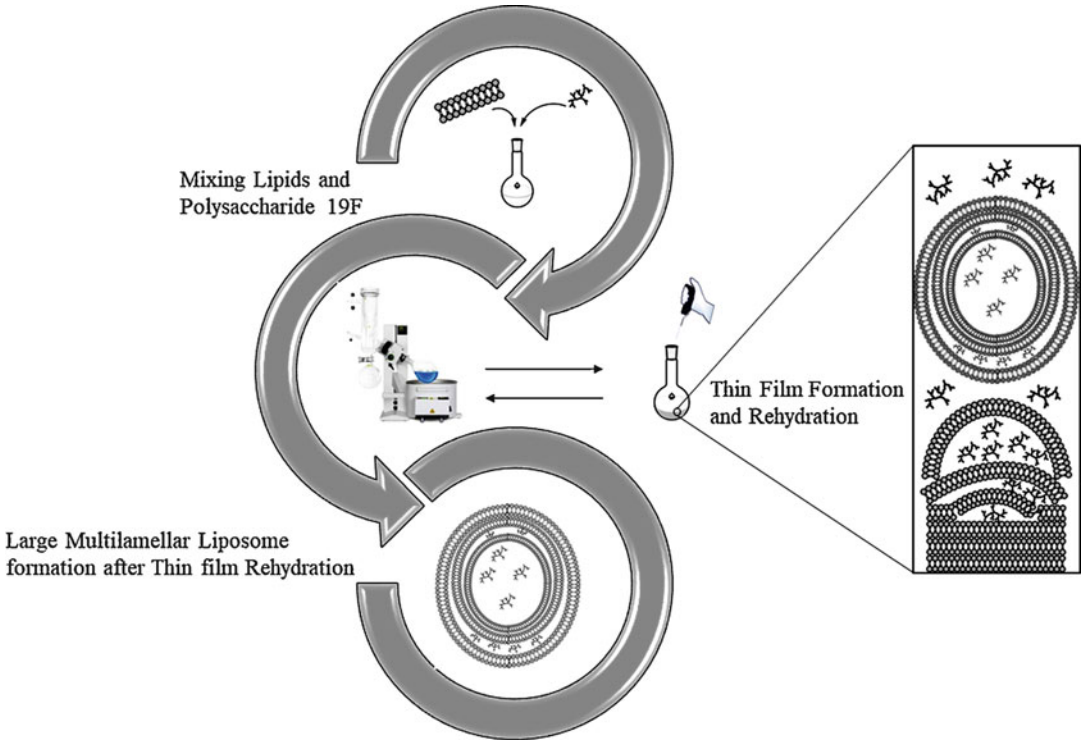

Fig. 1 Initial multilamellar liposome production

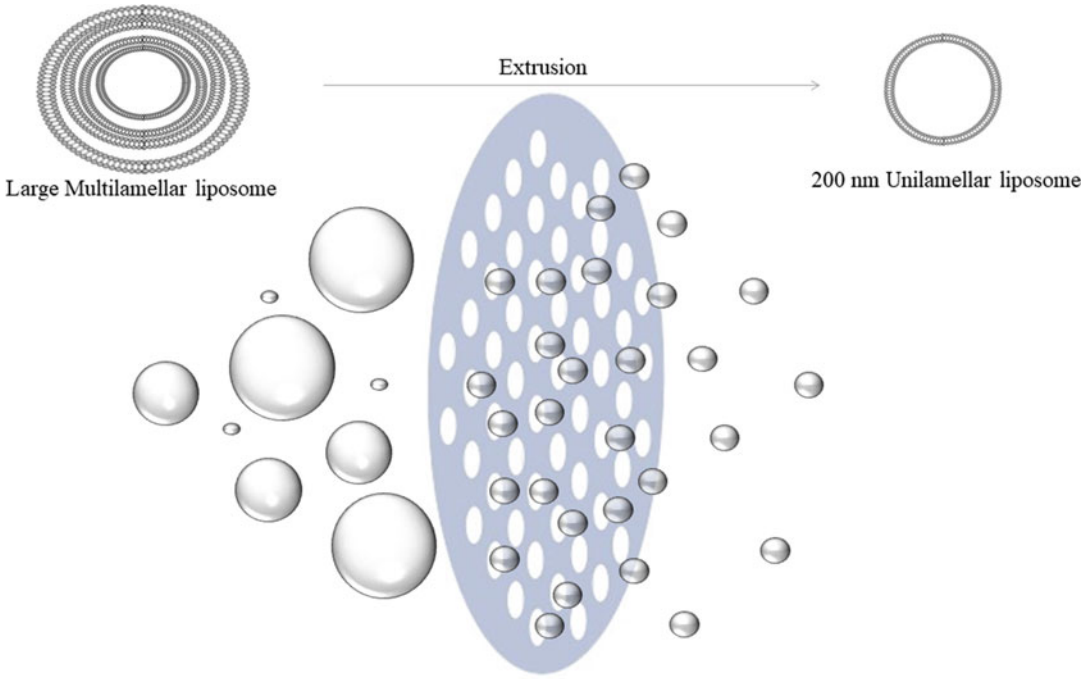

Fig. 2 Liposome extrusion schematic

7. Resuspend the purified liposome in up to 500 μL PBS and transfer the solution to a 1.5 mL centrifugal tube.
8. Add 140 μg of GFP to the liposome solution (*see* **Note 3**) and incubate for 30 min at room temperature (20–25 °C).
9. Transfer 500 μL of the sample to the 300 kDa microcentrifuge tubes and centrifuge for 5 min at 4 °C at 12,000 rcf.
10. Resuspend the final purified liposomes in up to 500 μL of PBS and transfer to a microcentrifuge tube and repeat the centrifugation for 5 min at 4 °C at 12,000 rcf.
11. Resuspend the purified liposome in up to 500 μL PBS and transfer the solution to a 1.5 mL centrifugal tube (Fig. 3).

3.3 Measuring Protein Binding Efficiency

1. Transfer 50 μL of liposome-bound GFP to a 96-well plate. A negative control of the liposome without added GFP should be included. In addition, a positive control containing the amount of GFP initially added should be included.
2. Measure the fluorescence of the plate wells using a plate reader with at an excitation wavelength of 359 nm and an emission wavelength of 508 nm. The fluorescence of the liposome without added GFP (F_{blank}) and the fluorescence of the amount of GFP initially to the liposomes added F_{max} should be noted.

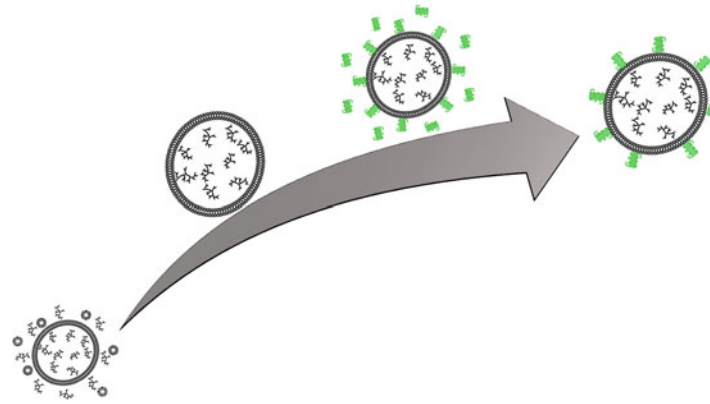

Fig. 3 Liposome polysaccharide encapsulation, surface protein attachment, and purification steps

3. Compare the fluorescence measurements to a GFP standard curve and determine the fraction bound by comparing to initial amount of introduced protein: Binding efficiency = $(F_{\text{sample}} - F_{\text{blank}})/(F_{\max} - F_{\text{blank}})$ * 100%.

3.4 Polysaccharide Encapsulation Efficiency

1. Add 0.6 mL of fully assembled LEPS construct to a glass vial. In addition, a negative control containing a LEPS construct without polysaccharides should be included. Furthermore, a standard curve of polysaccharides ranging from 0 to 0.6 mg/mL should be included.
2. Next, add 0.3 mL of phenol 5% (w/v) to the vial.
3. Add 1.5 mL concentrated sulfuric acid to the mixture.
4. After adding each component, mix the solution by vortexing for 5 s.
5. Following mixing, transfer 250 μL of sample to a microplate.
6. Seal the microplate and then place it into a sealable plastic bag.
7. Incubate the microplate in a 92 °C water bath for 15 min (*see* **Note 4**).
8. Transfer the microplate to a room temperature water bath (20–25 °C) and incubate for an additional 15 min.
9. Measure the absorbance at 480 nm using a plate reader (*see* **Note 5**). Utilize a standard curve to determine the concentration of polysaccharide in each well.
10. Compare the measured values to that obtained for the initial concentration of introduced polysaccharide (0.6 mg, $A_{\max}$) and the blank containing no polysaccharides (A_{blank}) to determine encapsulation efficiency using the following calculation: Encapsulation efficiency = $(A_{\text{sample}} - A_{\text{blank}})/(A_{\max} - \text{A}_{\text{blank}})$ * 100%.

3.5 Characterization of Liposome Size, Shape, and Charge

1. Dynamic light scattering on a Zetasizer Nano ZS90 (Malvern) was used to evaluate the particle diameter and zeta potential of liposomes at 25 °C. All experiments were conducted using a 4-mW, 633-nm HeNe laser as the light source at a fixed measuring angle of 90° to the incident laser beam.
2. Images of the LEPS particles were obtained through JEOL JSM-CXII transmission electron microscopy analysis at 100 kV, with samples prepared by dip-coating a 200-mesh formvar and a carbon-coated grid (FCF-200-Cu-TB, Electron Microscopy Sciences), followed by negative staining using a 1% solution of uranyl acetate.

3.6 Murine Immunization with LEPS

1. Following assembly and characterization, the LEPS vaccine can then be used for animal immunizations. Standardize dosage of LEPS to include 2.2 μg of the 19F polysaccharide.
2. Prior to the first immunization, collect serum from the mice via retro-orbital bleeding followed by centrifugation to establish baseline antibody and cytokine levels.
3. Immunize mice (female CD-1 mice) in groups of 6 using subcutaneous (SC) injections of 200 μL. Include a sham vaccination group that are immunized with PBS as a negative control.
4. After the passage of 14 days, collect serum from the mice as described above to measure antibody and cytokine values post-primary immunization.
5. After collecting the post-primary immunization serum, immunize the mice a second time using the same conditions.
6. After the passage of 14 days, collect serum from the mice as described above to measure antibody and cytokine values post-secondary immunization.

3.7 Quantification of the Immune Response

1. To characterize relative antibody titers associated with delivery optimization, perform an enzyme-linked immunosorbent assay (ELISA). First, coat a 96-well Costar high-binding polystyrene plate with polysaccharide 19F (10 μg/mL) in tris-buffered saline (TBS) at 4 °C overnight.
2. Next, block the plate with 3% bovine serum albumin in TBS–Tween 20 (TBS-T) for 1 h at 22 °C.
3. Add sera that has been diluted into TBS-T in ratios of 1:1000, 1:5000, 1:7500, and 1:10,000 to the plate.
4. Incubate the plates at 37 °C with mild agitation for 3 h.
5. Add the secondary antibody [anti-mouse IgG, IgA, IgM (H + L), IgE, highly X-adsorbed (biotin)] to the wells in a 1:1000 ratio and agitate for 2 h.

6. Add streptavidin conjugated with alkaline phosphatase to each well in a 1:1000 ratio and shake for 30 min.
7. Develop the signal with *p*-nitrophenylphosphate, and quench the reaction using 0.75 M NaOH.
8. Measure the absorbance at 405 nm for each well using a plate reader spectrophotometer. Relative antibody titers should be determined through comparison to the sham vaccine control.
9. Cytokine measurements should be determined using ELISA kits (R&D Systems) following the manufacturer's instructions.

4 Notes

1. The 0.6 mg/mL concentration is when liposomes with the current lipid composition show the maximum encapsulation efficiency for polysaccharide 19F [5].
2. Large vesicles, due to several reasons such as multilamellarity and small core volume, are not ideal candidates for the purpose of the study [11, 12]. By extruding the large multilamellar vesicles with size of around 1–5 μm, while passing through the polycarbonate membrane, their lipid bilayers disrupt and immediately reform to unilamellar vesicles with a size distribution related to the membrane pore size (which, here, is 200 nm), exit the membrane, and result in liposomes with defined size on the other side with more stability and improved encapsulation efficiency (due to a larger core volume).
3. The GFP that has been purified from recombinant *E. coli* must be evaluated for purity and concentration (in our case, by using SDS-PAGE and Bradford analysis, respectively).
4. To protect the sealed microplate during the polysaccharide detection assay, it is placed in a small, sealable plastic bag prior to addition to the surface of a 92 °C water bath.
5. Considering differences in monosaccharide compositions of polysaccharides [13, 14], it is necessary to optimize the colorimetric assay to find the maximum absorbance wavelength for each polysaccharide. In this case, the corresponding peak for polysaccharide 19F was observed at 480 nm.

References

1. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, Jha P, Campbell H, Walker CF, Cibulskis R, Eisele T, Liu L, Mathers C, Child Health Epidemiology Reference Group of WHO, Unicef (2010) Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet 375 (9730):1969–1987. https://doi.org/10.1016/S0140-6736(10)60549-1
2. Vergison A, Dagan R, Arguedas A, Bonhoeffer J, Cohen R, Dhooge I, Hoberman A, Liese J, Marchisio P, Palmu AA, Ray GT, Sanders EA, Simoes EA, Uhari M, van Eldere J, Pelton SI (2010) Otitis media and its

consequences: beyond the earache. Lancet Infect Dis 10(3):195–203. https://doi.org/10.1016/S1473-3099(10)70012-8

3. Walker CLF, Rudan I, Liu L, Nair H, Theodoratou E, Bhutta ZA, O'Brien KL, Campbell H, Black RE (2013) Global burden of childhood pneumonia and diarrhoea. Lancet 381(9875):1405–1416. https://doi.org/10.1016/S0140-6736(13)60222-6
4. Adetifa IMO, Adamu AL, Karani A, Waithaka M, Odeyemi KA, Okoromah CAN, Bello MM, Abubakar IS, Inem V, Scott JAG (2018) Nasopharyngeal pneumococcal carriage in Nigeria: a two-site, population-based survey. Sci Rep 8(1):3509. https://doi.org/10.1038/s41598-018-21837-5
5. Jones CH, Zhang G, Nayerhoda R, Beitelshees M, Hill A, Rostami P, Li Y, Davidson BA, Knight P 3rd, Pfeifer BA (2017) Comprehensive vaccine design for commensal disease progression. Sci Adv 3(10):e1701797. https://doi.org/10.1126/sciadv.1701797
6. World Health Organization (2017, February 27) WHO publishes list of bacteria for which new antibiotics are urgently needed. https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed
7. Papadatou I, Spoulou V (2016) Pneumococcal vaccination in high-risk individuals: are we doing it right? Clin Vaccine Immunol 23(5):388–395. https://doi.org/10.1128/CVI.00721-15
8. Saokaew S, Rayanakorn A, Wu DB, Chaiyakunapruk N (2016) Cost effectiveness of pneumococcal vaccination in children in low- and middle-income countries: a systematic review. Pharmacoeconomics 34(12):1211–1225. https://doi.org/10.1007/s40273-016-0439-3
9. Hill AB, Beitelshees M, Nayerhoda R, Pfeifer BA, Jones CH (2018) Engineering a next-generation glycoconjugate-like *Streptococcus pneumoniae* vaccine. ACS Infect Dis 4:1553. https://doi.org/10.1021/acsinfecdis.8b00100
10. Li Y, Hill A, Beitelshees M, Shao S, Lovell JF, Davidson BA, Knight PR, Hakansson AP, Pfeifer BA, Jones CH (2016) Directed vaccination against pneumococcal disease. Proc Natl Acad Sci U S A 113(25):6898–6903
11. Foteini P, Pippa N, Naziris N, Demetzos C (2019) Physicochemical study of the protein-liposome interactions: influence of liposome composition and concentration on protein binding. J Liposome Res 29:313–321. https://doi.org/10.1080/08982104.2018.1468774
12. Nele V, Holme MN, Kauscher U, Thomas MR, Doutch JJ, Stevens MM (2019) Effect of formulation method, lipid composition, and PEGylation on vesicle lamellarity: a small-angle neutron scattering study. Langmuir 35(18):6064–6074. https://doi.org/10.1021/acs.langmuir.8b04256
13. Kim JS, Laskowich ER, Arumugham RG, Kaiser RE, MacMichael GJ (2005) Determination of saccharide content in pneumococcal polysaccharides and conjugate vaccines by GC-MSD. Anal Biochem 347(2):262–274. https://doi.org/10.1016/j.ab.2005.09.022
14. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, Lee YC (2005) Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem 339(1):69–72. https://doi.org/10.1016/j.ab.2004.12.001

Chapter 27

Thin-Film Freeze-Drying Is a Viable Method to Convert Vaccines Containing Aluminum Salts from Liquid to Dry Powder

Riyad F. Alzhrani, Haiyue Xu, Chaeho Moon, Laura J. Suggs, Robert O. Williams III, and Zhengrong Cui

Abstract

Aluminum salts are used as an adjuvant in many human and veterinary vaccines. However, aluminum salt-adjuvanted vaccines are sensitive to temperature change and must be stored at 2–8 °C. Inadvertently exposing them to slow freezing temperatures can cause irreversible aggregation of aluminum salt microparticles and loss of potency and/or immunogenicity of the vaccines. There have been efforts to overcome this limitation by either adding stabilizing agents to the liquid vaccine or converting the vaccine from a liquid to a dry powder. Thin-film freeze-drying (TFFD) has proven to be an effective process to convert aluminum salt-adjuvanted vaccines from liquid to dry powder without causing particle aggregation or loss of immunogenicity upon reconstitution. This chapter provides a review of the TFFD process and examples for preparing stable aluminum salt-adjuvanted vaccine dry powders using TFFD.

Key words Aluminum salts, Vaccine, Thin-film freezing, Lyophilization, Dry powder

1 Introduction

Insoluble aluminum salt-adjuvanted vaccines make up more than half of the vaccines approved for human use [1–3]. Vaccines including hepatitis B, human papillomavirus, and diphtheria–tetanus–pertussis vaccines are adjuvanted with aluminum salts [2–4]. The World Health Organization (WHO) recommends that all vaccines (a total of 16), except oral polio vaccine, must to be stored at 2–8 °C, especially the ones containing aluminum salts [5]. Aluminum salt-adjuvanted vaccines are known to be sensitive to slow freezing [5]. Vaccines adjuvanted with aluminum salts are suspensions of aluminum salt microparticles with antigens adsorbed on them [6]. When aluminum salt-adjuvanted vaccines are exposed to freezing temperatures, the aluminum salt particles aggregate, and the extent of aggregation is dependent on parameters such as the

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rate of freezing and the number of freeze–thaw cycles [7, 8]. Overall, subjecting vaccines adjuvanted with aluminum salts to slow freezing can significantly comprise their immunogenicity [7–10]. Unfortunately, it is estimated that 75–100% of vaccines are exposed to freezing temperatures before being administered [11]. These vaccines are either discarded under the best practice, or inadvertently administered to humans, leading to costly waste to the health care system and poor or no immune protection [12–17]. For instance, it was reported that the difference in the level of hepatitis B surface antigen-specific antibody between human subjects living in rural areas (70%) versus urban areas (94%) in Mongolia is due mostly to the exposure of hepatitis B vaccine they received to freezing temperatures during transportation [18].

Strategies to overcome this problem include adding stabilizing agent(s) to the liquid vaccines and/or converting the liquid vaccines to dry powder. In the liquid vaccine formulation, water is the main component that exists between and within the aluminum salt particles. This water layer helps to maintain the physical state and shape of the antigen-adsorbed aluminum salt particles when stored at the recommended storage conditions. However, during a slow freezing process, the ice crystals that are generated cause distortion to the hydration shell and eventually lead to aggregation of the aluminum salt particles [4, 8]. Inhibiting ice crystal formation using certain excipients (e.g., polyols) could minimize the particle aggregation and preserve the immunogenicity of the vaccine [7, 8, 19]. For example, Braun and colleagues showed that adding 50% (v/v) of polyethylene glycol (PEG)300, propylene glycol (PG), or glycerin to the hepatitis B vaccine that contains insoluble aluminum salts inhibited particle aggregation after the vaccine was exposed to multiple freeze–thaw cycles (−20 °C), and the vaccine maintained its potency and induced an antibody level similar to that induced by the freshly prepared vaccine [19].

Another approach to improve the stability of aluminum salt-adjuvanted vaccines is by converting the liquid vaccine into dry powder using methods such as spray freeze-drying and spray-drying [8, 20–23]. We reported that thin-film freeze-drying (TFFD) can be used to convert vaccines containing insoluble aluminum salts from liquid to dry powder without causing particle aggregation or decreasing the immunogenicity after reconstitution [24, 25]. During TFFD, liquid droplets (~2–4 mm in diameter) of a vaccine are first applied onto a cryogenically cooled surface (Fig. 1). Upon impacting the cryogenically cooled surface, the droplets spread and freeze rapidly (e.g., in ~70–1000 ms), generating frozen thin films that are ~100–400 μm thick [20, 26]. The solvent in the frozen thin film is then removed by lyophilization. The applicability of the TFFD technology was confirmed using model vaccines prepared in-house as well as commercial human and veterinary vaccines that contain insoluble aluminum salts such

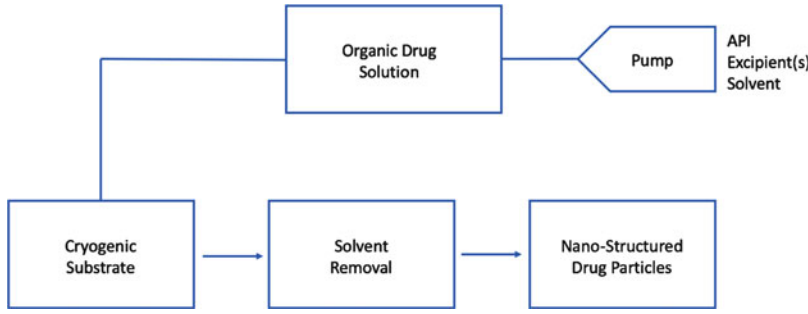

Fig. 1 Schematic of the thin-film freeze-drying (TFFD) process. (Reproduced from Overhoff et al. [26] with permission from Elsevier)

as aluminum (oxy)hydroxide, aluminum (hydroxy)phosphate, aluminum potassium sulfate, at a relatively high aluminum concentration (~1%, w/v). Trehalose alone at a concentration as low as 2% (w/v) is sufficient as the excipient during the TFFD process [24]. It is worth noting that although the freezing of the thin films in the thin-film freezing (TFF) process is rapid, with an estimated cooling rate of >100 K/s (e.g., also reported at >1000 K/s), it is not as fast as that in the spray freeze-drying process (e.g., about 10^6 K/s) [20, 26, 27]. It is speculated that what makes TFF so effective is that, due to the relatively large size of the droplets before they impact the cryogenically cooled surface and the thin films form thereafter, the overall air-liquid interface of the falling droplets and the spreading films generated during TFF are smaller, in comparison to the large gas-liquid interface generated when atomized droplets are sprayed through liquid nitrogen vapor into liquid nitrogen [20, 26, 27]. Thin-film freeze-dried vaccine powder is not sensitive to freeze–thaw cycles and can be stored at temperatures as high as 40 °C for months without compromising its immunogenicity [25]. For a longer-term storage, a method to minimize the humidity in the container, and thus moisture content in the powder, need to be used. The following describes a procedure for preparing aluminum salt-adjuvanted vaccine dry powder using the TFFD method.

2 Materials

2.1 Reagents

1. 2% (w/v) Alhydrogel® (aluminum content, 9–11 mg/mL).
2. Gardasil®.
3. Engerix-B®.
4. 5 N sodium hydroxide (NaOH) solution (pH 12–14).
5. 5 N acetic acid (CH_3COOH) solution.
6. 0.9% (w/v) sodium chloride (physiological normal saline).

7. 1% (w/v) Thimerosal [2-(ethylmercuriomercapto)benzoic acid sodium salt] solution, if needed.
8. Trehalose (i.e., α-D-glucopyranosyl-α-D-glucopyranoside).
9. Liquid nitrogen.

2.2 Equipment and Supplies

1. Syringes, pipettes, and needles (e.g., 17 G).
2. Trays and beakers.
3. Desiccators.
4. Silanized glass vials.
5. Centrifuge.
6. Cold room (2–8 °C).
7. pH meter,
8. Freezer (−80 °C).
9. VirTis Advantage bench top freeze dryer apparatus.

2.3 Thin-Film Freezing (TFF) Apparatus

A typical TFF apparatus (Fig. 2a) has a rotating stainless steel cylinder filled with liquid nitrogen, and its surface temperature is monitored via an external thermocouple (*see* **Notes 1** and **2**) [28]. The rotating speed of the drum is adjusted to enable one thin-film formation at a time (*see* **Note 3**) [28, 29]. It is recommended to have a stainless steel blade mounted along the drum surface to facilitate removal of the frozen thin films and collecting them in a reservoir below the rotating drum, filled with liquid nitrogen [28, 29]. The TFF apparatus is operated in a room with low relative humidity (at least <~15%) to avoid the condensation of water vapor on the drum surface [29].

3 Methods

3.1 Aluminum Salt-Adjuvanted Vaccines

Aluminum salts such as amorphous aluminum hydroxyphosphate sulfate, aluminum (oxy)hydroxide, and aluminum (hydroxy)-phosphate are the most common salt types used as adjuvants in human and veterinary vaccines [6]. Different aluminum salts have different physicochemical properties that could directly affect their adjuvanticity. Based on the antigens, different conditions (e.g., pH, binding efficiency, salt concentration) should be carefully investigated before preparing aluminum salt-adjuvanted vaccines. Readers are referred to other methods of preparing aluminum salt containing vaccines in reference [6]. However, vaccines prepared by in situ adsorption of protein antigens (e.g., ovalbumin (OVA)) onto aluminum salt particles will be described below as an exemplary method to prepare aluminum salt-adjuvanted vaccines for TFFD. Additionally, we include a method of converting two commercially available vaccines from liquid to dry powder using the TFFD method.

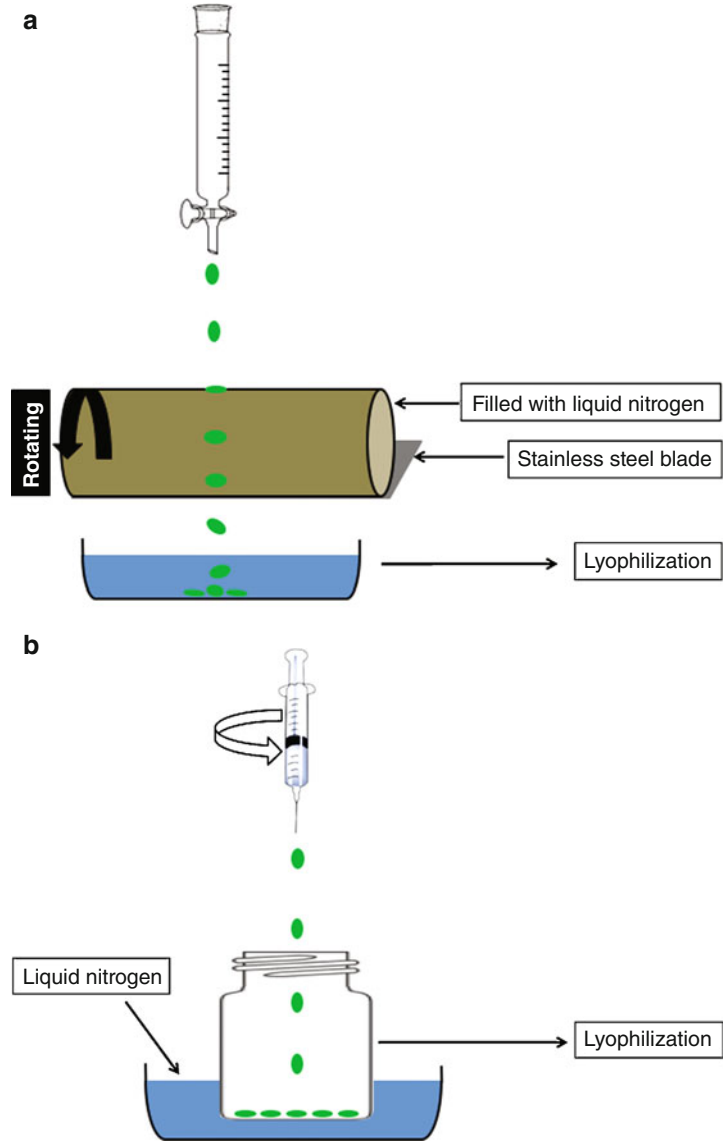

Fig. 2 Schematic of thin-film freezing of vaccine in liquid using a TFF apparatus (**a**) or in a single vial (**b**) followed by lyophilization

3.1.1 Preparation of In-House Vaccine: In Situ Adsorption of OVA onto Alhydrogel®

Alhydrogel® is a commercially available 2% (w/v) aluminum hydroxide wet gel. It is recommended to identify the optimal binding efficiency between the antigen of interest and the aluminum (oxy)hydroxide particles in Alhydrogel® to prepare a vaccine [6].

1. Vortex Alhydrogel® suspension briefly to break up the large coagulates.
2. Use 5 N NaOH or 5 N acetic acid to adjust the pH to a desired value, if needed.

3. Prepare an OVA antigen solution in a normal saline solution and mix the OVA solution and the Alhydrogel® suspension at a desired rate while stirring.
4. Incubate the mixture for at least 20 min while stirring in a cold room.
5. Adjust the pH of the final vaccine formulation, if needed.
6. Add trehalose powder directly to the vaccine formulation to reach a concentration of 2% (w/v) (*see* **Note 4**).

3.1.2 Preparation of Commercially Available Vaccines, Gardasil® and Engerix-B®

Gardasil® is an FDA-approved vaccine for the prevention of diseases caused by the human papillomavirus virus (HPV). Gardasil-4® is composed mainly of quadrivalent capsid L1 proteins (HPV-6, 11, 16 and 18) adsorbed onto amorphous aluminum hydroxyphosphate sulfate (225 μg of aluminum salt per a 500 μL dose) [30].

1. Dilute the vaccine tenfold in a normal saline solution.
2. Mix the vaccine gently by pipetting up and down.
3. Add trehalose powder directly to the diluted vaccine formulation to reach a concentration of 2% (w/v).

Engerix-B® is a human vaccine against hepatitis B virus. The vaccine is a suspension of hepatitis B virus surface antigen (HBsAg) adsorbed onto aluminum hydroxide (500 μg per 1 mL dose) [31].

1. Shake the vaccine vials gently.
2. Add trehalose powder directly to the vaccine to reach a concentration of 2% (w/v).

3.2 Preparation of Aluminum Salt-Adjuvanted Vaccine Dry Powder

The TFFD process can be divided into two steps, thin-film freezing and drying. The liquid vaccine droplets are converted to frozen thin films initially followed by drying via lyophilization [29]. The application of the TFFD have been largely demonstrated using the TFF apparatus (Fig. 2a) [24–29], but a method of TFF in a single vial (Fig. 2b) has also been developed [25].

3.2.1 Thin-Film Freezing Process Using the TFF Apparatus

1. Place a stainless-steel reservoir containing liquid nitrogen below the rotating cryogenic cylinder (*see* **Note 5**).
2. Monitor the cylinder temperature (*see* **Note 2**) and adjust the speed at which the cylinder rotates to be 5–10 rotations per minute (rpm) (*see* **Note 3**).
3. Load vaccine formulation in an applicator (*see* **Note 6**) and apply dropwise to the rotating cryogenically cooled cylinder surface. The stainless-steel blade mounted along the surface of the rotating cylinder will facilitate the removal of the thin films, which will then drop into the stainless-steel reservoir below.

4. Transfer the collected frozen films to the lyophilizer (*see* **Note 7**).
5. Subject the thin films to lyophilization, collect the lyophilized powder in sealed glass vials, and then store it in a desiccator at room temperature before further analysis (*see* **Note 8**).
6. Characterize the powder (e.g., moisture content, particle size distribution after reconstitution, antigen-binding efficacy, immunogenicity).

3.2.2 TFF in a Single Vial

1. Immerse a salinized glass vial into liquid nitrogen to create a cryogenically cooled surface in the inner bottom of the vial (*see* **Notes 9** and **10**).
2. Using a pipette or syringe, add the vaccine formulation dropwise to the bottom of the vial so that the droplets, upon impact of the surface, can rapidly freeze into thin films (*see* **Notes 6** and **11**).
3. Transfer the glass vial into a lyophilizer (*see* **Note 7**).
4. Subject the thin films to lyophilization, add a rubber stopper and aluminum cap to the vial and store it in a desiccator at room temperature before further analyses (*see* **Note 8**).
5. Characterize the powder.

3.2.3 Lyophilization

Drying is the next important step to obtain vaccine dry powder and it could be achieved using lyophilization. Under certain pressure and temperature, the water could be removed from the frozen-film (sublimation), without going through the liquid phase. Any remaining solvent might lead to loss of immunogenicity and/or particle aggregation after reconstitution. To conduct the drying using a lyophilizer, a VirTis Advantage bench top tray lyophilizer or any other compatible tray lyophilizer can be used [24, 29].

1. Set the VirTis Advantage bench top tray lyophilizer shelf temperature at −40 °C.
2. Prepare the collected reservoir or vials for the lyophilizer.
3. Start the lyophilization cycle: −40 to 25 °C for 40 h (primary drying), 20 °C for 26 h (secondary drying), at a pressure < 200 mTorr.

3.2.4 Particle Size Distribution Measurement

The particle size of the antigen-adsorbed aluminum salt particles in the vaccine is critical for the vaccine immunogenicity [2]. The particle size distribution of the reconstituted vaccine may be measured using a Sympatec Helos laser diffraction instrument (Sympatec GmbH, Germany) equipped with an R3 lens [24].

3.2.5 Moisture Content

The moisture content in the dry powder is measured using a Karl Fisher Titrator Aquapal III from CSC Scientific Company (Fairfax, VA) [24].

3.2.6 Antigen-Binding Efficiency

Free, unbound antigens before and after the vaccine is subjected to TFFD and reconstitution can be measured by centrifuging the vaccine for 5 min (4500 × rcf) and measuring the protein content in the supernatant [24]. The integrity of the antigen can be determined using SDS-PAGE after it is desorbed from the aluminum salts [6, 32].

4 Notes

The following notes are listed to provide further description of the exemplary adjuvanted vaccine dry powder and methods. The reader can find supporting details described in the notes from the following references [20, 24–29].

1. The TFF apparatus requires a mechanical stirrer with the flexibility of adjusting the speed of the system. A Heidolph mechanical stirrer (Model RZR2041) connected to speed controller (Model GS6013G) is used in our apparatus. Any other compatible model could be used to rotate the cylinder.
2. The cylinder surface temperature should be confirmed to be around −40 to −50 °C before starting to drop the vaccine liquid onto the cylinder.
3. The rotating cylinder speed should be adjusted to avoid any overlap between droplets over the cylinder. For our apparatus model, a rotating speed of 5–7 rpm is optimal to achieve one frozen film at a time.
4. Trehalose at a final concentration of 2% (w/v) has proven to be sufficient for a successful TFFD. Its concentration may need to be increased or optimized if a different vaccine is used.
5. The liquid nitrogen should be added to the reservoir to keep the thin films frozen during and after the TFF process. Otherwise, the thin films will melt, and this could lead to a failure in the entire TFF process.
6. Different designs may be used to apply the liquid.
7. The collected frozen films should be transferred to a −80 °C freezer if they are not lyophilized immediately.
8. The dry powder is hygroscopic and fluffy. Therefore, the lyophilized powder should be stored immediately in a desiccator to reduce moisture absorption.
9. The neck and mouth of the silanized glass vial should not be immersed in the liquid nitrogen. Yet the bottom of the vial

should be completely immersed in the liquid nitrogen (Fig. 2b).

10. Much attention should be paid in this step due to the vapor generated from the liquid nitrogen. This vapor could potentially freeze the vaccine formulation at the dropper or pipette tip, preventing the deposition of the rest of formulation into the vial. To avoid this issue, try to find the optimum distance from the bottom of the vial where it is possible to drop the vaccine without freezing. It is also recommended to use personal protective equipment during this process.
11. The volume of the vaccine formulation should not exceed 250 μL in a 20 mL glass vial to avoid formation of overlapped thin films.

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References

1. Kristensen D, Chen D, Cummings R (2011) Vaccine stabilization: research, commercialization, and potential impact. Vaccine 29 (41):7122–7124
2. Clausi A, Cummiskey J, Merkley S, Carpenter JF, Braun LJ, Randolph TW (2008) Influence of particle size and antigen binding on effectiveness of aluminum salt adjuvants in a model lysozyme vaccine. J Pharm Sci 97 (12):5252–5262
3. Shi S, Zhu H, Xia X, Liang Z, Ma X, Sun B (2019) Vaccine adjuvants: understanding the structure and mechanism of adjuvanticity. Vaccine 37(24):3167–3178
4. Chen D, Kristensen D (2009) Opportunities and challenges of developing thermostable vaccines. Expert Rev Vaccines 8(5):547–557
5. World Health Organization (2006) In: Galazka AM (ed) Temperature sensitivity of vaccines. World Health Organization, Geneva
6. Thakkar SG, Cui Z (2017) Methods to prepare aluminum salt-adjuvanted vaccines. In: Vaccine adjuvants, Methods in molecular biology, vol 1494. Springer, New York, pp 181–199
7. Zapata MI, Peck GE, Hem SL, White JL, Feldkamp JR (1984) Mechanism of freeze-thaw instability of aluminum hydroxycarbonate and magnesium hydroxide gels. J Pharm Sci 73 (1):3–8
8. Maa YF, Zhao L, Payne LG, Chen D (2003) Stabilization of alum-adjuvanted vaccine dry powder formulations: mechanism and application. J Pharm Sci 92(2):319–332
9. Diminsky D, Moav N, Gorecki M, Barenholz Y (1999) Physical, chemical and immunological stability of CHO-derived hepatitis B surface antigen (HBsAg) particles. Vaccine 18 (1–2):3–17
10. Boros CA, Hanlon M, Gold M, Roberton D (2001) Storage at −3 °C for 24 h alters the immunogenicity of pertussis vaccines. Vaccine 19(25–26):3537–3542
11. Matthias DM, Robertson J, Garrison MM, Newland S, Nelson C (2007) Freezing temperatures in the vaccine cold chain: a systematic literature review. Vaccine 25(20):3980–3986
12. Nygaard UC, Samuelsen M, Aase A, Løvik M (2004) The capacity of particles to increase allergic sensitization is predicted by particle number and surface area, not by particle mass. Toxicol Sci 82(2):515–524

13. Davaalkham D, Ojima T, Wiersma S, Lkhagvasuren T, Nymadawa P, Uehara R, Watanabe M, Oki I, Nakamura Y (2007) Administration of hepatitis B vaccine in winter as a significant predictor of the poor effectiveness of vaccination in rural Mongolia: evidence from a nationwide survey. J Epidemiol Community Health 61(7):578–584
14. Menon P, Sahai G, Joshi V, Murthy R, Boparai M, Thomas A (1976) Field trial on frozen and thawed tetanus toxoid. Indian J Med Res 64(1):25–32
15. Wirkas T, Toikilik S, Miller N, Morgan C, Clements CJ (2007) A vaccine cold chain freezing study in PNG highlights technology needs for hot climate countries. Vaccine 25 (4):691–697
16. Lydon P, Zipursky S, Tevi-Benissan C, Djingarey MH, Gbedonou P, Youssouf BO, Zaffran M (2013) Economic benefits of keeping vaccines at ambient temperature during mass vaccination: the case of meningitis A vaccine in Chad. Bull World Health Organ 92(2):86–92
17. Nelson CM, Wibisono H, Purwanto H, Mansyur I, Moniaga V, Widjaya A (2004) Hepatitis B vaccine freezing in the Indonesian cold chain: evidence and solutions. Bull World Health Organ 82:99–105
18. Edstam JS, Dulmaa N, Tsendjav O, Dambasuren B, Densmaa B (2004) Exposure of hepatitis B vaccine to freezing temperatures during transport to rural health centers in Mongolia. Prev Med 39(2):384–388
19. Braun LJ, Tyagi A, Perkins S, Carpenter J, Sylvester D, Guy M, Kristensen D, Chen D (2009) Development of a freeze-stable formulation for vaccines containing aluminum salt adjuvants. Vaccine 27(1):72–79
20. Overhoff KA, Johnston KP, Tam J, Engstrom J, Williams R III (2009) Use of thin film freezing to enable drug delivery: a review. J Drug Deliv Sci Technol 19(2):89–98
21. Clausi AL, Morin A, Carpenter JF, Randolph TW (2009) Influence of protein conformation and adjuvant aggregation on the effectiveness of aluminum hydroxide adjuvant in a model alkaline phosphatase vaccine. J Pharm Sci 98 (1):114–121
22. Clausi AL, Merkley SA, Carpenter JF, Randolph TW (2008) Inhibition of aggregation of aluminum hydroxide adjuvant during freezing and drying. J Pharm Sci 97(6):2049–2061
23. Cox JC (1999) Spray dried vaccine preparation comprising aluminium adsorbed immunogens. US 5,902,565, 11 May 1999
24. Li X, Thakkar SG, Ruwona TB, Williams RO 3rd, Cui Z (2015) A method of lyophilizing vaccines containing aluminum salts into a dry powder without causing particle aggregation or decreasing the immunogenicity following reconstitution. J Control Release 204:38–50
25. Thakkar SG, Ruwona TB, Williams RO, Cui Z (2017) The immunogenicity of thin-film freeze-dried, aluminum salt-adjuvanted vaccine when exposed to different temperatures. Hum Vaccin Immunother 13(4):936–946
26. Overhoff KA, Engstrom JD, Chen B, Scherzer BD, Milner TE, Johnston KP, Williams RO III (2007) Novel ultra-rapid freezing particle engineering process for enhancement of dissolution rates of poorly water-soluble drugs. Eur J Pharm Biopharm 65(1):57–67
27. Engstrom JD, Simpson DT, Cloonan C, Lai ES, Williams RO 3rd, Barrie Kitto G, Johnston KP (2007) Stable high surface area lactate dehydrogenase particles produced by spray freezing into liquid nitrogen. Eur J Pharm Biopharm 65(2):163–174
28. Engstrom JD, Lai ES, Ludher BS, Chen B, Milner TE, Williams RO, Kitto GB, Johnston KP (2008) Formation of stable submicron protein particles by thin film freezing. Pharm Res 25(6):1334–1346
29. Williams RO III, Watts AB, Miller DA (2016) Formulating poorly water soluble drugs, vol 22. Springer, New York
30. Siddiqui MAA, Perry CM (2006) Human papillomavirus quadrivalent (types 6, 11, 16, 18) recombinant vaccine (Gardasil®). Drugs 66 (9):1263–1271
31. Keating GM, Noble S (2003) Recombinant hepatitis B vaccine (Engerix-B®). Drugs 63 (10):1021–1051
32. Xu H, Ruwona TB, Thakkar SG, Chen Y, Zeng M, Cui Z (2017) Nasal aluminum (oxy) hydroxide enables adsorbed antigens to induce specific systemic and mucosal immune responses. Hum Vaccin Immunother 13 (11):2688–2694

Chapter 28

Application of Cryogenic Transmission Electron Microscopy for Evaluation of Vaccine Delivery Carriers

Hui Qian, Yimei Jia, and Michael J. McCluskie

Abstract

Cryogenic transmission electron microscopy (Cryo-TEM) enables visualizing the physicochemical structure of nanocarriers in solution. Here, we demonstrate the typical applications of Cryo-TEM in characterizing archaeosome-based vesicles as antigen carriers, including the morphology and size of vaccine carriers. Cryo-TEM tomography, incorporated with immunogold labeling for identifying and localizing the antigens, reveals the antigen distribution within archaeosomes in three dimensions (3D).

Key words Cryo-TEM, Plunge freezing, Electron Tomography, Immunogold labeling, Vaccine delivery carriers

1 Introduction

Cryofixation not only preserves the biomolecular structure near to its native state but also enables the structural study in a transmission electron microscope (TEM) for biological materials in aqueous solution [1]. Along with the advanced EM instrumentation and computing technology development, nowadays the structural resolution of biomolecules is revealed down to sub-Å using cryogenic TEM (Cryo-TEM) single particle analysis (SPA) [2, 3]. Cryo-TEM has also been widely used for characterization of drug delivery nanocarriers, such as inorganic colloid nanoparticles, lipid-based nanoparticles, or virus-like nanoparticles (VLP) (Fig. 1) [4]. Since the physicochemical characteristics, including size, shape, plasticity, and complex nanostructure, play a significant role in vaccine delivery effectiveness [5], each procedure of Cryo-TEM (Fig. 2) is critical to properly characterize nanocarriers.

In general, after the production of nanocarriers, the samples need to be optimized suitable for TEM analysis, such as the nanoparticle concentration. Sometimes preparation may involve further treatment for nanocarriers. For instance, nanocarriers are negatively stained with heavy metals to enhance image contrast; buffer is

Blaine A. Pfeifer and Andrew Hill (eds.), *Vaccine Delivery Technology: Methods and Protocols*, Methods in Molecular Biology, vol. 2183, https://doi.org/10.1007/978-1-0716-0795-4_28,

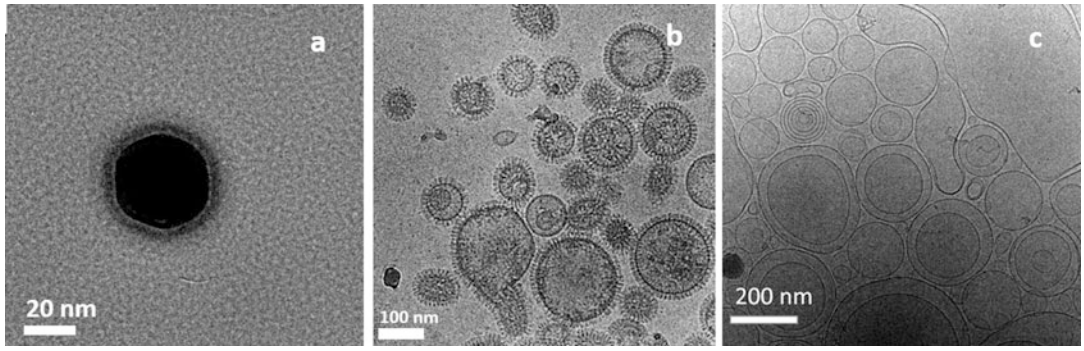

Fig. 1 Cryo-TEM images of: (**a**) silver nanoparticles coated with proteins; (**b**) M2e virus-like particles (VLPs); and (**c**) lipid vesicles embedded in vitreous ice

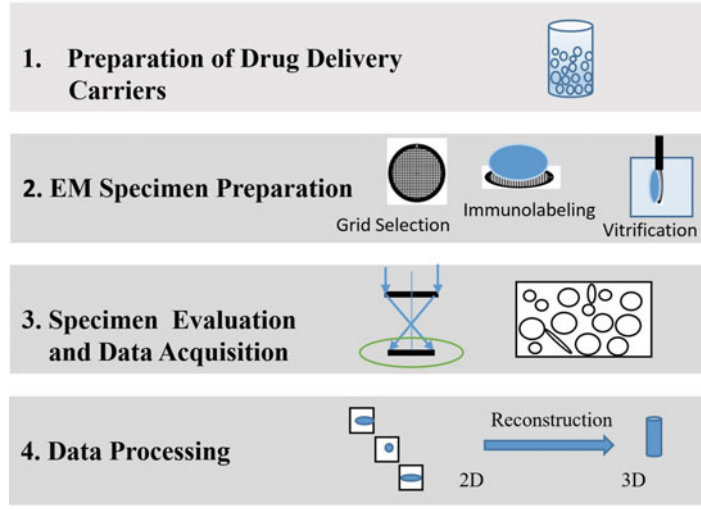

Fig. 2 The schematic general workflow of cryo-TEM for drug delivery carriers in aqueous solution

exchanged to enhance the quality of cryo-EM specimens; or site-specific labeling with immunogold of nanocarriers allows one to identify and locate proteins or macromolecules [6]. The selection of proper perforated film TEM grids, such as the material of the supporting film and the associated hole size (Fig. 3), may affect the quality of subsequent prepared cryo-EM specimens. The purity and temperature of the cryogen and the humidity and speed for freezing samples during cryofixation determines the crystallinity of ice. And the transferring and loading of frozen specimens to the TEM may also introduce ice phase change. Besides the quality of the cryo-EM specimen, the imaging condition settings in Cryo-TEM, such as using a low dose to minimize the electron beam damage, needs to be optimized for high quality images.

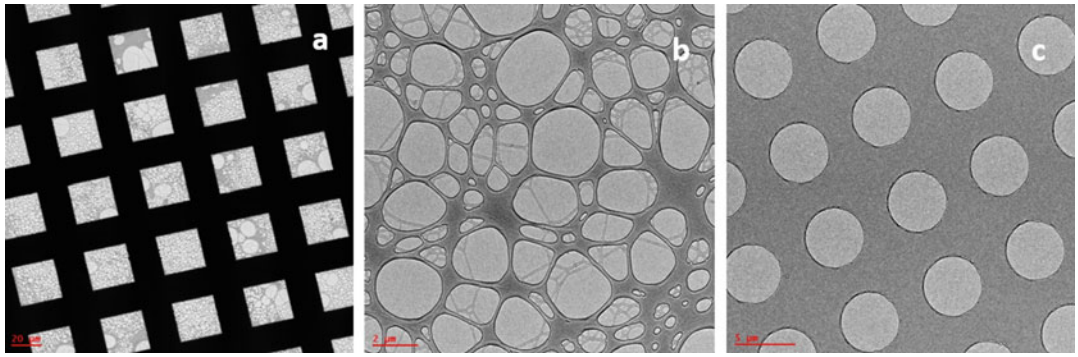

Fig. 3 (**a**) TEM image of carbon film TEM grids in low magnification. The grid bar can be copper, gold, nickel, or other metals; (**b**) the perforated film with holes in variable size; (**c**) the perforated film with hole arrays in uniformed size

Here, using archaeosomes as a model lipid-base nanoparticle and ovalbumin (OVA) as a model protein antigen, we describe the plunge freezing method for archaeosome-based formulations in aqueous solution and combine it with immunogold labeling techniques to identify OVA antigens in the formulation. Cryo-TEM bright field imaging and electron tomography using the single tilt-series method reveals the archaeosome morphology and the antigen distribution in 3D [7]. The techniques described herein can also be applied to other nanocarriers and antigens.

2 Materials

2.1 Vaccine Delivery Carriers

1. Sulfated lactosylarchaeol (SLA; 6′-sulfate-β-D-Galp-(1,4)-β-D-Glcp-(1,1)-archaeol) glycolipids were prepared in house [8].
2. Ovalbumin antigen (OVA): Type VI; Sigma Aldrich.
3. Ultrasonic water bath; Fisher Scientific, Nepean, ON, Canada.

2.2 Immunogold Labeling Reagents

1. Antigen Conjugates: polyclonal mouse anti-OVA IgG antibody is prepared in-house as primary antibody; Nanogold®-IgG, 1.4 nm (Nanogold conjugated secondary antibody goat anti-mouse IgG) is purchased from Nanoprobes Inc., Yaphank, NY, USA.
2. PBS-BSA buffer: 20 mM phosphate, 150 mM NaCl, pH 7.4, 0.5% BSA, 0.1% gelatin.
3. PBS buffer: 20 mM phosphate, 150 mM NaCl, pH 7.4.

2.3 TEM Specimen Preparation Materials

1. TEM grids: ultrathin carbon film (3–4 nm thick) supported TEM grids (400 mesh), lacey carbon film with an ultrathin formvar film (3–4 nm) Nickle (400 mesh), lacey carbon copper

TEM grids (400 mesh); Electron Microscopy Science (EMS), USA.

2. PELCO easiGlow™ Glow Discharge Cleaning System; Ted Pella, Inc., USA.
3. Cryogens: liquid nitrogen (N_2), ethane gas.
4. EMS-002 Rapid Immersion Freezer; EMS, USA.
5. TEM grids cryo-storage box, 50 L liquid N_2 Dewar, and 2 L liquid N_2 Dewar.

2.4 Instrumentation of Imaging Cryo-EM Specimen

1. Gatan cryo-transfer holder (model 910 with three specimen cartridge) with cryo-workstation, Gatan cryo-transfer holder (model 914) with cryo-workstation.
2. Gatan SmartSet (Model 900) Cold Stage Controller, Gatan temperature controller (Model 1905).
3. Specimen clamping rings and loading/unloading tools.
4. JEOL-2200FS TEM/STEM at 200 kV, with cryo-pole piece, Schottky electron source, in-column Ω energy filter.
5. Gatan Ultrascan 2k × 2k CCD camera.

2.5 Software

1. 2D electron micrograph acquisition: Gatan Microscopy Suite (GMS) V1.6.
2. 3D data reconstruction: TEMography™ package including 2D data collection (Recorder), 3D reconstruction (Composer) and visualization (Visualizer Kai); System in Frontier Inc., Japan.

3 Methods

3.1 Archaeosome-Based Vaccine Formulations

1. Sulfated lactosylarchaeol (SLA; 6′-sulfate-β-D-Galp-(1,4)-β-D-Glcp-(1,1)-archaeol) glycolipids are to be dissolved in chloroform–methanol and dried under N_2 gas with mild heating to form a thin film of lipid layer. The vacuum should be applied for at least 2 h to ensure total removal of trace solvents.
2. Two different archaeosome formulations (antigen entrapped and empty archaeosome) should be prepared according to the thin-film method described as Jia et al. [7].
   (a) For preparation of the entrapped Ag formulation, lipid film is hydrated with 1.0 ml of Milli-Q water containing ovalbumin at concentration of 10 mg/ml, and the dispersions are shaken for 2–3 h at 40–50 °C until completely suspended. Next a brief sonication is applied at 40 °C in an Ultrasonic water bath until a desired particle size (~100 nm) is obtained. Unentrapped antigen is removed by ultracentrifugation (223,000 × *g* for 1.5 h at 4 °C).

Pellet is washed twice with pyrogen-free water and resuspended with PBS buffer to desired volume.

(b) Empty archaeosomes are formed using the same processes above except that lipid film is hydrated in Milli-Q water without protein antigen.

3.2 Cryo-TEM Specimen Preparation

1. Select proper TEM grids to meet different imaging and analysis requirements (*see* **Note 1**). Continuous ultrathin carbon film supported TEM grids are used for negative staining or immunogold labeling antigens at room temperature in dried state. Lacey carbon film TEM grids are used for cryo-EM specimens. The lacey carbon film with an ultrathin formvar film Nickle grids are used for cryo-EM specimens with immunogold labeling.
2. Glow discharge TEM grids (*see* **Note 2**). Place the carbon side of TEM grids facing up in the discharge chamber. Set the pressure in glow chamber to 40 mbar at air atmosphere, discharging current of 15–20 μA and discharging time of 10–20 s.
3. Immunogold labeling archaeosomes based formulations (*see* **Note 3**).

   I. Labeling ovalbumin as positive control to check the effectiveness of immunogold labeling (Fig. 4).

      (a) Dilute aliquot OVA with 1× PBS to the concentration of 1 mg/ml. Dilute Au-IgG Ab 40× (1:40) in PBS buffer.

      (b) Place 4 μl of OVA aqueous solution on continuous carbon TEM grids and blot excess liquid after 1 min.

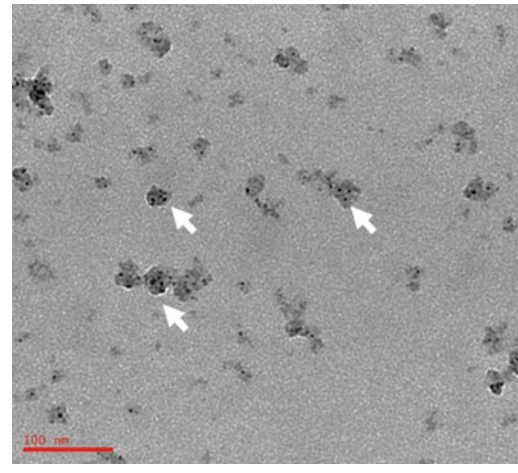

Fig. 4 Bright field TEM image of OVA antigens immunolabeled with primary Ab and 1.4 nm nanogold conjugated secondary Ab. Aggregates marked as white arrows

(c) Place one 10 μl droplet of primary antibody, diluted in PBS-BSA buffer to the concentration of 2 mg/ml, on paraffin films; then float TEM grids with OVA antigens side on the top of droplet; followed by incubation in a wet chamber at RT for 10–15 min.

(d) Wash the grids using 10 μl droplet of PBS buffer three times.

(e) Incubate the grids in a droplet of 10 μl Au-IgG Ab diluted in PBS buffer for 5–10 min at RT.

(f) Wash the grids with PBS buffer and then with Milli-Q water.

(g) Blot excess liquid and dry TEM grids at RT before imaging.

II. Labeling SLA archaeosome-based formulations: archaeosomes entrapped with and without OVA. The labeling of archaeosomes can be done in suspension although this is suboptimal (*see* **Note 4**) and thus labeling on TEM grids is preferred. For negative control, the **steps (b)–(d)** below are not necessary since the sample is labeled with secondary Au-IgG Ab only.

(a) Place a 6 μl droplet of archaeosome aqueous sample on a lacey carbon film with ultrathin formvar film TEM grid.

(b) Place a 2 μl droplet of PBS-BSA buffer on TEM grids and incubate in a wet chamber for 10 mins at 37 °C.

(c) Blot excess liquid; then float TEM grids on a 20 μl droplet of primary Ab and incubate in a wet chamber for about 1 h at 37 °C.

(d) Wash TEM grids using 10 μl droplet of PBS buffer three times.

(e) Place a 10 μl droplet of diluted Au-IgG Ab in PBS on grid for 30 min at RT or 1 h at 4 °C chamber (*see* **Note 5**).

(f) Wash TEM grids using 10 μl droplet of PBS buffer three times.

(g) Place 5 μl droplet of PBS on TEM grids which is ready for the subsequent plunge freezing.

4. Plunge-freeze the archaeosomes on TEM grids (*see* **Note 6**).

(a) Precool the cryogen cup in liquid N_2 for about 30 min.

(b) Liquefy the compressed ethane gas into a Falcon tube surrounded by liquid N_2.

(c) Pour liquid ethane into cryogen cup and wait until the bottom of the cup freezes.

(d) Apply 5 μl droplet of archaeosomes solution on the carbon side of the grid.

(e) Mount the tweezer securing the TEM grids loaded with aqueous solutions or the grids with the immunolabeled archaeosomes prepared in **step 3** onto the releasing anvil.

(f) Blot the TEM grid with filter paper from the back side of carbon film for about 4 s; press the anvil releasing button and plunge the grids into liquid ethane rapidly.

(g) Transfer the frozen grid from liquid ethane cup to the cryo-EM grid storage box immersed in liquid nitrogen.

(h) Transfer and store the TEM grid storage box in a large nitrogen cryo-storage Dewar (50 L). It can be stored for several days or weeks until transferred for imaging.

3.3 Cryo-TEM Imaging

For imaging frozen-hydrated specimens in a TEM, additional precautions and proper procedures should be followed to obtain high quality cryo-TEM micrographs. Ice contaminations or ice phase change during specimen transferring/loading and electron beam damage to frozen specimen are unavoidable but can be mitigated.

1. Loading cryo-EM specimen to TEM column (*see* **Note 7**).
   (a) Transfer the storage box containing frozen TEM grids from cryo-storage Dewar (50 L) to a 2 L liquid N_2 Dewar quickly.
   (b) Precool down the cryo-transfer holder in TEM column to −180 °C.
   (c) Cool down the cryo-workstation with liquid N_2 for about 30 min. Immerse tweezers and tools for fixing specimen in liquid N_2.
   (d) Transfer the precooled holder into the cryo-workstation and monitor the temperature below −160 °C.
   (e) Transfer storage box containing frozen grids from the liquid N_2 Dewar to the cryo-workstation.
   (f) Load the frozen specimen onto holder in liquid N_2 or just above liquid N_2 (*see* **Note 8**).
   (g) Close the anti-frosting cover on the cryo-holder and transfer it to the TEM column. Top up liquid N_2 to the Dewar on holder as needed (*see* **Note 9**).
   (h) Wait for about 20–30 min to let the temperature and stage stabilized before opening the beam.
2. Image acquisition using low dose imaging (*see* **Note 10**).
   (a) At low magnification, evaluate the quality of the cryo-EM specimen (*see* **Note 11**).

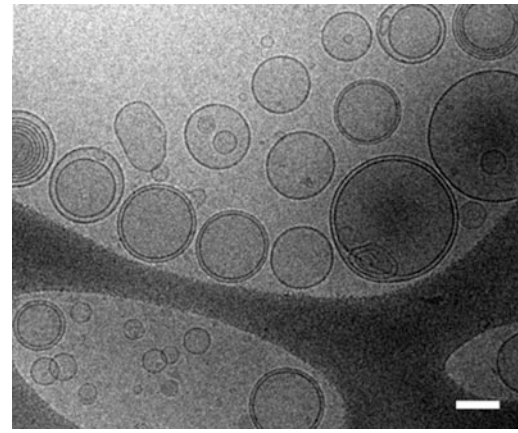

Fig. 5 Zero-loss energy filtered cryo-TEM image of archaeosomes embedded in vitreous ice. Scale bar is 100 nm

(b) Choose the proper magnification for imaging the archaeosomes (*see* **Note 12**).

(c) Adjust the electron emitter parameters and measure the beam current if necessary.

(d) Adjust and optimize the image contrast by inserting objective aperture and a 10 eV energy filter slit.

(e) Adjust the illumination area on the specimen. Optimize parameters in the setting for Gatan slow-scan 2k × 2k camera including the binning of 2 × 2 or 4 × 4 in search mode and 1 × 1 binning in acquisition mode. Set acquisition time to obtain the archaeosomes image with beam dose of 2 $e^{-}/Å^{2}$.

(f) Focus the specimen and blank the beam.

(g) Move the stage to the new area nearby (not previously exposed in the beam) and wait for several seconds to 1 min.

(h) Open the beam and acquire the image (Fig. 5).

3.4 Cryo-TEM Tomography

1. Tilt-series of 2D Electron Micrograph Acquisition.

(a) At low magnification, locate the interested specimen region. Check the tilting angle limitation (*see* **Note 13**).

(b) Switch to high magnification. Adjust the specimen to eucentric height using tilting method.

(c) Perform microscope alignment. Make sure the beam shift /tilt balance is well-adjusted and coma free.

(d) Select the recording magnification and adjust the image contrast using 10 eV energy filtered slit and object aperture. The defocus of −0.5 μm or −1 μm is generally used.

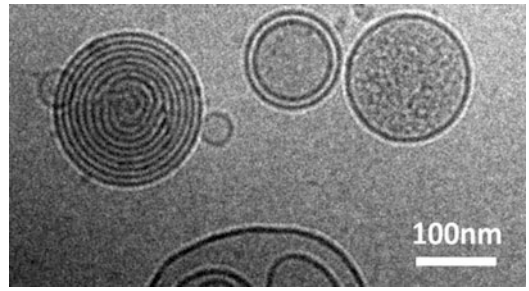

Fig. 6 Cryo-TEM bright field image of archaeosomes entrapped with OVA immunolabeled with 1.4 nm gold conjugated Ab. The archaeosomes in the field of view were used for tilt-series micrograph

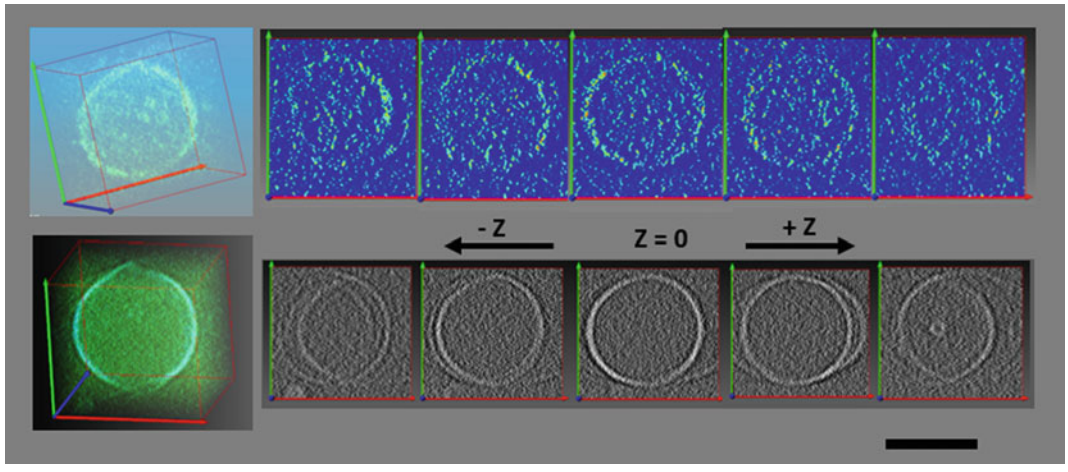

Fig. 7 The reconstructed 3D volume view (left) and the associated section views along the *z* axis of archaeosomes embedded in vitreous ice. The tilt-series images were obtained at ±64° with 4° increments. The top row is archaeosomes entrapped with OVA antigens, which are inside and within the wall of vesicles. The bottom row is empty archaeosomes. The double arcs in slice view is due to the missing wedge effect. Scale bar is 100 nm

(e) Set up data collection parameters and conditions in data collection software (Recorder). The low dose imaging mode should be applied, in which the position for search and focus is set in nonacquisition region.

(f) Perform the calibrations for beam brightness, compensators (beam shift/tilt balance and image shift), and stage condition etc. at recording magnification at 0°.

(g) Find a typical effective region where the antigen capsulated archaeosomes is in the field of view and also the tilting angle is in the range of ±70° (Fig. 6).

(h) Tilt specimen holder in *X*-axis to the maximum minus angle and start the image acquisition automatically till the tilt angle reaching the maximum plus.

(i) Stop the acquisition and save a series of images.

2. 3D Reconstruction and Visualization (*see* **Note 14**).
   (a) Open a tilt series of 2D images recorded above in Composer.
   (b) Check the effective data to be used for further alignment.
   (c) Select the interested region for prealignment.
   (d) Pick fiducial markers within the selected region and perform fine alignment (*see* **Note 15**).
   (e) Check the fine alignment results and perform the manual alignment if necessary.
   (f) Set reconstruction parameters and perform reconstruction. The filtered back projection (FBP) method was used in our case.
   (g) Export reconstructed result to Visualizer-kai. Perform rendering to display the volume and sections of reconstructed 3D data (Fig. 7).

4 Notes

1. The lacey carbon film copper TEM grid is the most popular and the cheapest grid to start with. The holes are variable which is good for accommodating nonhomogeneous nanoparticles, but the rigidity and stability of samples embedded in ice may not be strong enough for some analysis due to the weak carbon support between holes. Extracontinuous carbon or formvar thin film may therefore be needed for the enforcement of lacey TEM grids.
2. The freshly prepared carbon films are hydrophilic, but they become more hydrophobic over time. It is therefore necessary to restore the surface hydrophilicity so that the aqueous solutions spread over the grid film easily and evenly. This can be realized using plasma or glow discharging [9]. There are different types of glow dischargers on the market. But basically the discharge current, pressure level, atmosphere gas and discharging time need to be tested to obtain the desired surface property.
3. The successful application of immunogold labeling depends on the capacity of antibodies to infiltrate throughout the lipid membrane, and the specificity of recognition between antigen-primary antibodies. The low antigen encapsulation in archaeosomes also makes the labeling more challenging.
4. Labeling in a suspension method can be used. In this case, primary Ab was added to an aliquot of archaeosomes in a vial and incubated at 37 °C for about 1 h. However, the subsequent

removal of free unbound Ab is not very effective and the presence of extra unbound primary Ab in buffer may lead to binding of secondary Au-IgG Ab and thus limit the amount of secondary Ab available to bind to antigen entrapped in archaeosomes.

5. Au-IgG Ab easily form aggregates at 37 °C, so the incubation should be carried out at RT or at 4 °C. To avoid the collapse of archaeosomes, the aqueous sample on TEM grids should be kept in a humidity chamber during incubation.
6. In recent years, the design of plunge freezers has become more sophisticated and considerably improved to make the freezing process more efficient and reproducible. Nevertheless, whether using an automated or manual control, the basic design of the instrument is still the same and the production of high quality cryo-EM specimens still depends on the skill of the individual since the factors affecting cryo-EM, such as grid selection, temperature, humidity, blotting pressure, and blotting duration, need to be optimized for each specimen.
7. The atmosphere moisture can condense on the surface of cryo-TEM specimens as crystalline ice when transferring the frozen samples from storage Dewar to workstation and loading them to TEM holder or to the microscope. During transferring and loading, the temperature of the frozen specimen can increase and the ice phase can be changed from vitreous to cubic crystalline phase if the temperature is higher than −135 °C [1]. Shortening the exposure time of the frozen sample at atmosphere, immersing the frozen sample in liquid nitrogen and applying antifrosting shield on the holder can mitigate the ice contamination and recrystallization.
8. The three-specimen cartridge on Gatan cryo-transfer holder 910 is removable. So the cryo-EM specimen can be loaded onto the cartridge first before transferring the 910 holder from TEM to cryo-workstation and thus the time exposed to air can be shortened. This holder allows us to screen specimens prepared at different freezing conditions to obtain optimal conditions. Gatan cryo-transfer holder 914, with a narrow holder tip allowing for high angle tilting up to ±90°, is used for tomography experiments. The cryo-EM specimen loading direction is side entry so more caution should be paid to specimen damage or crystalline ice contamination from cryo-workstation.
9. To avoid the spill of liquid N_2 from the Dewar while inserting the holder to TEM column, the stage can be tilted to plus X direction at about 25°. Thus, it can prevent the liquid N_2 from splashing around and also the risk of the temperature rising. However, the proper procedures and precautions should be

followed as in the instrument operation procedure (SOP) while tilting the stage, such as holder type selection and tilting mechanical limit.

10. Electron-beam radiation damage is an inevitable activity in a TEM, especially to soft materials. It can introduce structural change (loss of crystallinity or radiolysis of matter, etc.) and mass loss [10]. Bubbling and charging induced by beam irradiation are the phenomena most frequently observed for biological materials embedded in water or vitrified ice [11]. Low dose imaging, minimizing the beam dose (beam current density times exposure time), is an approach that can be used to mitigate the radiation damage in a broad beam TEM imaging for frozen specimens.
11. The quality of cryo-TEM specimen, such as the ice crystallinity, thickness of vitreous ice, sample viscosity and concentration, uniformity spanning the holes and grids, and the immunogold labeling effectiveness, affects TEM imaging and analysis. The surface property of TEM grids, the hole size, the material of TEM grids, the cryofixation approach and conditions (plunge speed, temperature, blotting parameters and cryogen, etc.) and the procedure of cryo-EM specimen transferring are the main influencing factors.
12. This step is to optimize the imaging conditions which comprise spatial resolution, particles in the field of view and radiation damage to obtain high quality TEM micrographs for frozen specimens.
13. The Gatan cryo-transfer holder 914 allows the maximum tilt to $\pm 90^\circ$ at *X*-axis direction in our TEM column with a large gap polepiece. However, the shadow of holder tip and TEM grid bars limit the effective tilting angles to $\pm 75^\circ$. The position of the area of interest may also limit the effective tilting angle but nevertheless the maximum tilting should be tried to minimize the missing wedge.
14. Other reconstruction software, such as IMOD, an open source software, can also open the tilt-series of 2D micrograph, reconstruct and display the reconstructed 3D results.
15. Fiducial markers, such as colloid gold nanoparticles, are often applied while preparing cryo-EM specimen for tracking and alignment in tomography [12]. In our case, no extra fiducial markers were introduced since the free antibody conjugated with 1.4 nm nanogold was not removed during cryo-EM specimen preparation.

Acknowledgments

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References

1. Dubochet J et al (1988) Cryo-electron microscopy of vitrified specimens. Q Rev Biophys 21 (2):129–228
2. Bartesaghi A et al (2018) Atomic resolution cryo-EM structure of β-galactosidase. Structure 26:848
3. Merk A et al (2016) Breaking cryo-EM resolution barriers to facilitate drug discovery. Cell 165:1698–1707
4. Kuntsche J et al (2011) Cryogenic transmission electron microscopy (cryo-TEM) for studying the morphology of colloidal drug delivery systems. Int J Pharm 417:120–137
5. Venkataraman S et al (2011) The effects of polymeric nanostructure shape on drug delivery. Adv Drug Deliv Rev 63:1228–1246
6. Paul AL et al (2012) Immunoelectron microscopy: a reliable tool for the analysis of cellular processes. In: Applications of immunocytochemistry. InTech, Rijeka, Croatia, pp 65–96
7. Jia Y et al (2019) A comparison of the immune responses induced by antigens in three different archaeosome-based vaccine formulations. Int J Pharm 561:187–196
8. Whitfield DM et al (2016) Sulfated-glycolipids as adjuvants for vaccines. National Research Council of Canada. Patent No. WO/2016/004512
9. Aebi U et al (1987) A glow discharge unit to render electron microscope grids and other surfaces hydrophilic. J Electron Microsc Tech 7:29–33
10. Egerton RF (2013) Control of radiation damage in the TEM. Ultramicroscopy 127:100–108
11. Glaeser RM (1971) Limitations to significant information in biological electron microscopy as a result of radiation damage. J Ultrastruct Res 36:466–482
12. Lawrence MC (1992) Least-squares methods of alignment using markers. In: Frank J (ed) Electron tomography. Plenum, New York, pp 197–204

Chapter 29

Methods to Evaluate Immune Cell Recruitment and Cellular Uptake and Distribution of Antigen Following Intramuscular Administration of Vaccine to Mice

Gerard Agbayani, Felicity C. Stark, Bassel Akache, and Michael J. McCluskie

Abstract

An effective vaccine depends on the stimulation of the immune system to generate effective antigen-specific immune responses capable of neutralizing mediators of disease long after vaccination. However, the ability of the vaccine to enhance immune parameters such as cell activation, cell recruitment and antigen uptake shortly following administration contributes to the development of long-term responses directed toward the antigen. Here, we describe a flow cytometry-based method to identify changes in immune cell profile and assess cellular uptake and distribution of antigen following vaccination.

Key words Vaccines, Cell recruitment, Antigen uptake, Flow cytometry

1 Introduction

Intramuscular immunization is routinely used in the clinic due to its relative ease and consistency during vaccine administration. Emulating the clinical vaccination route when evaluating vaccine candidates in preclinical animal models is important, as the route can influence (1) the magnitude/quality of the immune response against a disease, (2) the site of immune activation (i.e., mucosal vs. systemic), and (3) the risk for local or systemic adverse effects. In addition to characterizing antigen-specific immune responses (humoral and/or cellular), it is possible to evaluate immune cell recruitment and cellular uptake of antigen at the vaccination site and in distant tissues, particularly draining lymph nodes (LNs), following intramuscular immunization.

Herein, we describe a mouse vaccination model using ovalbumin (OVA) as a model antigen and a novel class of archaeosome adjuvant. However, the methods described are appropriate for other antigens and adjuvants. OVA is a chicken egg-derived

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glycoprotein that is widely used for examining antigen-specific cellular and humoral responses in mice. It is an inherently weak immunogen when administered alone, but can induce strong antigen-specific responses when paired with the proper adjuvant. Archaeosomes are liposomes that are traditionally comprised of total polar lipids or semisynthetic glycerolipids derived from Archaebacteria. They have been shown to exhibit strong adjuvant activity in multiple preclinical models [1]. We recently developed an SLA archaeosome formulation composed of a single sulfated glycolipid named sulfated lactosylarchaeol (SLA; 6′-sulfate-β-D-Galp-(1,4)-β-D-Glcp-(1,1)-archaeol) that when admixed or used to encapsulate antigen results in the induction of strong, long-lasting cellular and humoral immune responses in mice with a favorable safety profile [2, 3].

In our vaccination model, we inject OVA alone or in combination with SLA to the tibialis anterior (TA) muscles (Fig. 1). At days 1 and 3 postvaccination, TA muscles and draining LNs (inguinal and popliteal) are harvested and processed for flow cytometric analysis of immune cell types. Antigen uptake is determined by calculating the number of cells positive for OVA conjugated with Alexa Fluor 647 fluorochrome (OVA-AF647) per immune cell type. The impact of immunization on immune cell recruitment can be assessed by quantifying cell number regardless of positivity for antigen. Using this method, we have demonstrated the impact of SLA-based adjuvants on the magnitude and kinetics of immune cell recruitment to the injection site and draining LNs and how this modulates cellular uptake and distribution of antigen [2–5].

2 Materials

2.1 Vaccine Formulation

1. Adjuvant: sulfated lactosylarchaeol (SLA; 6′-sulfate-β-D-Galp-(1,4)-β-D-Glcp-(1,1)-archaeol) (National Research Council Canada) (*see* **Note 1**).
2. Antigen: Ovalbumin (Hyglos GmbH, Bernried am Starnberger See, Germany) labeled with Alexa Fluor® 647-NHS Ester dye (Thermo Fisher Scientific, Pittsburgh, PA, USA) (OVA-AF647) (*see* **Note 2**).
3. Negative control: 1× phosphate-buffered saline (PBS) (pH 7.4) (*see* **Note 3**).

2.2 Intramuscular Injection

1. Insulin syringes, 0.5 cc.
2. Polyethylene (PE)-20 tubing.

2.3 Muscle Collection and Processing

1. R10 complete medium: RPMI containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% glutamine, and 55 μM 2-mercaptoethanol.

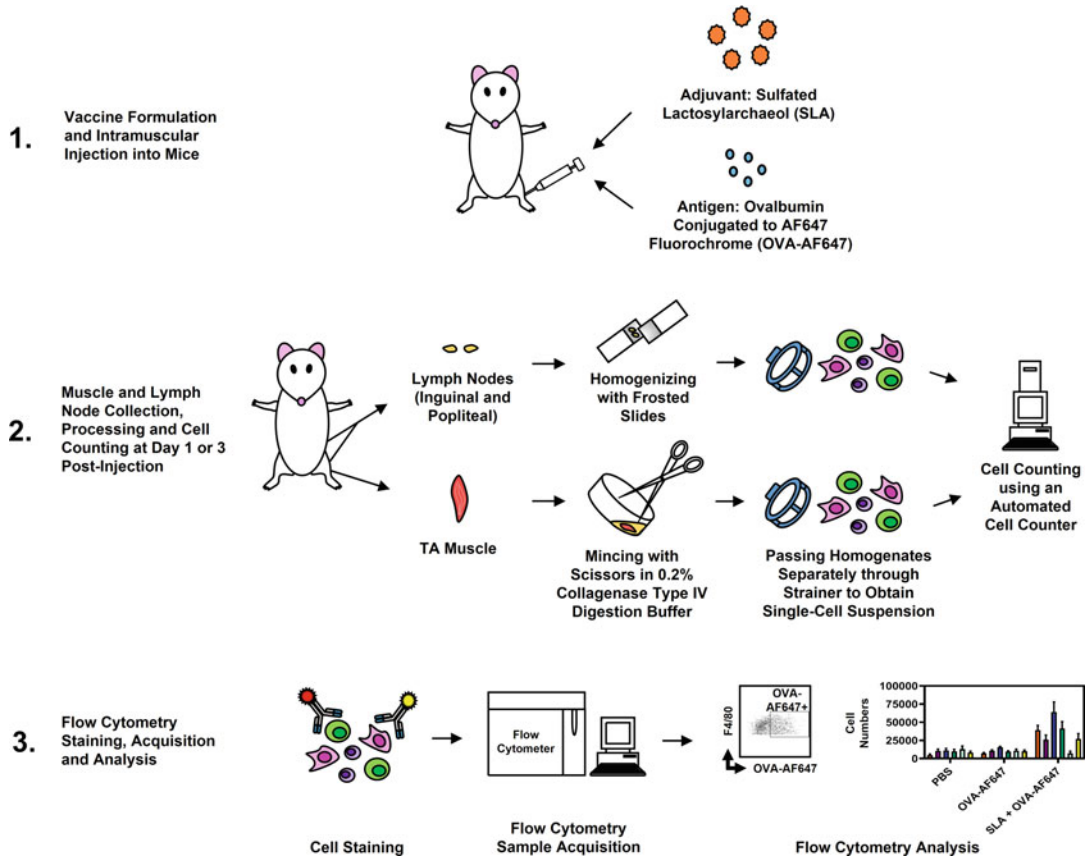

Fig. 1 Schematic overview of experimental procedures. Vaccine formulations are prepared according to optimal adjuvant and antigen concentrations and injected intramuscularly to mice. At days 1 and 3 postinjection, TA muscles and lymph nodes are collected for processing into single-cell suspensions and cell counting. Cells are first stained with LIVE/DEAD™ Fixable Dead Cell Stain, followed by cell surface antibodies to determine cell viability and immune cell phenotypes, respectively, during flow cytometry acquisition. Flow cytometry data are analyzed using FlowJo 10 (BD) software. The total numbers of each immune cell type and OVA-AF647^{+} cells per immune cell type are calculated based on their percentages relative to the total sample cell number. Lastly, analyzed data are graphed using GraphPad® Prism 8 software

2. 0.2% Collagenase Type IV digestion buffer: Dissolve 0.2 g in 100 mL 1× Hank's Balanced Salt Solution (HBSS) containing 2% FBS. Filter-sterilize using 0.2 μm membrane filter. Store at −20 °C. Keep on ice or at 4 °C upon thawing before use.
3. Sterilized surgical scissors.
4. Sterilized surgical forceps.
5. Sterilized petri dishes.
6. Microcentrifuge tubes, 1.5 mL.
7. Falcon™ conical centrifuge tubes, 15 mL.
8. Falcon™ conical centrifuge tubes, 50 mL.

2.4 Lymph Node Collection and Processing

1. R10 complete medium.
2. Sterilized frosted microscope slides.
3. Sterilized petri dishes.
4. Microcentrifuge tubes, 1.5 mL.

2.5 Cell Counting

1. Cellometer Auto 2000 cell viability counter (Nexcelom Bioscience).
2. Cellometer disposable counting chambers (Nexcelom Bioscience).
3. ViaStain™ Acridine Orange/Propidium Iodide (AO/PI) viability stain (Nexcelom Bioscience).

2.6 Flow Cytometry

1. Viability Dye: LIVE/DEAD™ Fixable Dead Cell Stain kit (Invitrogen).
2. Fluorochrome-conjugated antibodies: anti-mouse CD45 (30-F11), CD11b (M1/70), Ly-6G (1A8), Ly-6C (HK1.4), F4/80 (BM8), CD11c (HL3), CD45R/B220 (RA3-6B2), CD8a (53-6.7) (*see* **Note 4**).
3. UltraComp eBeads™ compensation beads (Invitrogen).
4. Staining buffer: 2% FBS and 3 mM EDTA in 1× PBS.
5. Fc block: 20 μg anti-mouse CD16/32 in 1 mL staining buffer.
6. Cytofix™ fixation buffer (BD Biosciences).
7. Round-bottom polystyrene tubes, 5 mL.
8. 96-Well DeepWell™ polypropylene microplates, 2 mL.
9. Flow Cytometer: LSRFortessa™ (BD Biosciences).

3 Methods

1. Prepare vaccine formulations (Table 1; *see* **Note 5**):

Table 1
Representative vaccine formulations

Group	Treatment	Concentration	Final volume
Vaccine (antigen + adjuvant)	SLA OVA-AF647 PBS	1 mg 20 μg Bring to final volume	50 μL
Antigen alone	OVA-AF647 PBS	20 μg Bring to final volume	50 μL
Naïve	PBS	–	50 μL

3.1 Vaccine Preparation

3.2 Anesthesia and Intramuscular Injection

1. Turn on the oxygen flowmeter of the isoflurane anesthesia machine and set the rate at 2 L/min (*see* **Note 6**).
2. Turn on the isoflurane vaporizer and set to 3% [6].
3. After 5 min, place the mouse in the anesthetizing chamber.
4. Once the mouse is completely anesthetized, disinfect the injection site above the TA muscle by spraying with 70% ethanol.
5. Draw 50 μL of antigen, vaccine or negative control using a 0.5 cc insulin syringe.
6. Insert the syringe needle into the polyethylene (PE)-20 tubing, and cut the tubing so that around 2 mm of the needle bevel is exposed when the other end of the tubing is flush against the plastic hub (this will help keep the injection depth consistent).
7. Slowly inject total volume into the TA muscle (*see* **Note 7**).
8. Transfer the mouse back into cage after injection to allow full recovery from anesthesia.
9. Turn off the isoflurane vaporizer and keep the oxygen on for 5 min to remove residual isoflurane in the anesthetizing chamber.
10. Turn off the oxygen flowmeter of the isoflurane machine.

3.3 Muscle Collection and Processing

1. Anesthetize the mouse using the isoflurane anesthesia machine as described previously.
2. Perform cervical dislocation once the mouse is completely anesthetized (*see* **Note 8**).
3. Carefully isolate the TA muscle using surgical scissors and forceps at day 1 or 3 postinjection. Keep TA muscle in 1 mL R10 complete medium on ice or at 4 °C until ready for tissue processing.
4. Thaw a frozen aliquot of 0.2% Collagenase Type IV digestion buffer on ice or prepare fresh (*see* **Note 9**). Transfer 2 mL of the digestion buffer onto a petri dish. Alternatively, keep the digestion buffer on ice until ready for tissue processing.
5. Tilt the petri dish until the 0.2% Collagenase Type IV digestion buffer settles onto the bottom end. Place the TA muscle in the digestion buffer and mince to approximately 1–2 mm in length using surgical scissors. Ensure that the TA muscle remains in the digestion buffer while mincing to prevent drying of the tissue.
6. Transfer the 0.2% Collagenase Type IV digestion buffer containing the minced TA muscle into a 15-mL Falcon™ conical

centrifuge tube, and place in a shaking incubator for 1 h at 37 °C and 100 rpm.

7. Inactivate Collagenase Type IV digestion buffer activity by adding 10 mL of ice-cold R10 complete medium.
8. Centrifuge cells at 500 × *g* for 5 min at 4 °C. Remove supernatant and resuspend cells in 1 mL R10 complete medium.
9. Pass cells through a 100-μm nylon cell strainer placed onto a 50-mL Falcon™ conical centrifuge tube prior to cell counting.

3.4 Lymph Node Collection and Processing

1. Isolate the inguinal and popliteal LNs using surgical scissors and forceps. Keep LNs in 1.5-mL microcentrifuge tubes containing 1 mL R10 complete medium on ice or at 4 °C until ready for tissue processing.
2. Place the LNs in 1 mL R10 complete medium onto a sterile petri dish and homogenize using the rough edge of sterilized frosted microscope slides.
3. Pass cells through a 100-μm nylon cell strainer placed onto a 50-mL Falcon™ conical centrifuge tube prior to cell counting.

3.5 Cell Counting

1. Dilute 15 μL of sample with 15 μL AO/PI viability stain (1:1 ratio).
2. Load 20 μL of sample stained with AO/PI viability stain onto a counting chamber and analyze on the Cellometer Auto 2000 Cell Viability Counter (*see* **Note 10**).

3.6 Flow Cytometry Staining and Acquisition

1. Prepare single-stain controls using the UltraComp eBeads™ compensation beads (*see* **Note 11**).
2. Reconstitute a fresh vial of LIVE/DEAD™ Fixable Dead Cell Stain by adding 50 μL of DMSO included in the kit.
3. Prepare a 1:100 working concentration of LIVE/DEAD™ Fixable Dead Cell Stain using 1× PBS. A total of 50 μL working concentration is required per sample (*see* **Note 12**).
4. Transfer up to 1×10^6 cells to a 5-mL polystyrene tube or to each well of a 96-well DeepWell™ polypropylene microplate (*see* **Note 13**).
5. Centrifuge cells at 500 × *g* for 5 min at 4 °C and remove supernatant (*see* **Note 14**).
6. Add 50 μL of the working concentration (1:100) of LIVE/DEAD™ Fixable Dead Cell Stain to each well or tube, and incubate for 20 min at 4 °C in the dark.
7. Wash cells by adding 1 mL 1× PBS and centrifuging at 500 × *g* for 5 min at 4 °C.
8. Remove supernatant and resuspend cells in 50 μL Fc block. Incubate for 10 min at 4 °C in the dark.

9. Add fluorochrome-conjugated antibodies at the optimal concentrations to each sample and incubate for 30 min at 4 °C in the dark (*see* **Note 15**).
10. Wash cells by adding 1 mL staining buffer and centrifuging at 500 × *g* for 5 min at 4 °C.
11. Remove supernatant and resuspend in 500 μL staining buffer prior to sample acquisition on the flow cytometer (*see* **Note 16**). Alternatively, cells can be fixed using Cytofix™ fixation buffer after removing supernatant if acquisition cannot be performed within 24 h (*see* **Note 17**).
12. Identify immune cell types on the flow cytometer according to cell surface marker expression (Fig. 2a–d) (*see* **Note 18**).

4 Notes

1. SLA is routinely used as a model adjuvant in our immunization studies. For testing other adjuvants, dose response studies must be performed to determine the optimal magnitude/quality of local or systemic immune responses.
2. OVA is routinely used as a model antigen in our immunization studies. The Alexa Fluor 647-NHS Ester dye is conjugated to OVA according to manufacturer guidelines [3]. The dye powder is first solubilized in DMSO to a final concentration of 10 mg/mL. A fivefold molar excess of the dye in 10% (v/v) carbonate buffer (pH 9.3) is then added to OVA in PBS (pH 7.4) while mixing. The reaction is incubated for 2 h at room temperature with slow mixing prior to removal of unreacted dye with an Amicon® Ultra-4 Centrifugal Filter Unit with a 10k cutoff membrane. Lastly, the dye-labeled OVA is resuspended in PBS (pH 7.4). This labeling procedure yields an optimal dye/protein ratio of approximately 1–2. For testing other antigens, dose response studies must be performed to determine the optimal magnitude/quality of local or systemic immune responses.
3. The appropriate negative control for vaccine formulations is the diluent used for the antigen. PBS is routinely used as the diluent for OVA in our immunization studies and therefore would be included as negative control. Similarly, adjuvant without antigen can be included as an additional negative control.
4. The specific fluorochromes and viability dye color selected for the staining panel should be customized to be compatible with the configuration of the flow cytometer to be used.

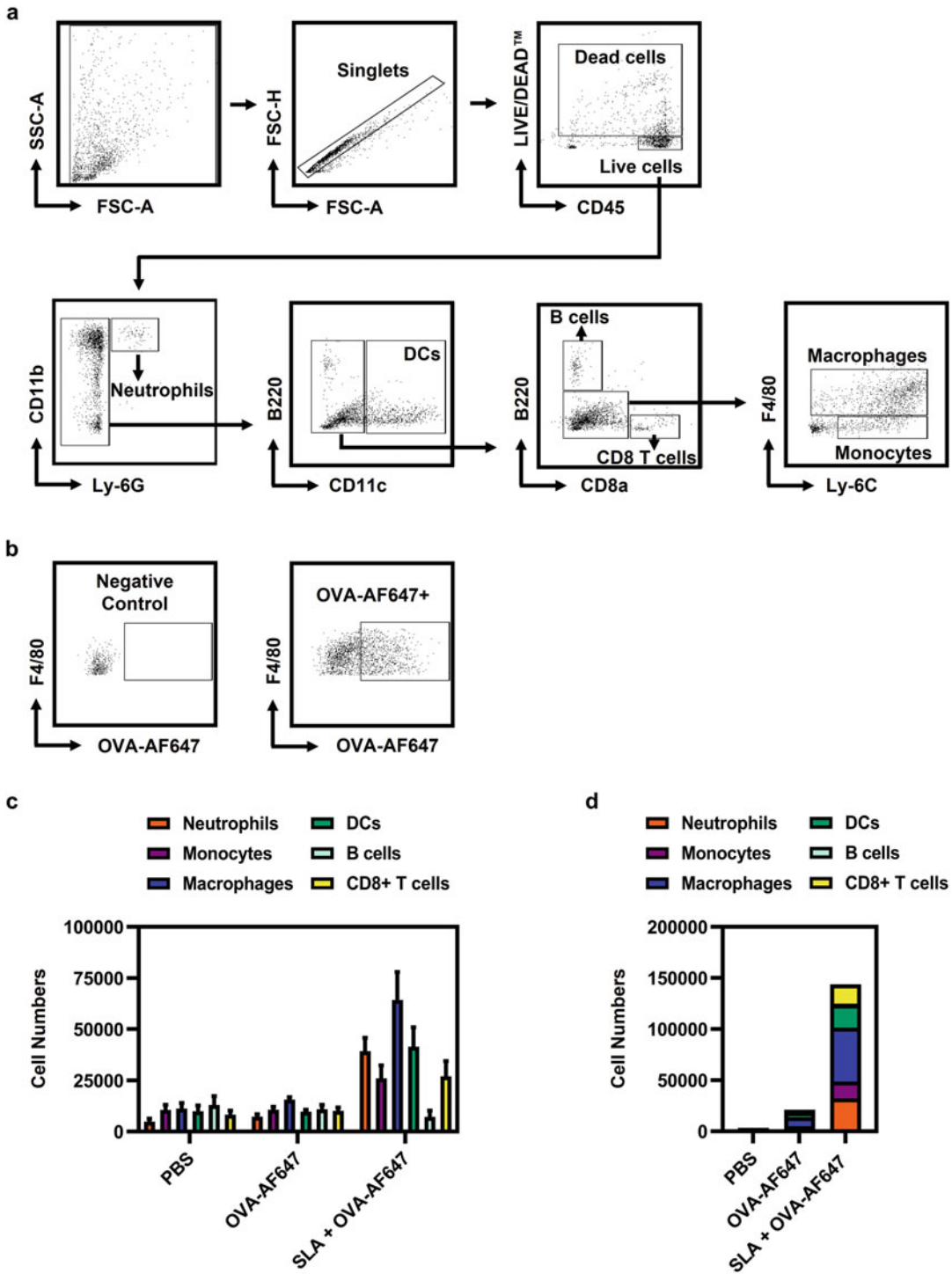

Fig. 2 Flow cytometry gating strategy for immune cell types and OVA-AF647^{+} cells. (**a**) Live immune cells were gated according to their forward scatter (FSC) and side scatter (SSC) properties, negative LIVE/DEAD™ cell stain signal and high CD45 expression. Immune cell types were identified according to cell surface expression of specific markers. (**b**) Representative dot plots showing the gating of OVA-AF647^{+} populations in macrophages from the negative and antigen alone groups. Representative bar graphs showing total (**c**) and OVA-AF647^{+} (**d**) cell numbers per immune cell type

5. Vaccine preparation methods will vary depending on the antigen and adjuvant used. The recommended maximum injection volume of 50 μL is routinely used in our immunization studies [7].
6. Isoflurane is routinely used for clinical anesthesia of mice in our studies. Other anesthetizing agents may also be used depending on availability and approval by the institutional animal care and use committee.
7. The typical sites of intramuscular injection in mice are the TA, gastrocnemius, and quadriceps leg muscle groups [8]. The TA muscle is routinely used in our immunization studies to evaluate local and systemic responses, particularly immune cell recruitment and the cellular uptake and distribution of antigen. Intramuscular injection should only be performed by trained personnel. Due to the small size of the TA muscle, extra care must be taken when performing intramuscular injection to minimize the risk of damage to surrounding tissue [7]. Practice injection technique using tracker dyes, such as India ink and 1% methylene blue [8, 9].
8. Mouse euthanasia through cervical dislocation should only be performed under anesthesia by trained personnel.
9. Avoid repeated freeze–thaw cycles to prevent degradation of Collagenase Type IV enzymatic activity.
10. Samples can be diluted further at 1:5 or 1:10 ratio with R10 complete medium in a fresh microcentrifuge tube prior to addition of dye if cell concentration is too high. Furthermore, cell counting can be performed using trypan blue and a hemocytometer if an automated cell counter is not readily available.
11. Label a tube for each fluorochrome-conjugated antibody used for staining and add one drop of UltraComp eBeads™, as recommended by the manufacturer. Add the appropriate fluorochrome-conjugated antibody (0.03–1.0 μg; quantity based on amount used to stain cells) to each tube and mix thoroughly. Incubate for 15–30 min at 4 °C in the dark. Perform the same wash steps as the stained cells and resuspend in 500 μL staining buffer.
12. Prepare negative and positive controls for LIVE/DEAD™ Fixable Dead Cell Stain. While the manufacturer-recommended working concentration of 1:1000 is a good starting point, increasing it to 1:100 provides a strong positive signal and allows clear distinction between live and dead cell populations in this setting. Furthermore, spleen cells can be used for the controls to avoid unnecessary usage of muscle and LN samples. Collect and process the spleen in the same manner as LNs. Bring the final volume of the single-cell suspension to 10 mL using R10 complete medium prior to counting the cells

using the Cellometer Auto 2000 cell viability counter. Transfer 1×10^6 cells into two tubes, and label one tube "negative control" and the second "positive control." Wash cells by adding 1 mL of PBS to each tube and centrifuging at $500 \times g$ for 5 min at 4 °C. Remove supernatant and fix cells by adding 100 μL Cytofix™ Fixation Buffer. Fixing the cells allows the viability dye to pass through the cell membrane and react with free amines both on the cell surface and in the cell interior to generate strong fluorescence that is characteristic of nonviable cells. Mix cells thoroughly and incubate for 15–30 min at 4 °C in the dark. Perform the same wash steps as described previously. Resuspend the negative control cells in 500 μL staining buffer. In contrast, stain the positive control cells with 50 μL of the working concentration (1:100) of LIVE/DEAD™ Fixable Dead Cell Stain for 20 min at 4 °C in the dark. Wash and resuspend positive control cells in 500 μL staining buffer. Lastly, add 150–200 μL of the negative control cell suspension into the positive control tube. This will allow simultaneous visualization of the live and dead cell populations in the positive control tube upon acquisition on the flow cytometer.

13. The 96-well DeepWell™ polypropylene microplate is ideal for staining a large set of samples and optimal washing of cells with buffer volumes of up to 2 mL/well.
14. Remove the supernatant in a round-bottom polystyrene tube after centrifugation by using a Pasteur pipette attached to a vacuum aspirator. Alternatively, carefully pour the supernatant into a waste receptacle. If using a 96-well DeepWell™ polypropylene microplate, quickly invert the microplate over a waste receptacle and gently tap once on a paper towel to remove residual volume.
15. Antibody titration must be performed to determine the optimal concentration of each antibody used. This can be determined by looking at the staining index over a range of concentrations [10]. The aim is to select a concentration that gives bright staining with minimum background. A starting concentration of $\leq$0.5 μg antibody per 1×10^6 cells in 50 μL staining buffer can be used for staining optimization.
16. Run the appropriate controls, such as single-stain compensation beads and Fluorescence Minus One (FMO) controls prior to sample acquisition. Single-stain compensation beads should be acquired every experiment for optimal correction of fluorochrome spectral overlap. In contrast, FMO controls are cells that are stained with all the fluorochromes except one fluorochrome. These cells are critical in setting up the cell gating and

can be acquired only during the initial optimization experiment.

17. Fix cells by adding either 100 or 250 μL of Cytofix™ Fixation Buffer per well or tube, respectively. Mix thoroughly by vortexing, and incubate for 15–30 min at 4 °C in the dark. Perform the same wash steps as described previously, and resuspend in 500 μL staining buffer prior to sample acquisition. Fixed cells can be stored at 4 °C in the dark for up to a week.
18. Flow cytometry data from our immunization studies are routinely analyzed using FlowJo™ 10 (BD). The total number of each immune cell type is determined using the formula: (percentage of immune cell type gated on live $CD45^+$ cells × total sample cell number obtained using the Cellometer Auto 2000 cell viability counter)/100. Similarly, the number of OVA-$AF647^+$ cells per immune cell type is calculated using the formula: (percentage of OVA-$AF647^+$ immune cell type gated on live $CD45^+$ cells × total sample cell number obtained using the Cellometer Auto 2000 cell viability counter)/100. Immune cell numbers are graphed and analyzed for statistical significance using GraphPad® Prism 8 (GraphPad, La Jolla, CA, USA).

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References

1. Haq K, Jia Y, Krishnan L (2016) Archaeal lipid vaccine adjuvants for induction of cell-mediated immunity. Expert Rev Vaccines 15:1557–1566
2. Akache B, Stark FC, Jia Y, Deschatelets L, Dudani R, Harrison BA, Agbayani G, Williams D, Jamshidi MP, Krishnan L et al (2018) Sulfated archaeol glycolipids: comparison with other immunological adjuvants in mice. PLoS One 13:e0208067
3. Akache B, Stark FC, Iqbal U, Chen W, Jia Y, Krishnan L, McCluskie MJ (2018) Safety and biodistribution of sulfated archaeal glycolipid archaeosomes as vaccine adjuvants. Hum Vaccin Immunother 14:1746–1759
4. Jia Y, Akache B, Deschatelets L, Qian H, Dudani R, Harrison BA, Stark FC, Chandan V, Jamshidi MP, Krishnan L et al (2019) A comparison of the immune responses induced by antigens in three different archaeosome-based vaccine formulations. Int J Pharm 561:187–196
5. McCluskie MJ, Deschatelets L, Krishnan L (2017) Sulfated archaeal glycolipid archaeosomes as a safe and effective vaccine adjuvant for induction of cell-mediated immunity. Hum Vaccin Immunother 13:2772–2779
6. Anesthesia Induction and Maintenance. 2019. JoVE, Cambridge, MA, JoVE science education database. Lab Animal Research. Ref Type: Audiovisual Material

7. Shimizu S (2004) Routes of administration. In: Hedrich HJ, Bullock G (eds) The laboratory mouse. Elsevier, Amsterdam, pp 527–542
8. Gruntman AM, Bish LT, Mueller C, Sweeney HL, Flotte TR, Gao G (2013) Gene transfer in skeletal and cardiac muscle using recombinant adeno-associated virus. Curr Protoc Microbiol. Chapter 14, Unit 14D.3
9. Fargas A, Roma J, Gratacos M, Roig M (2003) Distribution and effects of a single intramuscular injection of India ink in mice. Ann Anat 185:183–187
10. Maecker HT, Frey T, Nomura LE, Trotter J (2004) Selecting fluorochrome conjugates for maximum sensitivity. Cytometry A 62:169–173

Chapter 30

The Quantification of Antigen-Specific T Cells by IFN-γ ELISpot

Bassel Akache and Michael J. McCluskie

Abstract

The enzyme-linked immune absorbent spot (ELISpot) assay allows for the quantification of the number of cells producing a particular secreted analyte. As T lymphocytes secrete cytokines such as interferon (IFN)-γ upon binding of the T cell receptor with its cognate antigen epitope, IFN-γ ELISpot allows for the measurement of antigen-specific T cells in an immune sample. Immune cells are isolated from the vaccinated subject and incubated with the epitope/antigen of interest on polyvinylidene difluoride (PVDF)-lined microplates precoated with a capture antibody to IFN-γ. Cytokine spots are then detected utilizing an IFN-γ-specific detection antibody and an enzyme-linked conjugate. Here, we describe the quantification of OVA-specific CD8 and CD4 T cells from mouse splenocytes to measure vaccine-induced cellular responses.

Key words Vaccines, ELISpot, T lymphocyte, Interferon-γ, Ovalbumin

1 Introduction

The immune system employs a cellular immune response to fight various disease indications, such as cancer or viral infections. Malignant or infected cells are killed through direct interaction with lymphocytes [1]. As such, vaccine platforms and adjuvants capable of inducing strong antigen-specific CD8 T cell responses have been developed and evaluated for use in novel vaccines targeting these types of indications. To measure the activity of such vaccines/adjuvants, a reliable method to quantitate the number of antigen-specific T cells is required. Enzyme-linked immunosorbent spot (ELISpot) is a modification of a regular sandwich ELISA, where the assay input is live cells instead of protein-containing extract [2]. It is routinely used to measure the immunogenicity of vaccines in preclinical and clinical vaccine studies where cellular immune responses are thought to be an important correlate/contributor to vaccine efficacy [3–6]. In addition, these assays have been used to measure the number of T cells generated spontaneously by the body in response to a viral infection (RSV, influenza, etc.) or

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malignant tumor growth [7–9]. ELISpot can also be used to guide the proper application of viral gene therapy vectors by measuring cellular responses to the viral proteins [10].

Lymphocytes are a type of white blood cell that play a central role in the body's immune defense and include T, B and natural killer cells. T cells mediate the adaptive antigen-specific cellular immune responses and are subdivided into many subtypes including CD4+ T helper and CD8+ cyotoxic T cells. CD8+ and CD4+ T cells are specific to a particular epitope which they can bind in the context of a major histocompatibility complex (MHC) on the cellular surface [1]. Upon binding of their epitope, CD4+ and CD8+ T cells are activated resulting in the release of cytokines such as IFN-γ. Cytotoxic CD8+ T cells can also respond by releasing lytic granules with the aim of killing the undesired host cell which has been deemed to be a risk due to its presentation of a pathogen or cancer-specific epitope. While CD4+ cells predominantly support the immune response by producing and secreting cytokines, they can also have lytic activity [11]. In studies evaluating vaccine activity, the use of ELISpot to quantify antigen-specific T cells relies most commonly on detecting IFN-γ expression due to its (1) strong correlation with CD8 T cell lytic activity and (2) production in CD4 T cells involved in the activation of cytotoxic immune responses [3, 12]. Herein, we describe an ELISpot-based method to measure ovalbumin-specific CD4+ and CD8+ T cells in mouse samples (Fig. 1). Ovalbumin is routinely used as a model antigen when evaluating vaccine adjuvants in preclinical studies as (1) it is an inherently weak immunogen when administered alone and (2) the epitopes presented by specific laboratory mouse strains are well characterized. In a sandwich ELISA, the analyte of interest is already present in the test sample prior to addition to the ELISA plate to be bound by the capture and detection antibodies. With ELISpot, cells are added directly to the plate and stimulated with the antigen of interest to generate the test analyte after their addition to the plate. The capture antibodies on the well surface directly adjacent to the stimulated cell will bind the cytokine in real time as it is being produced. Roundish spots will form once the plates are developed, and based on the one spot–one cell principle, we can estimate the number of cells producing an analyte such as IFN-γ [13]. This method has allowed for the determination of the capability of a vaccine formulation to induce antigen-specific T cells and also study the kinetics and longevity of these cellular responses. It can be easily adapted to different antigens by replacing the stimulant peptides.

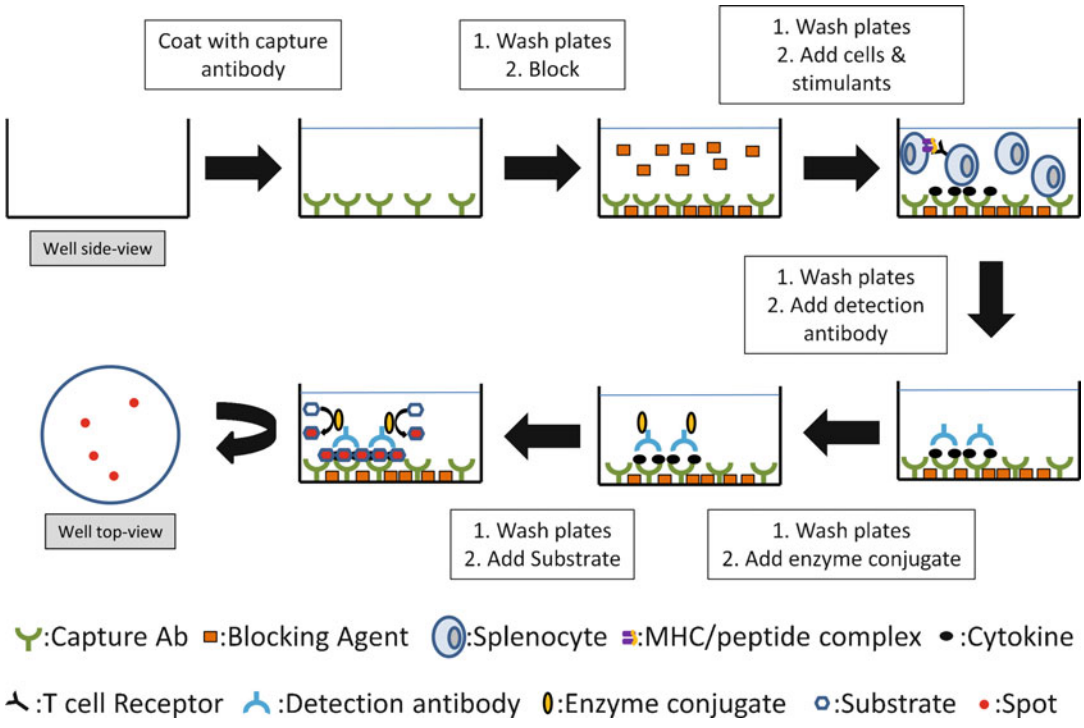

Fig. 1 Schematic overview of experimental procedures

2 Materials

Use ultrapure water to prepare all necessary buffers/solutions. The supplemented RPMI medium and ethanol solutions may be prepared in advance. However, the Diluent buffer and AEC substrate solution is to be prepared on the day of the assay as described below.

2.1 Solutions and Reagents

1. R10 medium: RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin/streptomycin, and 55 μM 2-mercaptoethanol: Stored at 2–8 °C.
2. Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium.
3. Diluent buffer: Dilute fetal bovine serum (FBS) solution 200-fold in DPBS to achieve a 0.5% FBS solution.
4. 3-Amino-9-ethylcarbazole (AEC) substrate set (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA): Stored at 2–8 °C.
5. 35% and 70% ethanol.
6. Dimethyl sulfoxide (DMSO).

2.2 Antibodies and Stimulants

1. Capture antibody to IFN-γ (Clone AN18; Mabtech, Nacka Strand, Sweden): Stored at 2–8 °C (*see* **Note 1**).
2. Biotinylated detection antibody to IFN-γ (Clone R4-6A2; Mabtech, Nacka Strand, Sweden): Stored at 2–8 °C.
3. Streptavidin-horseradish peroxidase (HRP) (Mabtech, Nacka Strand, Sweden): Stored at 2–8 °C.
4. Concanavalin A (conA) from jack bean: Dissolved in DPBS at a concentration of 5 mg/mL and dispensed into single use aliquots, which are then frozen at <−15 °C.
5. Synthetic peptides corresponding to specific ovalbumin residues (1) amino acids 257–264: SIINFEKL (CD8 epitope) and (2) amino acids 323–339: ISQAVHAAHAEINEAGR (CD4 epitope). Peptides are provided as lyophilized powder at >90% purity and have a trifluoroacetate counterion. They are dissolved in DMSO at a concentration of 2 mg/mL and dispensed into single use aliquots, which are then frozen at <−70 °C (*see* **Note 2**).

2.3 Equipment and Plasticware

1. Surgical scissors.
2. Forceps.
3. Sterile petri dish.
4. Sterile microscope glass slides with frosted end.
5. Hemocytometer.
6. ELISpot PVDF 96-well microplates, MSIP type (Millipore Sigma, Burlington, MA, USA).
7. Multichannel pipettes.
8. Reagent reservoirs.
9. 12-Channel reagent reservoirs.
10. P200 and P1000 pipette tips.
11. 15 and 50 mL conical tubes.
12. 70 μm cell strainer.
13. Large plastic container.
14. Orbital shaker.
15. Dissection microscope or automated ELISpot reader.

3 Methods

Steps in Subheadings 3.1–3.4 are to be performed aseptically in a biological safety cabinet. The procedures described in Subheading 3.5 (plate development) do not need to be performed aseptically.

3.1 Antibody Coating

1. Determine the number of plates required to test desired samples (each sample is usually tested in duplicate).
2. Prepare sufficient quantities of coating solution (~10 mL per plate) by diluting capture antibody to a final concentration of 15 μg/mL in DPBS (*see* **Note 3**).
3. Add 15 μL of 35% ethanol per well of the PVDF ELISpot plate and incubate for 1 min.
4. Wash each plate well with 200 μL sterile water 5× using multichannel pipette. Dump wash solutions by flicking plates over a wide plastic container within the biosafety cabinet to maintain the plate's sterility. Tap plate on paper towel after final wash to remove excess water and add 100 μL of coating solution per well (*see* **Note 4**).
5. Incubate overnight for ~16–20 h at 2–8 °C.

3.2 Blocking of Plates

1. On the following day, wash the plates 5× with 200 μL per well of DPBS solution using multichannel pipette (*see* **Note 5**). Again, washes are dumped within the biosafety cabinet and plates are tapped on paper towel after final wash to remove excess DPBS.
2. Add 200 μL of R10 to each well and incubate at room temperature for at least 30 min prior to addition of cells and stimulants prepared below.

3.3 Spleen Collection and Processing

1. Anesthetize mouse (previously immunized with adjuvanted ovalbumin vaccine formulation) and euthanize by cervical dislocation (*see* **Note 6**).
2. Place mouse on counter, right flank down. Spray left flank with 70% ethanol.
3. Make small incision with surgical scissors across left flank (~1/3 of the way from base of tail to neck) to access abdominal cavity.
4. Locate spleen and gently pull out in an exterior direction through the incision using tweezers. Detach entire spleen using surgical scissors and place in 50 mL conical tube containing 5 mL of sterile chilled R10 media and place spleen on ice (*see* **Note 7**).
5. Pour contents of tube (i.e., spleen and R10 media) in a sterile petri dish. Homogenize tissue by placing spleen in between the frosted edges of two sterilized microscope slides and sliding them back and forth gently. Continue rubbing the slides against each other until the entire tissue has been reduced to cellular suspension.
6. Place 70 μm cell strainer on same 50 mL conical tube that contained spleen in **step 4** above and transfer splenocyte

suspension back into tube through strainer using a serological pipette. Place cells on ice.

7. Determine concentration of samples following dilution (e.g., tenfold) and addition of appropriate live/dead stain (e.g., trypan blue) by counting cells using a hemocytometer or automated cell counter (*see* **Note 8**).

3.4 Preparation of Sample Dilutions and Stimulants

1. Using measured cell concentrations, calculate appropriate amount of cells from each sample to add to 1 mL of R10 media to obtain a 4×10^6 cells/mL cell suspension (*see* **Note 9**). Vortex cell suspension gently and add appropriate volume of each sample to a single well of a multichannel reservoir. Once all cell samples have been added, dispense 1 mL of R10 media to each well to adjust cells to a final concentration of 4×10^6 cells/mL.
2. Thaw stimulants at room temperature. Prepare following 2× stimulant solutions by pipetting the appropriate amount of stimulant to a conical tube containing R10 media. 0.2 mL of each solution will be required for each sample.
   (a) Media alone: 2 μL DMSO per mL of R10.
   (b) 2× CD8 epitope solution (4 μg/mL SIINFEKL): 2 μL 2 mg/mL SIINFEKL per mL of R10.
   (c) 2× CD4 epitope solution (4 μg/mL ISQAVHAAHAEINEAGR): 2 μL 2 mg/mL ISQAVHAAHAEINEAGR per mL of R10.
   (d) Positive control (2× conA solution (10 μg/mL)): 2 μL of 5 mg/mL conA per mL of R10. ConA is a mitogen that will induce IFN-γ secretion in a non-antigen-specific manner (*see* **Note 10**).
3. Dump R10 media from blocked ELISpot plates within biosafety cabinet. Immediately add 100 μL of stimulants per well of plates according to plate layout (*see* Fig. 2 for an example of a plate layout).
4. Resuspend cell suspension in multichannel reservoir by pipetting up and down 10–15 times. Add 100 μL of cells per well of plates according to plate layout (*see* **Note 11**).
5. Shake cells gently on orbital shaker for ~60 s to evenly disperse cells within well and place plates in 37 °C humidified incubator with 5% CO_2 overnight for ~20 h (*see* **Note 12**).

3.5 Plate Development

1. Dilute the biotinylated detection antibody with diluent buffer to a final concentration of 1 μg/mL. Each plate will require 10 mL.
2. Remove the plates from the incubator and dump cell suspension to a plastic container. With a multichannel pipette, add

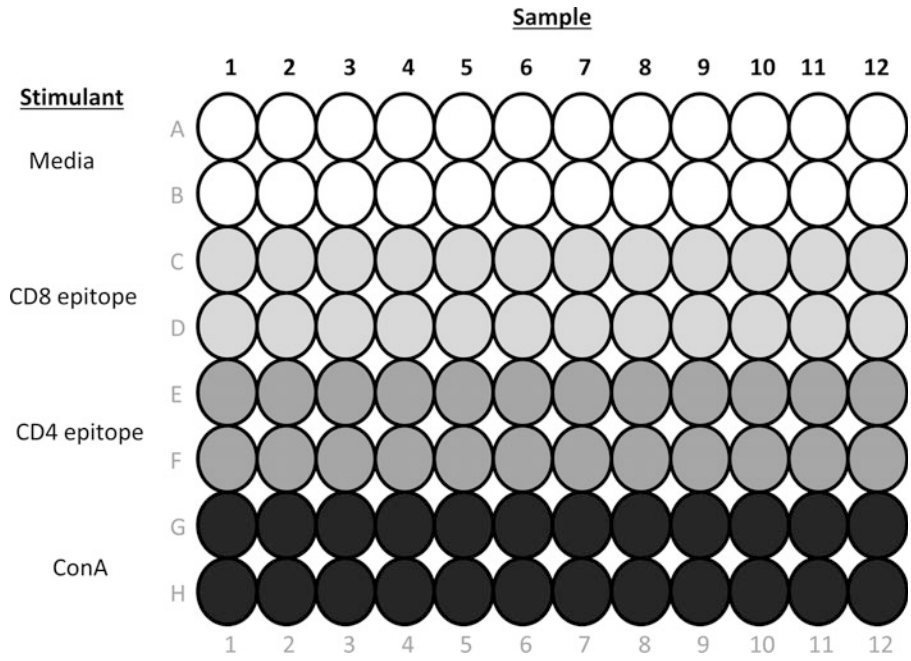

Fig. 2 Example of plate layout

200 μL of water to each well and incubate the plate at room temperature for 5 min (*see* **Note 13**).

3. Dump the water and again with a multichannel pipette, wash the plates 4× with 200 μL of DPBS per well.
4. Tap the plates on a paper towel to get rid of the excess DPBS and add 100 μL of the detection antibody solution to each well. Incubate the plates for 2 h at room temperature.
5. Dilute the 1000× streptavidin-HRP 1:1000 with diluent buffer to the appropriate final concentration. Each plate will require 10 mL.
6. Dump the solution from the plates and with a multichannel pipette, wash the plates 5× with 200 μL of DPBS per well.
7. Tap the plates on a paper towel to get rid of the excess DPBS and add 100 μL of the streptavidin-HRP solution to each well. Incubate the plates for 1 h at room temperature.
8. Just prior to the completion of the above incubation step, prepare the AEC substrate solution (*see* **Note 14**). The AEC substrate set contains separate AEC substrate and chromagen solutions. For each plate, add 10 mL of AEC substrate solution and 200 μL of the chromagen solution to a conical tube. Invert tube a few times to mix solution and keep at room temperature in the dark until ready to use.

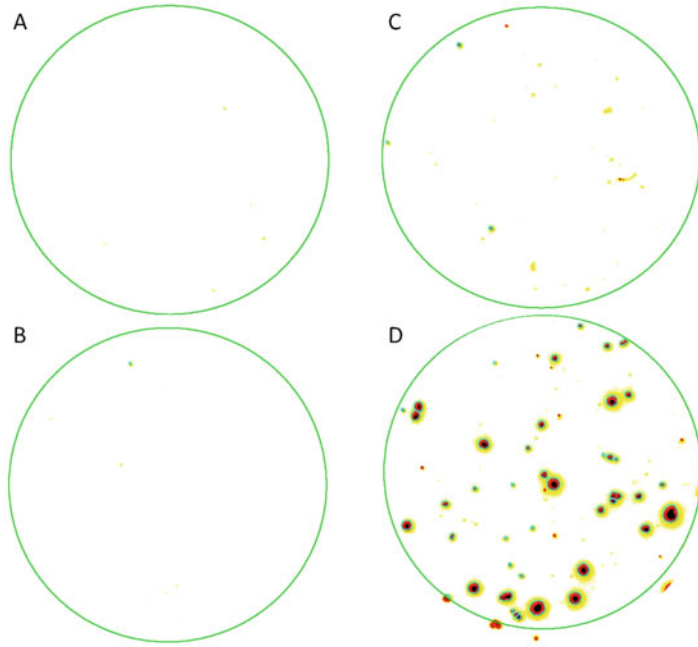

Fig. 3 IFN-γ + spot forming cells from splenocytes of immunized mice. C57Bl/6 mice were immunized intramuscularly with OVA (10 μg) alone or adjuvanted with alum (40 μg) + CpG 1826 (10 μg) on days 0 and 21. Spleens were collected on Day 28 with splenocytes analyzed for OVA-specific CD4 T cells by IFN-γ ELISpot. Images of representative wells are shown in Panels (**a**)–(**d**). Cells from a mouse immunized with OVA alone stimulated with media or 2 μg/mL ISQAVHAAHAEINEAGR in Panels (**a**) and (**b**), respectively. Cells from a mouse immunized with OVA + alum/CpG stimulated with media or 2 μg/mL ISQAVHAAHAEINEAGR in Panels (**c**) and (**d**), respectively

9. Dump the solution from the plates. With a multichannel pipette, wash the plates 5× with 200 μL of DPBS per well.
10. Tap the plates on a paper towel to get rid of the excess DPBS and add 100 μL of the AEC solution to each well. Incubate plates for 20 min at room temperature (*see* **Note 15**).
11. Stop the reaction by submerging the plate in a large plastic container being continuously filled with running tap water in a sink. Quickly remove and flick the plate to dump out the water in the sink. Resubmerge the plate ensuring that all wells are filled with the water. Repeat 4–6 times.
12. Remove the underdrain (plastic covering on bottom of plate) from the plate, and wash the plate again by submerging a few times as above in the water container (*see* **Note 16**).
13. Allow plates to dry at room temperature overnight.

14. Count spots using an automated ELISpot plate reader or a dissection microscope (Fig. 3). Results are routinely reported as spot forming cells (SFC)/million splenocytes (*see* **Note 17**).

4 Notes

1. If interested in measuring the secretion of cytokines other than IFN-γ, this ELISpot protocol can be adapted through the replacement of the capture and detection antibodies with appropriate equivalent antibodies specific to the analyte of interest.
2. Due to their widespread use, these peptides are available off the shelf from many different commercial suppliers. If using large quantities of these peptides, it may be beneficial to place a custom order with a peptide manufacturer. This usually reduces the overall cost and allows the user to determine characteristics such as purity and aliquot size. While the peptides described here are soluble enough in water to get working solutions, the solubility of other peptides in water can be low. As such, we routinely use DMSO as a solvent for our peptides. Solubility is usually higher, which can be quite useful when working with other antigens where there is a need to combine multiple peptides in a pool. However, DMSO can be toxic at higher concentrations. The final concentration of DMSO when added to the cells should not exceed 1% (ideally 0.5%). If adapting this protocol to other antigens where epitopes have not been defined, cells can be stimulated with whole protein (which tend to favor activation of CD4 cells [3]) or overlapping peptide libraries which cover the length of the protein (e.g., 15mer peptides overlapping by 11 amino acids).
3. When washing and coating the plates, it is important not to allow the wells to dry out after activation with the ethanol or coating may be uneven. While more expensive, some suppliers do offer precoated plates where application of coating solution has been standardized.
4. As live cells will be incubated on the ELISpot plates, it will be important to take steps to reduce the risk of microbial contamination. While certain steps described above (such as removing the media by flicking the plate upside down and tapping the plates on nonsterilized paper towels) may seem risky, they do not usually introduce any contamination in our experiments.
5. When pipetting into the plates, pay attention to orient the tips toward the walls of the well instead of straight down at the membrane. Direct contact with the membranes can introduce tears. In addition, the background color of the well can be

inconsistent/patchy if solution is added or removed from the membrane at high pressure/speed. This is also why the substrate is washed off by submerging the plates in water instead of running tap water directly over them.

6. C57Bl/6 mice are routinely used in ovalbumin vaccine/tumor studies, as the above mentioned epitopes bind strongly to the MHC haplotypes found in this mouse strain.
7. When removing the spleens, try to carefully trim off any large pieces of associated fat (whitish mass on spleen) with the surgical scissors prior to placing the spleens in the R10 containing tube. This will help in the homogenization process. Also, when homogenizing, darker foci can occasionally be found in a small number of spleens. They are more difficult to homogenize and can be discarded once the surrounding reddish tissue is placed into suspension.
8. If counting with a hemocytometer, please note that the cell suspension will contain both red and white blood cells (RBC/WBC). As they are the cells of interest, include only the white blood cells in your counts. They can be differentiated based on their size and morphology. Alternatively, if using an automated cell counter, some are compatible with nucleic acid-sensitive dyes such as acridine orange which will positively stain WBC but not RBC.
9. The cell amount added per well can be reduced based on availability of cells or strength of signal (i.e., number of spots is too high leading to saturation of the well). However, the number of SFCs obtained may not be linear (i.e., if you obtain ~100 spots with 400,000 splenocytes, you should not necessarily expect to obtain ~50 spots with 200,000 cells). As the number of lymphocytes is reduced, there will also be an impact on the number of antigen presenting cells and the degree of cell-to-cell contact. So users must confirm the linearity in the system if planning to use different cell concentrations. If responses found to be nonlinear, the level of response cannot be directly compared between experiments using different amounts of cells.
10. As conA is non–antigen-specific mitogen, it should stimulate all samples to some degree allowing the user to confirm that all samples contain viable cells. Even if a similar number of cells are stimulated with conA, the number of resulting SFCs may differ between mouse samples. Again, if the response with the positive control is so strong as to saturate the well, the number of cells can be reduced for this test condition only. In an effort to save space on the plate, it may not be necessary to include the conA control for all samples once the user is confident with the quality of their samples.

11. When adding cells, the same tips can be used to add the samples to multiple wells as long as they do not touch the wells or stimulant solution already in the plate. To help avoid contact, position the tips so that they hover above the wells/plate and eject the solution quickly. It is not an issue to point the tips toward the bottom of the well in this step as there is already solution in the well and the tips are not within the well.
12. Ensure the surface in the incubator on which the plates are placed is flat. Also, do not stack or move plates within the incubator once placed. The formation of a well-defined round spot depends on the cell remaining in place during the entire incubation. If the plates are moved or at a tilt, a cell producing IFN-γ may shift leading to the formation of a streak or multiple spots from a single cell. Also, altering the overnight incubation time may increase/decrease the number of detected spots, keeping it approximately the same (i.e., ~20 h) throughout a study will help ensure consistency.
13. The initial addition of water causes the cells to swell and burst through osmosis, making them easier to remove during the subsequent washes.
14. Tetramethylbenzidine (TMB) can be used in place of AEC as the substrate in this HRP-based system. TMB is generally more sensitive than AEC, resulting in more spots, but can also lead to higher background signals in the media alone wells.
15. Most spots should appear relatively quickly after addition of substrate. However, the development time with the substrate can be shortened or lengthened based on the size and number of spots obtained in your system. Again, to help maintain consistency, it is important to establish a specific development time and use it throughout a study.
16. Remove the underdrain with care and pay attention to not damage or tear the PVDF membranes as they will now be exposed from the bottom of the plate. A tear in the well can interfere with the counting of spots.
17. When counting spots, consider that cell-generated spots should be generally round with a dense center. Fibers, tears, and debris can lead to the formation of irregular-looking spots, which should be disregarded when counting.

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References

1. Klimpel GR (1996) Immune Defenses. In: Baron S, ed. Medical Microbiology. 4th ed. Galveston (TX): University of Texas Medical Branch at Galveston, https://pubmed.ncbi.nlm.nih.gov/21413332/
2. Cox JH, Ferrari G, Janetzki S (2006) Measurement of cytokine release at the single cell level using the ELISPOT assay. Methods 38:274–282
3. Akache B, Stark FC, Jia Y, Deschatelets L, Dudani R, Harrison BA, Agbayani G, Williams D, Jamshidi MP, Krishnan L et al (2018) Sulfated archaeol glycolipids: comparison with other immunological adjuvants in mice. PLoS One 13:e0208067
4. Calarota SA, Baldanti F (2013) Enumeration and characterization of human memory T cells by enzyme-linked immunospot assays. Clin Dev Immunol 2013:637649
5. Jia Y, Akache B, Deschatelets L, Qian H, Dudani R, Harrison BA, Stark FC, Chandan V, Jamshidi MP, Krishnan L et al (2019) A comparison of the immune responses induced by antigens in three different archaeosome-based vaccine formulations. Int J Pharm 561:187–196
6. Plotkin SA (2008) Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis 47:401–409
7. Currier JR, Kuta EG, Turk E, Earhart LB, Loomis-Price L, Janetzki S, Ferrari G, Birx DL, Cox JH (2002) A panel of MHC class I restricted viral peptides for use as a quality control for vaccine trial ELISPOT assays. J Immunol Methods 260:157–172
8. Junker N, Thor SP, Andersen MH, Svane IM (2011) Characterization of ex vivo expanded tumor infiltrating lymphocytes from patients with malignant melanoma for clinical application. J Skin Cancer 2011:574695
9. Patton K, Aslam S, Lin J, Yu L, Lambert S, Dawes G, Esser MT, Woo J, Janetzki S, Cherukuri A (2014) Enzyme-linked immunospot assay for detection of human respiratory syncytial virus f protein-specific gamma interferon-producing T cells. Clin Vaccine Immunol 21:628–635
10. Britten CM, Walter S, Janetzki S (2013) Immunological monitoring to rationally guide AAV gene therapy. Front Immunol 4:273
11. Stevenson PG (2020) Immune control of gamma-Herpesviruses. Viral Immunol 33 (3):225–232
12. Slota M, Lim JB, Dang Y, Disis ML (2011) ELISpot for measuring human immune responses to vaccines. Expert Rev Vaccines 10:299–306
13. Mobs C, Schmidt T (2016) Research techniques made simple: monitoring of T-cell subsets using the ELISPOT assay. J Invest Dermatol 136:e55–e59

Chapter 31

Measurement of Antigen-Specific IgG Titers by Direct ELISA

Bassel Akache, Felicity C. Stark, and Michael J. McCluskie

Abstract

Direct ELISA allows for the measurement of antibody levels to a particular antigen. Serum or plasma from the vaccinated subject are incubated on high-binding capacity microplates precoated with the antigen of interest and detected utilizing an enzyme-linked secondary antibody. Herein, using influenza hemagglutinin as model antigen, we describe the quantification of antigen-specific IgG titers in mouse serum to measure vaccine-induced humoral responses.

Key words Vaccines, Immunoglobulin, Antibody, Humoral response, ELISA

1 Introduction

For many infectious disease indications, effective vaccines rely on the generation of antibodies capable of binding and neutralizing the pathogen [1]. As such, methods to accurately measure antigen-specific antibodies are widely employed during vaccine development, whether in (1) the preclinical setting when optimizing vaccine formulations or (2) clinically, when correlating immunological readouts to vaccine efficacy in human subjects [2–5]. In addition, such assays are used widely for infectious disease diagnosis or the detection of autoantibodies in an autoimmune disease setting [6, 7]. The levels of antigen-specific antibodies are generally assessed by enzyme linked immunosorbent assay (ELISA) or fluorescent bead-based immunoassays. While bead-based assays offer many advantages to ELISA such as a wider dynamic range and the ability to multiplex (i.e., simultaneous measurement of antibodies to multiple antigens in a single sample) [5], they are more laborious to optimize and require specialized equipment. Due to its relative ease to execute and adopt, the ELISA-based method is more generally employed to quantify vaccine-specific responses in a preclinical setting.

Of the various immunoglobulin types (i.e., A, D, E, G, M), immunoglobulin G (IgG) is most commonly the isotype of interest

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in a nonmucosal vaccine setting. This monomeric immunoglobulin is the predominant isotype in blood and extracellular fluid [8]. While antigen-specific IgM dominates the humoral response immediately following antigen exposure, it is succeeded by IgG as antibody isotype class-switching occurs during maturation of the antibody response [9]. Herein, using influenza hemagglutinin as a model antigen, we describe an ELISA-based method to measure antigen-specific IgG titers in mouse samples. While a sandwich ELISA generally relies on two different antibodies to capture and detect a protein of interest, this direct antibody ELISA utilizes the antigen of interest and an enzyme-linked secondary antibody to capture and detect the IgG molecules (Fig. 1). This method has allowed for the determination of the impact of (1) various adjuvants on the immunogenicity of vaccine formulations, (2) repeated vaccine administration, and (3) longevity of the immune response. It can be easily adapted to different antigens or antibody types (different isotypes, subtypes and/or species).

2 Materials

Use ultrapure water to prepare all necessary buffers/solutions. The Wash buffer, Citrate buffer and Stop solution may be prepared in advance. However, the Blocking buffer, Diluent buffer, and Substrate solution are to be prepared on the day of the assay as described below.

2.1 Antigens and Antibodies

1. Influenza A H1N1 hemagglutinin: Dissolved and dispensed into single use aliquots, which are then frozen at <-15 °C.
2. Goat anti-mouse IgG-horseradish peroxidase (Southern Biotech, Birmingham, AL, USA): Stored at 2–8 °C (*see* **Note 1**).
3. Goat anti-mouse IgG1-horseradish peroxidase (Southern Biotech): Stored at 2–8 °C.
4. Goat anti-mouse IgG2a-horseradish peroxidase (Southern Biotech): Stored at 2–8 °C.

2.2 Solutions and Reagents

1. Fetal Bovine Serum (FBS).
2. Wash Buffer (10× stock solution): Add indicated amounts of following chemicals to 3 L of ultrapure water: 8.0 g monopotassium phosphate (KH_2PO_4), 24.4 g disodium phosphate (Na_2HPO_4), 8.0 g potassium chloride (KCl), and 320.0 g sodium chloride (NaCl). Once dissolved, add 20 mL of Tween 20 and bring up volume to 4 L with ultrapure water. Dilute this 10× stock solution tenfold in ultrapure water to achieve the 1× solution used below for preparation of diluent

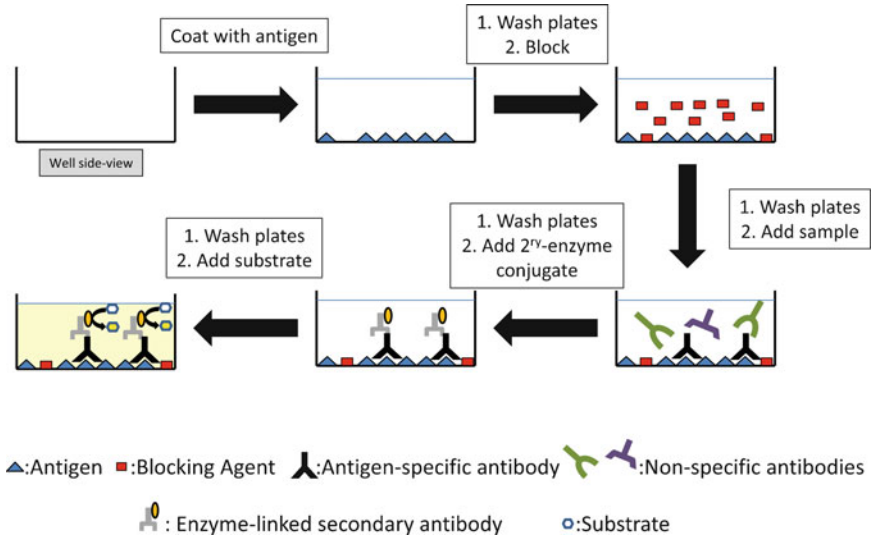

Fig. 1 Schematic overview of experimental procedures

buffer (*see* Subheading 2.3, **item 3**) and plate washes (*see* Subheading 3) (*see* **Note 2**).

3. Blocking Buffer: Dilute fetal bovine serum (FBS) solution tenfold in Dulbecco's phosphate buffered saline (without calcium and magnesium) to achieve a 10% FBS solution.
4. Diluent buffer: Dilute FBS solution tenfold in Wash buffer to achieve a 10% FBS solution.
5. Citrate buffer: Prepare 0.1 M citric acid as follows: Dissolve 21.01 g of citric acid, monohydrate ($C_6H_8O_7 \cdot H_2O$) in 1 L water. Separately, prepare 0.1 M sodium citrate solution as follows: Dissolve 29.41 g of sodium citrate tribasic dehydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) in 1 L water. Autoclave both solutions at ~110 °C for 15 min. Prepare citrate buffer solution by combining 205 mL 0.1 M citric acid, 0.1 M 295 mL sodium citrate and 500 mL water. Store the citrate buffer solution, 0.1 M citric acid and 0.1 M sodium citrate at 4 °C.
6. Stop solution (4 N H_2SO_4): Slowly dilute 50 mL of 36 N H_2SO_4 in 400 mL of water (*see* **Note 3**).
7. O-phenylenediamine dihydrochloride (OPD) substrate tablets (5 or 30 mg/tablet).
8. Hydrogen peroxide solution (30%w/w).

2.3 Equipment and Plasticware

1. Serum separator Microtainer.
2. Microcentrifuge tubes, 1.5 mL.
3. 1.2 mL Cluster tubes, 8-tube strip, racked.
4. Sealing mats, round cap for 1.2 mL tubes.

5. Enzyme immunoassay flat-bottom 96-well microplates (*see* **Note 4**).
6. Multichannel pipettes.
7. 96-Well dilution plates (vinyl or polypropylene).
8. Microcentrifuge.
9. Centrifuge with plate rotor.
10. P200 and P1000 pipette tips.
11. 15 and 50 mL conical tubes.
12. Plastic wrap.
13. Microplate washer.
14. Microplate absorbance spectrophotometer.

3 Methods

3.1 Sample Collection

1. Collect blood from immunized mouse in a serum separator tube (*see* **Note 5**).
2. Incubate blood for 30 min at room temperature (RT) to allow blood to clot.
3. Place tubes in a microcentrifuge and centrifuge blood at 13,000 × *g* for 5 min at RT.
4. Collect the serum using a pipette and transfer to 1.2 mL cluster tubes or 1.5 mL microcentrifuge tubes (*see* **Note 6**).
5. Serum can be frozen at <−15 °C until ready for analysis.

3.2 Antigen Coating

1. Determine the number of plates required to test desired samples (each plate usually contains 12 test samples).
2. Prepare sufficient quantities of antigen solution (~10 mL per plate) by diluting coating antigen (e.g., hemagglutinin) to a final concentration of 1 μg/mL in PBS (*see* **Note 7**).
3. Dispense 100 μL of antigen solution to each well using multichannel pipette.
4. Place plates in stacks of up to ten plates and cover stack with plastic wrap. Incubate overnight at RT.
5. In preparation for Subheading 3.4, thaw serum samples at 4 °C overnight. Alternatively, samples can be thawed at room temperature during the blocking step (Subheading 3.3, **step 3**).

3.3 Blocking of Plates

1. On the following day (16–20 h postincubation), wash the plates 5× with 200 μL per well of 1× Wash Buffer solution using the automated plate washer. Washes can also be done by hand using a multichannel pipette (*see* **Note 8**).

2. Add 200 μL of Blocking Buffer to each well.
3. Stack plates and cover with plastic wrap and incubate for 1 h at 37 °C.

3.4 Preparation of Sample Dilutions

1. Centrifuge samples briefly to separate any potential solid debris from sample. If samples are in a 96-well cluster tube box, spin samples briefly at 500 × *g* for 5 min in the swinging bucket centrifuge, using the plate rotor. If samples are stored in microcentrifuge tubes, spin for 1 min at 10,000 × *g* in microcentrifuge.
2. For each set of ten samples, one dilution plate (either a non-treated U-bottom 96-well disposable plate or 0.5 mL deep-well assay block plate, depending on required sample volume) will be loaded with the appropriate volume of diluent buffer and be used to perform a serial dilution of each serum sample. Eight data points are generated per sample using a half log serial dilution to enable the determination of antibody titer (Fig. 2).
3. The required sample dilution volumes will depend on the number of antigens or antibody isotypes that will be tested simultaneously. While routine testing may include a single antibody isotype (e.g., IgG), multiple isotypes (e.g., IgG, IgG1, and IgG2a) may be tested in parallel, for example to evaluate the Th1 vs. Th2 bias of the humoral response (*see* **Note 9**). Alternatively, a single sample's ability to cross-react with multiple viral serotypes can be evaluated on separate plates.
4. For a single antibody isotype–antigen combination, pipette 150 μL of dilution buffer in rows B-H of a U-bottom 96-well disposable plate. For row A, 180 μL of dilution buffer is added to each well. Add 20 μL of the test serum sample to a well in Row A to create a starting 1:10 dilution (*see* **Note 10**). If available, place positive control (e.g., serum from previously tested animal) in well A11 and negative control in well A12 (e.g., serum from nonimmunized animal) and dilute as above. Once all samples have been loaded, use a multichannel to serially dilute 69.4 μL of diluted serum sample 7 times between rows B-H by pipetting up and down 4–6 times (*see* **Note 11**).
5. If running three plates per sample, use a 0.5 mL deep-well assay block plate and add 400 and 540 μL of dilution buffer to rows B-H and A, respectively. 60 μL of the test serum would be added to Row A to create a starting 1:10 dilution and the serial dilutions would be performed by pipetting 185 μL of sample down the rows.

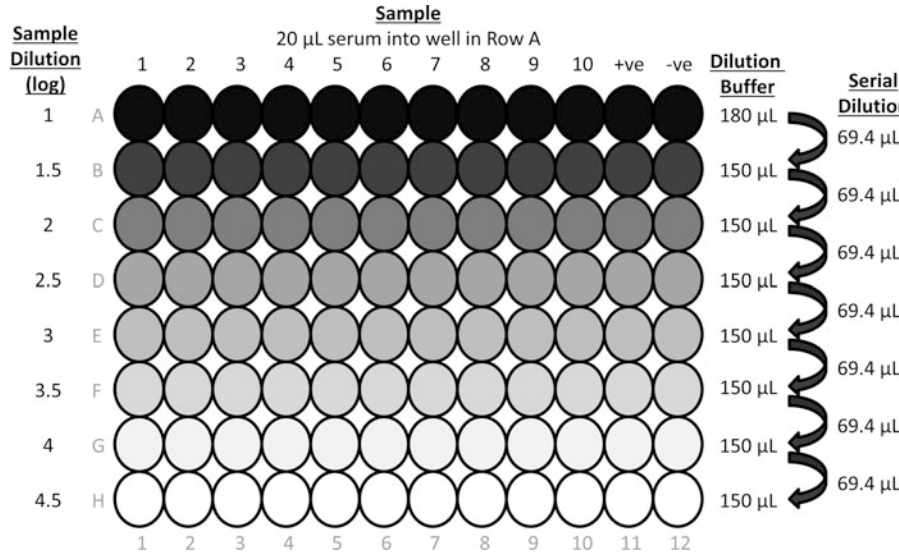

Fig. 2 Schematic of strategy for preparation of sample dilutions

6. Prior to addition of samples, wash the plates 5× with 200 µL per well of Wash Buffer solution using the automated plate washer.
7. Starting with the most dilute sample dilution (i.e., row H in Fig. 2) transfer 100 µL of the diluted samples to the appropriate wells of each ELISA plate. Continue to transfer each row moving upward on the dilution plate without need to change multichannel tips between rows finishing with the least diluted (i.e., row A in Fig. 2) (*see* **Note 11**).
8. Stack plates and cover with plastic wrap and incubate for 1 h at 37 °C.

3.5 Addition of Secondary Antibody and Plate Development

1. Dilute the appropriate HRP-labeled secondary antibodies (e.g., anti-IgG2a, anti-IgG1, and/or anti-IgG) with diluent buffer at a concentration of 1:4000. As an example, each plate requires 10 mL; therefore, dilute 2.5 µL in 10 mL of diluent buffer (*see* **Note 12**).
2. Remove the plastic wrap and wash the plate 5× with 200 µL per well of Wash Buffer solution using the automated plate washer.
3. Add 100 µL of the appropriate secondary antibody to each well of the corresponding plate.
4. Stack plates and cover in plastic wrap and incubate for 1 h at 37 °C.
5. Just prior to the completion of the above incubation step, prepare the OPD substrate solution at 10 mL per plate. For each 10 mL of citrate buffer pH 5.0, add 30 µL of hydrogen peroxide and a 5 mg OPD tablet. (If preparing large volumes of

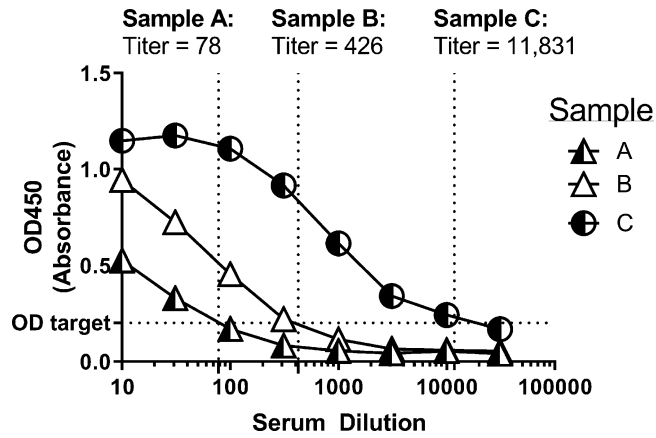

Fig. 3 Antibody titers in serum of immunized mice. BALB/c mice were immunized intramuscularly with HA (2 μg) on days 0 and 21. Animals were bled on Day 35 with serum analyzed for anti-HA IgG Abs by ELISA

substrate solution, 30 mg tablets may also be used in place of 6 × 5 mg tablets).

6. Remove plastic wrap and wash the plates 5× with 200 μL per well of Wash Buffer solution using the automated plate washer.
7. Add 100 μL of OPD substrate solution to each well (*see* **Note 13**).
8. Put the plates in a dark drawer at room temperature for 30 min.
9. After the 30-min incubation, add 50 μL of Stop Solution to each well in the same order the substrate was added.
10. Read the plate at an optical density of 450 to 490 nm (OD_{450} to OD490) using a microplate reader (*see* **Note 14**).
11. Using a data analysis software, construct a curve for each sample plotting (OD_{450nm} vs. sample dilution (Fig. 3)). Titers are determined by extrapolating the sample dilution that would result in a target OD reading (e.g., 0.2) (*see* **Notes 15–17**). Alternatively, a standard curve could be constructed using a positive sample and values normalized to the standard curve.

4 Notes

1. While we routinely use these types of secondary antibody–enzyme conjugates, conjugates based on antibodies raised in other species may be used if more readily available. However, some optimization will be required to establish a working concentration suitable for your system.
2. While the 10× Wash buffer can be prepared in advance and stored until use, microbial contamination may arise over time.

To minimize the risk of microbial growth, the solution may be autoclaved or stored at 2–8 °C. As salt precipitates may form with the latter option, it is important to warm up the 10× solution to ensure complete dissolution of the buffer components prior to dilution with water to obtain the working 1× Wash buffer solution.

3. Exercise great caution when diluting the sulfuric acid for the stop solution. Be sure to work in a chemical hood with proper ventilation and follow all appropriate safety precautions. Also, dispense acid slowly into the bottle already containing the entire volume of water so as not to generate too much heat/energy. Allow solution to cool down before firmly tightening the lid. The stop solution should be stored in an appropriate acid cabinet.
4. Enzyme immunoassay plates are used as they are designed to maintain assay consistency and improve binding of the coating antigen to the plate surface. As this is a colorimetric assay, transparent plates are used to allow measurement of the solution OD by the spectrophotometer.
5. If preferred, plasma samples may also be used to measure antigen-specific antibody titers. While a strong correlation in titers against some antigens has been demonstrated with plasma and serum collected simultaneously [10], it would be prudent to select one sample type in a particular experiment if possible.
6. The ELISA will be conducted in a 96-well format, where multichannel pipettes will be employed to dilute the serum. Therefore, when dealing with a large number of samples, it is more efficient to collect and store samples in a format compatible with a multichannel pipette (e.g., 8 or 12- cluster strips of 1.2 mL). The samples can be arranged to match your final layout (e.g., leave columns 11 and 12 empty to have room in the plate for controls). If transferring the samples with a multichannel pipette, confirm that all tips contain equal volume before loading onto the dilution plate.
7. Coating concentration will need to be optimized to your particular antigen type/source. A trial run can be conducted with various concentrations of antigen. Ideally, you should select a concentration where the signal is close to saturation with the positive samples (i.e., increasing coating concentration will not boost your signal significantly) and where the negative control results in a low background signal ($OD_{450} < 0.1$).
8. Once the washing step is complete, tap plates while upside down onto paper towel to remove any residual wash solution remaining in the wells prior to addition of the subsequent solution (Blocking Buffer, samples, etc.).

9. Measuring IgG subtypes is sometimes performed as an indicator of helper T (Th) cell bias, where IgG2 (Th1) and IgG1 (Th2) responses have been associated with a strong cytotoxic T cell and humoral response, respectively. For example, inclusion of TLR agonists such as CpG in a vaccine formulation will shift antibodies to the IgG2 subtype while simultaneously inducing strong CD8 T cell responses [11]. This is not a hard and fast rule, as other adjuvants (e.g., sulfated lactosyl archaeol glycolipids) have been shown to induce high levels of antigen-specific CD8 T cells and predominantly antigen-specific IgG1 antibodies [3].
10. Depending on the vaccine regimen and/or formulation, the antibody titers in your sample may be too high to measure accurately with a starting 1:10 dilution. A starting dilution of 1:100 or 1:1000 may be required to properly capture the target OD in the range of tested serum dilutions. Adjust serum and dilution buffer volumes in Row A to achieve desired dilution and then serially dilute as described above. A predilution could be required to more precisely achieve a 1:1000 starting dilution (i.e., 1:20 followed by a 1:50).
11. Prior to executing/transferring serial dilutions, it is best to ensure all tips are tightly attached to the multichannel pipette. With the large number of repetitive pipetting steps, a tip may come loose in the middle of the process if not strongly attached to the pipette.
12. If evaluating IgG subtypes such as IgG2 in mice, it is important to note the strain of mouse from which the samples were collected. Commonly used mouse strains such as C57Bl/6 and BALB/c express the IgG2c and IgG2a forms of this antibody, respectively. Keep this in mind when selecting the appropriate secondary antibody for your system.
13. This assay protocol can be adapted to run with other substrates (e.g., 3,3′,5,5′-tetramethylbenzidine) or secondary antibodies with different enzymatic moieties (e.g., alkaline phosphatase). However, some optimization will be required to establish a number of parameters (secondary antibody concentrations, development time, target OD, etc.). In addition, the plates will need to be read at the optical density appropriate for the substrate used.
14. As plates are stacked during incubations, condensation may form on the underside of some of the plates. Wipe the underside of the plate to remove any condensation prior to loading in the spectrophotometer as the water droplets may interfere with the absorbance readings.
15. The selection of an appropriate target OD will depend on the background and peak signals observed in your assay. We

routinely use 0.2 as it is sufficiently higher than the absorbance reading we normally obtain in background wells with no or naïve sample (i.e., 0.05). It is also >fourfold lower than the peak values we obtain when signal levels may be saturated. If a particular sample does not reach the target OD, we suggest assigning the value of the lowest tested dilution for that particular sample for analysis/presentation purposes.

16. Titers should not be directly compared between different assays (different coating antigens, antibody isotypes, subtypes, etc.). The titers within a particular assay depend on the concentrations/activities of various assay reagents, such as coating protein and detection antibodies. This assay will allow you to rank antibody titers within a particular assay, but will not provide fixed concentrations or amounts of antibody. In order to do this, a standard curve is required using samples with a known antibody concentration. Another possible advantage of including a standard curve is the potential to assay more samples per plate. As the aim of the ELISA shifts from calculating a target OD to finding a value that falls within the range of a standard curve, a smaller number of sample dilutions can be tested per sample.
17. A "hook effect" may be observed at the lower end of the dilution range where OD values appear lower than in more diluted samples before they start decreasing as would be expected with subsequent dilutions. This may be due to inhibitory effects of the serum matrix when added at high concentrations or when all available binding sites have been saturated.

Acknowledgments

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References

1. Plotkin SA (2008) Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis 47:401–409
2. Akache B, Stark FC, Iqbal U, Chen W, Jia Y, Krishnan L, McCluskie MJ (2018) Safety and biodistribution of sulfated archaeal glycolipid archaeosomes as vaccine adjuvants. Hum Vaccin Immunother 14(7):1746–1759
3. Akache B, Stark FC, Jia Y, Deschatelets L, Dudani R, Harrison BA, Agbayani G, Williams D, Jamshidi MP, Krishnan L et al (2018) Sulfated archaeol glycolipids: comparison with other immunological adjuvants in mice. PLoS One 13:e0208067
4. Jia Y, Akache B, Deschatelets L, Qian H, Dudani R, Harrison BA, Stark FC, Chandan V, Jamshidi MP, Krishnan L et al (2019) A comparison of the immune responses induced by antigens in three different archaeosome-based vaccine formulations. Int J Pharm 561:187–196
5. Pavliakova D, Giardina PC, Moghazeh S, Sebastian S, Koster M, Pavliak V, McKeen A, French R, Jansen KU, Pride M (2018)

Development and validation of 13-plex Luminex-based assay for measuring human serum antibodies to *Streptococcus pneumoniae* capsular polysaccharides. mSphere 3. https://doi.org/10.1128/mSphere.00128-18

6. Castro C, Gourley M (2010) Diagnostic testing and interpretation of tests for autoimmunity. J Allergy Clin Immunol 125:S238–S247
7. Storch GA (2000) Diagnostic virology. Clin Infect Dis 31:739–751
8. Vidarsson G, Dekkers G, Rispens T (2014) IgG subclasses and allotypes: from structure to effector functions. Front Immunol 5:520
9. Stavnezer J, Guikema JE, Schrader CE (2008) Mechanism and regulation of class switch recombination. Annu Rev Immunol 26:261–292
10. Siev M, Yu X, Prados-Rosales R, Martiniuk FT, Casadevall A, Achkar JM (2011) Correlation between serum and plasma antibody titers to mycobacterial antigens. Clin Vaccine Immunol 18:173–175
11. McCluskie MJ, Weeratna RD, Evans DM, Makinen S, Drane D, Davis HL (2013) CpG ODN and ISCOMATRIX adjuvant: a synergistic adjuvant combination inducing strong T-cell IFN-gamma responses. Biomed Res Int 2013:636847

Chapter 32

A Method to Evaluate In Vivo CD8$^+$ T Cell Cytotoxicity in a Murine Model

Felicity C. Stark, Renu Dudani, Gerard Agbayani, and Michael J. McCluskie

Abstract

Herein, a method to measure in vivo CD8$^+$ T cell cytotoxicity in a murine model is presented. The activation of a strong CD8$^+$ T cell response is paramount when designing vaccines to tackle intracellular infections and for cancer therapy. CD8$^+$ T cells can directly kill infected and transformed cells and are directly associated with beneficial protection in many disease models. CD8$^+$ T cell cytotoxicity can be measured using multiple methods including measuring IFNγ production by ELISPOT or measuring intracellular cytokines or cytotoxic granules by flow cytometry. However, to determine the ability of CD8$^+$ T cells to kill their target in the context of its cognate receptor and in their native environment, the in vivo cytotoxic T cell assay (in vivo CTL) is ideal. The in vivo CTL assay provides a snapshot of the whole ability of the host to kill "Target" cells by measuring the loss of injected target cells relative to "Non-target" cells. The assay involves isolating splenocytes from donor mice, forming "Target" and "Non-target" cellular samples and injecting them intravenously into naïve and experimental mice at a chosen time-point in the experiment. Mice are humanely sacrificed 20 h later, and their spleens are excised and processed for flow cytometric analysis. The extent of "Target" cell killing relative to "Non-target" cells is determined by comparing the surviving proportions of these cells among experimental mice relative to naïve mice. The in vivo CTL assay is a rapid, sensitive, and reliable method to measure the potency of CD8$^+$ T cells in their host to kill their target.

Key words In vivo CTL, Cytotoxic T lymphocyte, CTL, Vaccines, Cytotoxicity, Antigen-specific CD8$^+$ T cell killing, Killing assay, Cellular immunity, T cell killing

1 Introduction

When evaluating a candidate vaccine in preclinical studies, it is key to design assays that can detect the activation of both humoral and cell-mediated immunity. Historically, the induction of a potent antibody response was sufficient to advance a new vaccine formulation toward a clinical trial. However, as vaccinologists tackle tougher, more complex pathogens (influenza, HIV, HCV, cancer, etc.), new vaccine formulations are frequently also required to show evidence of the activation of cell mediated immunity [1–4]. In this

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context, cell mediated immunity refers to the induction of a potent $CD8^+$ T cell response that can indirectly kill pathogens by targeting infected or transformed cells. The activation of $CD8^+$ T cells can similarly be assessed by flow cytometry using fluorochrome-conjugated antibodies, while antigen-specific $CD8^+$ T cells can be assessed using tetramer technology [5]. However, these techniques do not inform on the cytotoxic abilities of a $CD8^+$ T cell to kill its target in its host. $CD8^+$ T cell cytotoxicity assays were initially developed as in vitro assays to assess ex vivo splenocytes (containing activated $CD8^+$ T cells) for their ability to kill target cells (coated with peptide antigen and ^{51}Cr or CFSE) in a controlled cell culture system.

While this assay is highly useful for tracking $CD8^+$ T cell cytotoxicity over time as well as assessing the potency of the cytotoxic $CD8^+$ T cell (CTL) response by varying the ratio of splenocytes to target cells, a limitation of this assay is that the $CD8^+$ T cells are required to kill their target in an artificial environment (culture dish or well) and not in the host where other soluble or cellular factors might be available that could modulate the response. Assessing CTL cytotoxicity in vivo by intravenously injecting target cells allows for the detection of CTL killing in their natural environment in the whole organism [6].

To assess in vivo cytotoxicity in the mouse model, it is important to have a well-characterized antigen with a known $CD8^+$ T cell epitope. To generate target cells capable of being recognized by the $CD8^+$ T cell TCR, donor splenocytes must be incubated with antigenic peptides that have a good affinity for the MHC class I complex. We have used the model antigen ovalbumin protein for immunization containing the well-characterized $CD8^+$ T cell epitope, SIINFEKL. A good adjuvant is also required to induce strong CTL responses. We have used archaeosomes composed of a single sulfated glycolipid namely sulfated lactosylarchaeol (SLA; 6-′-sulfate-β-D-Galp-(1,4)-β-D-Glcp-(1,1)-archaeol) that when admixed or used to encapsulate antigen results in the induction of strong, long-lasting cellular and humoral immune responses [7]; however, the method described is applicable to other adjuvants. Herein, a method is described to assess CTL killing in vivo by using target cells coated with SIINFEKL peptide and labelled with a fluorescent cytoplasmic dye CFSE.

1.1 Example Experimental Overview

1. C57BL/6NCrl (C57BL/6) mice are immunized intramuscularly or subcutaneously with a vaccine formulation containing 20 μg ovalbumin and 1 mg SLA (*see* **Note 1**).
2. At various time-points after immunization, peptide-pulsed CFSE-stained syngeneic donor splenocytes, known as "Target" cells (Fig. 1), are injected intravenously into immunized C57BL/6 mice (*see* **Note 2**).

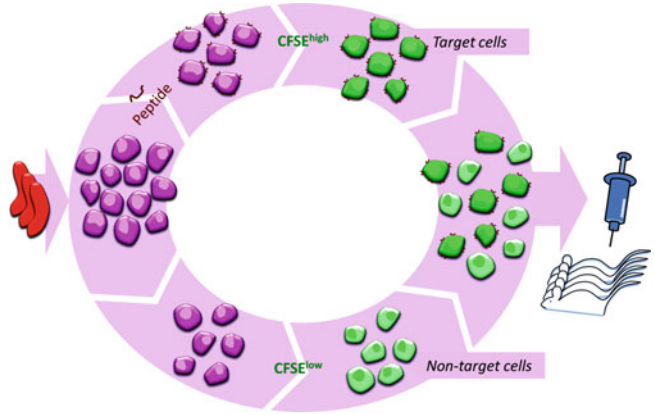

Fig. 1 Illustration of in vivo CTL cellular preparation workflow. Spleens are harvested from donor C57BL/6 mice and processed into a single cell suspension. Cells are divided into two equal portions, one group is identified as "Target" cells and incubated with the $CD8^+$ T cell peptide epitope of the ovalbumin protein, SIINFEKL; the other group of cells identified as "Nontarget" cells is left untouched. Next, "Target" cells are stained with a high concentration of CFSE and "Nontarget" cells are stained with a low concentration of CFSE. "Target" and "Nontarget" cells are combined and injected intravenously into experimental syngeneic C57BL/6 mice

3. Twenty hours after target cell injection, spleens are harvested from immunized mice, processed to single-cell suspensions and acquired by flow cytometry to identify target cells and determine CTL killing (Fig. 2).
4. % Target cell killing in an individual mouse is determined relative to the presence of nontarget cells in that same mouse and is also compared to the average of all naïve mice, Target cells to Nontarget cells (Fig. 3).

2 Materials

2.1 Vaccine Formulation and Immunization

1. Adjuvant: Sulfated lactosylarchaeol (SLA; 6′-sulfate-β-D-Galp-(1,4)-β-D-Glcp-(1,1)-archaeol) archaeosomes (National Research Council Canada).
2. Antigen: Ovalbumin (Hyglos GmbH, Bernried am Starnberger See, Germany).
3. Negative control: Phosphate-buffered saline (PBS) (pH 7.4).
4. Insulin syringes, 1.0 cc.

2.2 Spleen Collection and Processing

1. H-2Kb OVA (257–264) peptide, SIINFEKL (JPT Peptide Technologies, Berlin, Germany) (*see* **Note 3**).
2. Fetal bovine serum (FBS).

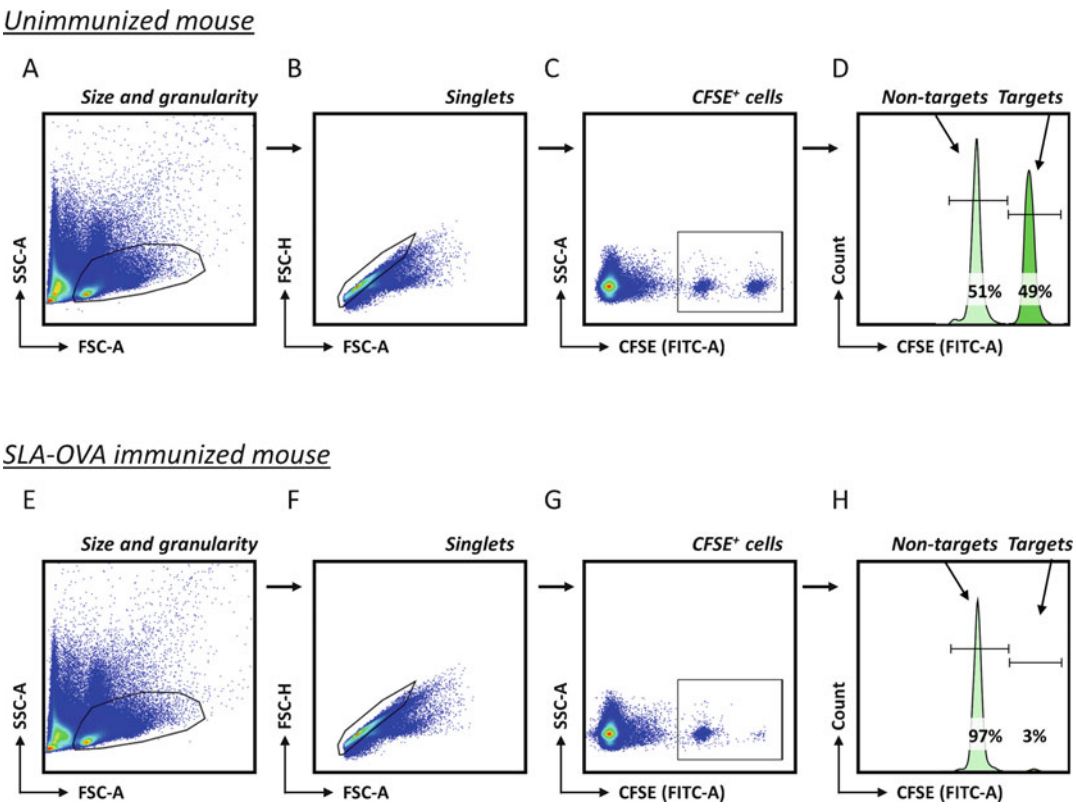

Fig. 2 Gating strategy to determine % CTL killing. Representative FACS dot plots of splenocytes retrieved from mice that were injected with 20×10^6 CFSE stained "Target" and "Nontarget" cells 20 h prior. Gating strategy to locate lymphocytes based on size and granularity (**a**, **e**), single cells (**b**, **f**) and $CFSE^+$ cells (**c**, **g**) followed by histograms to measure the percent of "Target" and "Nontarget" cells remaining in each mouse (**d**, **h**). The top panels (**a**)–(**d**) represent a naïve unimmunized mouse, both "Target" and "Nontarget" cells are found in equal proportion as expected. The bottom panels (**e**)–(**h**) represent an SLA-immunized mouse with a strong SIINFEKL specific $CD8^+$ T cell response with a near complete killing of "Target cells"

A

$$100 - \left[\left(\frac{(T/NT)_{exp.}}{\bar{x}\,(T/NT)_{naive}} \right) \times 100 \right]$$

B

$$100 - \left[\left(\frac{(3/97)}{\frac{(49/51) + (50/50) + (52/48)}{3}} \right) \times 100 \right] = 96.95\%\ \text{Killing}$$

Fig. 3 Example calculation to determine % CTL killing for an individual mouse. (**a**) T/NT: percent "Target" cells divided by percent "Nontarget" cells in an experimental mouse. $\bar{x}$: Average. $\bar{x}\,(T/NT)_{naïve}$: The average (T/NT) of all naïve mice used in the experiment. (**b**) An example calculation relating to Fig. 1: An experimental mouse had a T/NT ratio of 3/97. This value was divided by the average T/NT ratio of three naïve mice: 49/51, 50/50, and 52/48, multiplied by 100 then subtracted from 100, generating a 96.95% killing for the SLA-immunized experimental mouse

3. RPMI 1640 buffer: RPMI 1640 (Life Technologies, Carlsbad, CA).
4. R10: RPMI containing 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine.
5. PBS (pH 7.4), kept at room temperature (20–25 °C) without Ca^{2+} or Mg^{2+} (*see* **Note 4**).
6. FACS buffer: 2% FBS and 3 mM EDTA in PBS (*see* **Note 5**).
7. HBSS: Hank's balanced salt solution (Thermofisher, Waltham, MA, USA).
8. Dimethyl sulfoxide (DMSO), sterile (MilliporeSigma, Burlington, MA, USA).
9. T75 flasks.
10. Petri dishes.
11. Microcentrifuge tubes, 1.5 ml.
12. Falcon™ conical centrifuge tubes, 50 ml.
13. Round-bottom polystyrene tubes, 5 ml.
14. 96-Well DeepWell™ polypropylene microplates, 2 ml.
15. Multiscreen 60 μm nylon mesh ×96-well filter plate (MilliporeSigma, Burlington, Massachusetts).
16. U bottom ×96-well plate.
17. Carboxyfluorescein diacetate N-hydroxysuccinimidyl ester (CFDSE), a cell-permeant non-fluorescent pro-dye that becomes cleaved intracellularly to the membrane impermeant fluorescent dye carboxyfluorescein succinimidyl ester (CFSE).
18. BD LSR Fortessa™ flow cytometer, or other flow cytometer with a Blue laser (488 nm) and a FITC detector for CFSE's peak emission of 521 nm.

3 Method

3.1 Preparation of Target Cells

1. Spleens are collected from C57BL/6 donor mice. Ten donor spleens should be collected for every ~25 mice receiving injected cells.
2. Anesthetize the mice using isoflurane and perform cervical dislocation, then harvest the spleens (*see* **Note 6**).
3. Aseptically mash pooled spleens in a petri dish in R10 between the frosted ends of two glass slides to obtain a single-cell suspension. Use approximately 40 ml of R10 for every ten donor spleens (*see* **Note 7**).
4. Filter splenocytes through a 75 μm cell strainer into a new 50 ml Falcon tube.
5. Centrifuge cells for 5 min at 400 × *g*, 4 °C in a swinging bucket rotor to pellet cells, then discard supernatant.

6. Resuspend cells in approximately 25 ml of R10 (*see* **Note 8**). Remove a small aliquot for cell counting and count the cells using trypan blue or a similar cell counting protocol.
7. Add R10 to adjust the cell concentration to 10×10^6 cells/ml. Consider moving the cells to a T-75 flask instead of multiple Falcon tubes to accommodate larger volumes; however, cells would have to be transferred back to tubes for centrifuging.
8. Divide the cells into two equal portions in 50 ml Falcon tubes or T-75 flasks. Label one "Target" and the other ""Nontarget."
9. Prepare peptide solution: Make a working solution of peptide of 2 mg/ml in DMSO (s*ee* **Note 9**).
10. Peptide pulse: To the "Target" cell sample tube only, add OVA peptide (SIINFEKL) to a final concentration of 10 μg/ml in R10 and gently agitate the solution to mix (s*ee* **Note 10**).
11. Incubate both "Target" and "Nontarget" cells for 30 min in a water-jacketed incubator, 37 °C, 5% CO_2 (s*ee* **Note 11**).
12. Centrifuge cells for 5 min at $400 \times g$, 4 °C in a swinging bucket rotor to pellet cells, then discard supernatant.

    In the following section all cells will be stained with a CFSE pro-dye solution (high or low concentration) to a final cell concentration of 10×10^6 cells/ml and a final CFSE pro-dye concentration of 2.5 or 0.25 μM.
13. Prepare CFSE pro-dye solutions at a 2× concentration as follows: (*see* **Notes 2** and **12**).
    (a) $CFSE^{high}$ solution: 5.0 μM CFSE in room temperature (20–25 °C) PBS.
    (b) $CFSE^{low}$ solution: 0.5 μM CFSE in room temperature PBS.
14. Separately resuspend "Target" and "Nontarget" cells in room temperature PBS to a concentration of 20×10^6 cells/ml. Cover each tube/flask with tinfoil to prevent photobleaching (*see* **Note 13**).
15. To the splenocytes, add an equal volume of $CFSE^{high}$ or $CFSE^{low}$ as follows:
    (a) $CFSE^{high}$ solution is added to "Target" cells to obtain a final conc. of 2.5 μM CFSE pro-dye.
    (b) $CFSE^{low}$ solution is added to "Nontarget" cells to obtain a final conc. of 0.25 μM CFSE pro-dye.
16. Gently agitate the cells for 8 min at room temperature (*see* **Note 14**).
17. Add an equal volume of ice-cold FBS to each tube and place on ice for 5 min (*see* **Note 15**).

18. Centrifuge cells for 5 min at 400 × *g*, 4 °C in a swinging bucket rotor to pellet cells, then discard supernatant.
19. Separately resuspend "Target" and "Nontarget" cells in 10 ml of HBSS. Take a 10 μl aliquot of each for cell counting.
20. Centrifuge cells for 5 min at 400 × *g*, 4 °C in a swinging bucket rotor to pellet cells, then discard supernatant.
21. Separately resuspend "Target" and "Nontarget" cells in HBSS to a final concentration of 1×10^8 cells/ml.
22. Combine equal volumes of "Target" cells and "Nontarget" cells and keep on ice. Take into consideration how many mice will need to be injected. For instance, injecting 25 mice with 200 μl each requires a minimum volume of 6.0 ml (includes extra 1.0 ml volume for syringe loading) (*see* **Note 16**).
23. Keeping the cells on ice at all times, inject 20×10^6 cells in 200 μl intravenously per mouse (*see* **Note 17**).

3.2 Detection of "Target" and "Nontarget" Cells in Mice

1. Euthanize all mice injected with target cells at approximately 20 h postinjection. Anesthetize the mice using isoflurane anesthesia, perform cervical dislocation and harvest the spleens (*see* **Note 18**).
2. Process each spleen separately. Mash the spleen between the frosted ends of two glass slides in 4 ml of R10.
3. Create a 96-well table to record where each spleen sample will be aliquoted. Transfer 400 μl from each sample to a 96-well deep-well plate (*see* **Note 19**).
4. Centrifuge cells for 5 min at 400 × *g*, 4 °C in a swinging bucket rotor to pellet cells, then discard supernatant.
5. Resuspend cells in 200 μl of FACS buffer.
6. Filter the cell solution to remove clumps immediately before acquisition by flow cytometry. Transfer the cell solution through a Multiscreen 60 μm nylon mesh 96-well filter plate, then aliquot the samples into a U bottom 96-well plate (*see* **Note 20**).
7. Use a plate reader attachment, such as the High-Throughput Sampler (HTS) configured to a BD LSR Fortessa™ flow cytometer, to acquire samples on a U-bottom 96-well plate. Take care to ensure that CFSE positive cells (two peaks) are visible on the FITC detector (Fig. 2d, h) (*see* **Note 21**).
8. Calculate the % CTL killing for each experimental mouse by comparing the % "Target" cells of all $CFSE^+$ cells to the % "Nontarget" cells of all $CFSE^+$ cells (T/NT) using the formula shown in Fig. 3A.

   The (T/NT)naïve would be the average of all naïve mice used in the study, and the (T/NT)exp. would represent the

fraction of Target cells remaining in one mouse. *See* Fig. 3B for an example calculation.

4 Notes

1. The number of mice used per group can vary depending on the comparisons to be made in the experiment. We typically use ten mice per group when comparing vaccine formulations and repeat studies at least once. SLA is routinely used as an adjuvant in our immunization studies. For testing other adjuvants, manufacturer's recommendations should be followed. We routinely immunize with 50 or 100 μl of vaccine formulation by intramuscular injection in the tibialis anterior or by subcutaneous injection at the base of the tail, respectively.
2. A total of 20 million donor cells are needed for each experimental mouse. Depending on the age of the donor mouse, spleen cell numbers will vary. The typical cell yield from one mouse after processing is 40–60 million cells.
3. SIINFEKL peptide was chosen because it is the immunodominant $CD8^+$ T cell epitope for OVA protein in C57BL/6 mice. If using a different protein or mouse strain, a $CD8^+$ T cell peptide epitope must be chosen for that protein in that host.
4. It is important to use room temperature PBS as cold PBS can negatively affect cell viability when staining with CFSE.
5. FACS buffer (also known as cell staining buffer) commonly includes a protein carrier such as FBS or bovine serum albumin (BSA) to reduce nonspecific binding of antibodies, but it also provides a nutrient for cells in solution during prolonged tissue processing. EDTA is also included to prevent cell clumping.
6. Isoflurane is routinely used for clinical anesthesia of mice in our studies. Other anesthetizing agents may also be used depending on availability and approval by the institutional animal care and use committee.
7. The frosted end of a glass slide is typically abrasive, similar to sandpaper, and has enough grit to aid in the manual dissociation of the spleen. Not all frosted glass slides have the abrasive frosted end and other methods of mechanical spleen cell dissociation are acceptable [8].
8. The volume specified here is to achieve a solution of well-dispersed cells that can be accurately divided into two equal portions.
9. Some peptides are difficult to dissolve in aqueous solutions; therefore, we use the polar aprotic solvent DMSO to make all peptide solutions. The final concentration of DMSO in the cell

solution is 0.5% and in our experience, this has little to no effect on cell viability.

10. Depending on the peptide chosen, its concentration and incubation duration will vary. It is important to take into consideration whether the peptide will be soluble in a cell culture solution by looking at the chemical and physical properties of the amino acid sequence.
11. If using Falcon tubes, use a tube rack and keep the lids loosened. If using T75 flasks with ventilated caps, tighten the lid and keep the flask upright.
12. Prepare a volume based on the number of cells. For instance, suspend 500×10^6 cells in 25 ml of room temperature PBS to a concentration of 20×10^6 cells/ml. A 25 ml 2× solution of CFSE would then be combined with the cells to obtain a final concentration of 10×10^6 cells/ml.
13. The CFSE pro-dye solution (CFDSE) is not fluorescent and does not require protection from light, but once it is combined with cells the resulting fluorescence can be negatively affected by exposure to light. The following steps will include the rapid addition of other buffers; ensure that there is enough room in each tube for the addition of 4× the existing volume. For instance, if you have 20 ml of cells in a 50 ml Falcon tube, divide it among two more 50 ml Falcon tubes so that there is enough space for the subsequent steps. Be sure to transfer the labels "Target" or "Nontarget"; there is no way to recover the cells if a mixup occurs.
14. Since CFSE pro-dye rapidly stains cells, the cells and staining solution are combined in equal volumes to ensure all cells are exposed to CFSE pro-dye at the same concentration. Unequal staining can not only affect cell viability (higher CFSE pro-dye concentration is associated with cell death) but also the intensity of the fluorescent peaks, which can become wider and less distinct and make it difficult to discern between the $CFSE^{high}$ and $CFSE^{low}$ peaks. The duration of CFSE staining ranges from 5 to 20 min according to published protocols. We obtain consistent staining with 8 min of staining.
15. These steps should be completed quickly. FBS is added to stop CFSE staining, also known as quenching the reaction. If CFSE pro-dye is left too long with the cells, it can become toxic to the cells and in some cases a slightly higher cell death is observed for cells stained with $CFSE^{high}$ solution. If this becomes an issue, next time consider starting with slightly more cells for the "Target" cell population in **step 8**.
16. Combining an equal proportion of "Target" and "Nontarget" cells is ideal but exact equality is not required. "Target" cell killing is calculated relative to "Nontarget" cells in the same

host but it is also compared to the proportions of cells detected after injection into naïve animals. Variation in Target–Nontarget cell ratios such as 55:45 (or similar) are normal; it is not necessary to discard "Target" cells to ensure that an exact equal number of cells are injected. The most important criteria is to maintain a consistency in the number of "Target" cells injected within an experiment or preferably as a standard for all experiments.

17. Cells will be injected at a concentration of 100 million cells/ml (20 million cells/200 µl/mouse). "Target" cells and "Nontarget" cells are counted separately to take note of any extreme differences in cell count. Any extreme loss of cells (>50%) could be a result of CFSE staining toxicity and more care would be taken during these steps.
18. Typically, mice are euthanized 24 h after cell injection; however, for logistical purposes, we euthanize at 20 h and still observe the same extent of target cell killing.
19. If samples cannot be acquired immediately, cover the deep-well plate at this step and refrigerate until ready (up to 4 h). Since live splenocytes can readily reform clumps, it is best to filter the cells immediately before acquisition by flow cytometry.
20. If a plate reader is not available or if there are only a few samples, cells can be individually transferred to 5 ml round-bottom polystyrene tubes and acquired manually.
21. Flow cytometry data from our immunization studies are routinely analyzed using FlowJo™ 10 (BD Biosciences, Franklin Lakes, NJ, USA). $CFSE^+$ cells are graphed and analyzed for statistical significance using GraphPad® Prism 8 (GraphPad, La Jolla, CA, USA).

References

1. Hollingsworth RE, Jansen K (2019) Turning the corner on therapeutic cancer vaccines. NPJ Vaccines 4:7
2. Garçon N, Di Pasquale A (2016) From discovery to licensure, the adjuvant system story. Hum Vaccin Immunother 13:19–33
3. De Gregorio E, Caproni E, Ulmer JB (2013) Vaccine adjuvants: mode of action. Front Immunol 4:214
4. Landi A, Law J, Hockman D et al (2017) Superior immunogenicity of HCV envelope glycoproteins when adjuvanted with cyclic-di-AMP, a STING activator or archaeosomes. Vaccine 35:6949–6956
5. Wooldridge L, Lissina A, Cole DK et al (2009) Tricks with tetramers: how to get the most from multimeric peptide–MHC. Immunology 126:147–164
6. Barber DL, Wherry EJ, Ahmed R (2003) Cutting edge: rapid in vivo killing by memory CD8 T cells. J Immunol 171:27–31
7. Jia Y, Akache B, Deschatelets L et al (2019) A comparison of the immune responses induced by antigens in three different archaeosome-based vaccine formulations. Int J Pharm 561:187–196
8. Jungblut M, Oeltze K, Zehnter I et al (2008) Preparation of single-cell suspensions from mouse spleen with the gentleMACS Dissociator. J Vis Exp (22):1029

Chapter 33

Testing Anti-Pneumococcal Antibody Function Using Bacteria and Primary Neutrophils

Manmeet Bhalla, Shaunna R. Simmons, Essi Y. I. Tchalla, and Elsa N. Bou Ghanem

Abstract

Antibodies against *Streptococcus pneumoniae* (pneumococcus) following vaccination are crucial for host protection against invasive pneumococcal infections. The antibodies induced by pneumococcal vaccines act as opsonins to mediate bacterial uptake and killing by host phagocytic cells, especially polymorphonuclear leukocytes (PMNs) also called neutrophils. Therefore, it is important to measure not only the levels of antibodies induced by a pneumococcal vaccine candidate but their actual functional capacity in mediating bacterial opsonization and killing by PMNs. Here, we describe a protocol to demonstrate effective deposition of vaccine-induced antibodies on the surface of *S. pneumoniae* by flow cytometry and subsequent opsonophagocytic killing (OPH) by murine bone-marrow derived PMNs.

Key words *Streptococcus pneumoniae*, Opsonophagocytic killing, Antibodies, Neutrophils, Flow cytometry

1 Introduction

Streptococcus pneumoniae (pneumococcus) are Gram-positive bacteria with >90 serotypes classified based on the composition of the capsular polysaccharides [1]. These bacteria typically reside asymptomatically in the nasopharynx [1]. However, in certain susceptible individuals, *S. pneumoniae* can cause invasive pneumococcal diseases (IPD) including meningitis, pneumonia and bacteremia [2]. Increased mortality rates associated with IPD are often seen in high-risk populations such as young children, the elderly, and immunocompromised patients [2].

Two vaccines covering common disease causing bacterial serotypes that rely on production of anti-capsular antibodies (Ab) are available [3]. The pneumococcal polysaccharide vaccine (PPSV or Pneumovax®) covers 23 serotypes and consists of repeating polysaccharides that can directly cross-link B cell receptors leading to antibody production independent of T-cells [4]. The

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pneumococcal conjugate vaccine (PCV or Prevnar-13®) contains polysaccharides from 13 serotypes covalently linked to a nonpathogenic diphtheria toxoid that triggers a T-cell dependent antibody response [3]. The pneumococcal capsule is known to inhibit phagocytosis by immune cells [1]; therefore, one way antibodies induced following administration of vaccines can protect the host is by opsonizing *S. pneumoniae* and facilitating their uptake and killing by host cells [5].

Vaccine responses are typically assessed by measuring the levels of serotype-specific anticapsular antibodies against pneumococci using standard enzyme-linked immunosorbent assays (ELISA) [6]. However, antibody levels are not always indicative of vaccine efficacy since they do not reflect functionality, defined as the ability to opsonize and enhance phagocytic uptake of bacteria [6]. For example, in vulnerable populations, such as the elderly, postvaccination sera had significantly reduced opsonophagocytic activity against *S. pneumoniae* [7, 8] when compared to young adults despite the two populations having similar anti-polysaccharide antibody levels. Therefore, the ability of immune sera to act as an efficient opsonin has routinely been determined with in vitro opsonophagocytosis assays using phagocytic cell lines such as HL-60 cells [9]. While a great tool for directly assessing antibody function, these cell lines are not the ones mediating bacterial killing in vivo.

One of the most important defense mechanisms against pneumococci is polymorphonuclear cells (PMNs) also known as neutrophils. These cells rapidly migrate to sites of infection and are required for host defense [10]. Neutropenic individuals or mice predepleted for neutrophils are highly susceptible to IPD [10–13]. PMNs are viewed as effectors of vaccine responses. In other words, vaccination triggers antibodies and one of the ways antibodies protect the host against infection is by binding pneumococci and promoting their clearance via enhancing uptake and killing by PMNs [5]. There is evidence that age-related decline in the intrinsic PMN function may contribute to impaired PPSV efficacy in the elderly. Pneumococci that were opsonized with sera from young PPSV immunized donors were killed less efficiently by PMNs from elderly donors than by young controls suggesting that Ab-mediated opsonophagocytic killing of *S. pneumoniae* by PMNs is impaired in the elderly [5]. Therefore examining both antibody as well as PMN function would give a more complete assessment of vaccine efficacy in a given host.

The mouse model has extensively been used to demonstrate the immunogenicity and protective efficacy of *S. pneumoniae* vaccines in vivo [14, 15]. However, with the different serotypes present in the vaccine formulations and the possibility of variation in antibody and phagocytic cell responses, in vitro assays for measuring vaccine responses are more economical and are needed for dissection of mechanisms. Here we describe a one-stop protocol for in vitro

assessment of vaccine efficacy (Fig. 1). This protocol provides details of methods to produce immune sera in a mouse model, quantify the ability of antibodies to bind bacterial surfaces by flow cytometry and subsequently evaluate the opsonophagocytic capacity of immune sera using bacterial killing assays by primary bone marrow-derived mouse PMNs.

2 Materials

2.1 For Immune/ Hyperimmune Sera Generation

1. Appropriate mice strain. We routinely produce immune sera using male and female 8- to 12-weeks-old C57BL/6 mice (Jackson Laboratories, USA), housed in a fully certified Lab Animal Facility (LAF). All experiments are performed in accordance with the Laboratory Animals Welfare Act and the Guide for the Care and Use of Laboratory Animals.
2. Vaccine: Prevnar® (Wyeth pharmaceuticals Inc., USA) or Pneumovax® (Merck and Co Inc., USA).
3. Dissection tools: Suitable forceps, fine tipped scissors, dissection board.
4. 1 ml syringe; 27- and 18-G needles.
5. Microtainer tubes for blood collection.

2.2 For Bacterial Cultures

1. Bacteria: *S. pneumoniae* TIGR4 strain is used for all the methods outlined in this chapter. Other strains of interest can also be used.
2. Liquid media: Todd-Hewitt broth (Bacto™ BD 249240) and Oxyrase® (Oxyrase OB0050).
3. Blood agar plates (Tryptic soy agar supplemented with 5% sheep blood).

2.3 For Bacterial Binding Assays

1. FACS buffer: Hanks' balanced salt solution with magnesium and calcium (HBSS+) with 1% (wt/vol) heat-inactivated fetal bovine serum (FBS) and 0.1% (wt/vol) sodium azide. Add 5 ml FBS and 500 mg of sodium azide to a total of 495 ml of HBSS +. Filter-sterilize the buffer and store it at 4 °C for up to 1 year.
2. APC-conjugated anti-mouse IgG antibody (Polyclonal antibody, Invitrogen 17-4010-82).
3. 5 ml polystyrene round bottom 12 × 75 mm FACS tubes.
4. Non-tissue culture treated round bottom 96-well plate.
5. Flow cytometer. We have a BD Fortessa™ in our facilities. Any other cytometer can also be used.
6. Flow cytometry analysis software. We use FlowJo® for analysis.

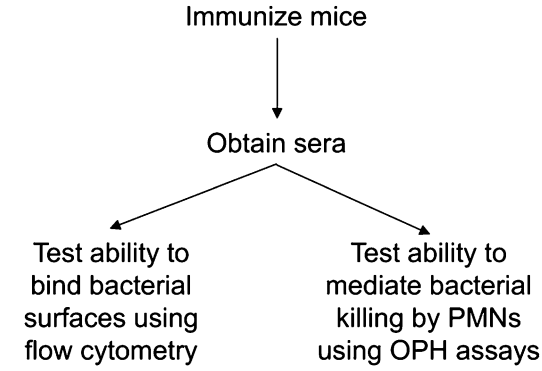

Fig. 1 Schematic depicting assay procedures

2.4 For PMN Bacterial Opsonophagocytic Killing (OPH) Assay

1. Histopaque 1119 (Sigma 11191) and Histopaque 1077 (Sigma 10771).
2. 15- and 50-ml sterile plastic conical Falcon tubes.
3. Sterile petri dishes, 60 × 15 mm.
4. Phosphate buffer saline (PBS, 1×).
5. 2% Gelatin solution: Prepare a solution of 2% by dissolving 1 g in 50 ml Hanks' balanced salt solution without magnesium and calcium (HBSS−). Bring to a boil and place at 37 °C in 50 ml tube until use. Should be used within a week of preparation.
6. +++ and −−+ buffers: HBSS+ and HBSS− respectively supplemented with 0.1% gelatin (dilute 1 ml of 2% gelatin in 19 ml corresponding HBSS). Prepare fresh each time.
7. RBC Lysis buffer: Dissolve 8.29 g of NH_4Cl (ammonium chloride), 1 g of $NaHCO_3$ (sodium bicarbonate) and 0.038 g of EDTA in a final volume of 1 L of distilled water. Filter-sterilize the buffer and store it at 4 °C.
8. RP-10 medium: RPMI-1640 medium supplemented with 10% heat-inactivated FBS.
9. RP-10 supplemented with 2 mM EDTA: Add 20 ml of 50 mM EDTA prepared in 1× PBS to a volume of 480 ml of RP-10 medium.
10. 10 ml syringe and 25-G needle.
11. Non-tissue culture treated flat bottom 96-well plate.

2.5 General Equipment

1. Incubator at 37 °C + 5% CO_2.
2. 37 °C shaker.
3. Tabletop centrifuge (we use the Eppendorf Centrifuge 5418 model to spin the bacteria at 1700 rcf and Microtainer tubes at 7607 rcf).
4. Benchtop centrifuge (we use the Beckman Coulter Allegra™ 6R model to centrifuge PMNs at 328 rcf at 4 °C).

3 Methods

This section has further been divided into subsections to include the outlined techniques for evaluating the vaccine-induced antibody response in vitro. These include culturing bacteria, immunizing mice, collecting and storing immune sera, quantifying antibody binding to bacterial surfaces using flow cytometry, isolating PMNs from mouse bone marrow, and OPH assays.

3.1 Growing Bacteria

1. A day prior, streak a blood agar pate with *S. pneumoniae* and keep overnight at 37 °C in 5% CO_2.
2. The following day, grow *S. pneumoniae* at 37 °C in 5% CO_2 in Todd-Hewitt broth supplemented with Oxyrase until the culture reaches mid-exponential phase (OD 0.7–0.8).
3. Centrifuge the culture at 1700 rcf, remove the supernatants and resuspend in the growth media with 20% (v/v) glycerol.
4. Flash freeze the cells on dry ice and transfer to −80 °C for storage.
5. Prior to use, thaw aliquots, wash and resuspend in PBS to obtain the appropriate concentration. Confirm the bacterial number by serial dilution and plating on blood agar plates. Incubate the plates at 37 °C, 5% CO_2 overnight and count the number of colony forming units (CFU) the next day [10].

3.2 Immune Sera Production

1. Prevnar 13® is heat labile and should be kept on ice at all times. Avoid exposure to high temperature or direct sunlight. Shake the vaccine vial well to disrupt any visible precipitates (*see* **Note 1**).
2. Using an 18 G needle, fill the 1.0 ml syringe with required amount of vaccine.
3. Discard the 18 G needle and cap the syringe with a 27 G needle. Place the syringe on ice and proceed with the intramuscular (*i.m.)* injection immediately.
4. *I.m.* injections require the assistance of another person. One individual should scruff the mouse near the ears and extend the hind leg to stretch it out. The second individual should wipe the area with a 70% ethanol swab and insert the needle slowly into the caudal thigh muscle, bevel up. Make sure to insert the needle pointing toward the tail (not toward the head). This helps ensure you do not hit the sciatic nerve that runs along the femur (*see* **Note 2**).
5. Slowly inject the 50 μl volume of the vaccine. Remove the needle gently and observe for any sign of bleeding or leakage.

6. Allow at least 4 weeks before bleeding the mice for immune sera. This time is required for isotype-switching of the antibodies from IgM to IgG (*see* **Note 3**).

3.3 Hyperimmune Sera Production

1. For intranasal colonization of *S. pneumoniae* thaw a frozen bacterial aliquot (Subheading 3.1) on ice.
2. Centrifuge at 1700 rcf for 5 min at room temperature (RT), discard the supernatant and wash once with 1 ml of sterile PBS to get rid of the glycerol-containing media.
3. Resuspend and dilute the bacterial pellet in sterile PBS to achieve 5×10^6 CFU/10 μl and proceed with intranasal challenge in mice (*see* **Note 4**).
4. Plate the bacterial suspension on blood agar to confirm the dosage. Perform the inoculation three times, each 7 days apart.
5. A week after three intranasal *S. pneumoniae* inoculations, vaccinate mice with Prevnar 13® as in Subheading 3.2.
6. Four weeks after Prevnar 13®, euthanize mice and collect hyperimmune sera (*see* **Note 5**).

3.4 Sera Collection and Storage

1. Euthanize the mouse using CO_2 euthanasia. Immediately, spray ethanol on the chest area and insert a 1.0 ml syringe with 25-G needle into the heart and draw blood. Alternatively, snip the portal vein and collect the blood using a pipette (*see* **Note 6**). Transfer the blood to Microtainer tubes.
2. Immediately, spin the Microtainer tubes for 2 min at 7607 rcf at room temperature in a tabletop centrifuge.
3. Transfer the sera (the clear part on top of the gel) to a sterile 15 ml falcon tube to pool the sera from all the mice.
4. Aliquot in small volumes of 50–100 μl in microcentrifuge tubes to avoid repeated freeze-thawing of large volumes of immune sera.
5. Save at −80 °C until ready to use (*see* **Note 7**).

3.5 Quantification of Antibodies Bound to the Bacterial Surface by Flow Cytometry

1. Thaw an aliquot of the *S. pneumoniae* TIGR4 strain. If available, use a bacterial strain expressing GFP-tag (*see* **Note 8**).
2. Once the aliquot of *S. pneumoniae* is thawed, spin the tube at 1700 rcf for 5 min at room temperature. Discard the supernatant. Resuspend the pellet in +++ buffer (similar buffer used for OPH assays) at a final concentration of 4×10^7 CFU/ml. Use 25 μl per well so as to have 10^6 bacteria per reaction. The amount of bacteria is important to balance feasible detection without diluting the antibodies present in the immune sera.
3. Add the 25 μl of diluted bacteria to the appropriate wells of a round bottom 96-well plate. Use different volumes of immune-sera to opsonize the bacteria. Use +++ buffer to

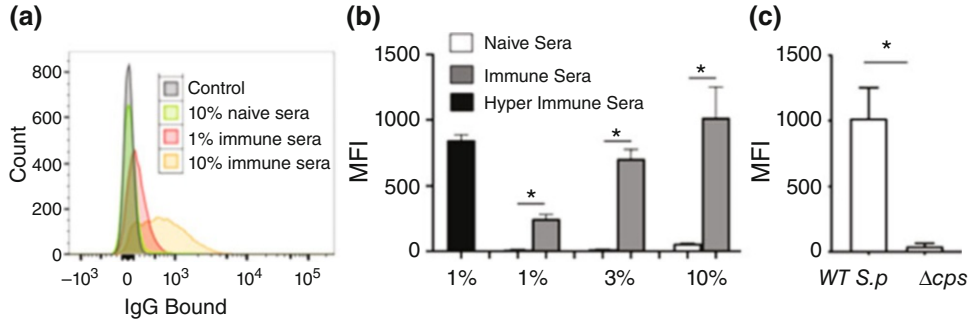

Fig. 2 IgG binding to live *S. pneumoniae* after incubation with mouse sera. Sera were collected from naïve or PCV immunized mice 4 weeks postvaccination or from hyperimmune mice. (**a**, **b**) Wild type *S. pneumoniae* TIGR4 were incubated with the indicated sera for 30 min or buffer only (control). The cells were washed and stained with fluorescently labeled anti-mouse IgG. (**a**) is a representative histogram and (**b**) is quantification of the amounts of bound antibodies determined by flow cytometry. MFI indicates the mean fluorescent intensity. (**c**) Wild type (WT) or a capsule deletion mutant (*Δcps*) *S. pneumoniae* TIGR4 were incubated with the 10% immune sera and stained with fluorescently labeled anti-mouse IgG. The MFI of bound Abs was determined by flow cytometry. Bars represent the mean ± SD. * denote significant differences between indicated groups by Student's *t*-test

bring up the volume per reaction to 50 µl (*see* **Note 9**) (Fig. 2a, b).

4. Shake the plate at 37 °C for 30 min at 78 rpm (this mimics the conditions of opsonization for the OPH assay).
5. Following incubation, wash two times with FACS buffer to remove unbound sera by adding 100 µl of FACS buffer, spinning plate at 2060 rcf at 4 °C for 5 min to pallet the bacteria and carefully remove the supernatant using a pipette.
6. To detect antibodies bound to bacteria, incubate the reaction mix with an appropriate secondary antibody. For detection of IgG we used a goat anti-mouse secondary antibody tagged with APC. Dilute the secondary antibody 1:250 in FACS buffer and add 50 µl per well.
7. Incubate the plate for 30 min on ice (in the dark). After incubation, wash twice with FACS buffer (**step 6**). Finally resuspend the pellet in 100 µl of FACS buffer and transfer the content in FACS tubes prefilled with 150–200 µl of FACS buffer. Keep cells on ice and in the dark (covered in foil) until ready to run the samples (*see* **Note 10**) (Fig. 2c).

3.6 PMN Isolation from Mouse Bone Marrow

1. Before starting: Prefill a 10 ml syringe with RP-10/EDTA, one petri dish with 5 ml RP-10, one petri dish with 5 ml 70% ethanol, and three petri dishes with 5 ml PBS. Place all items along with one 15 ml conical tube and one 50 ml conical tube in an ice bucket. Bring the Histopaque solutions to room temperature (*see* **Note 11**).

2. Euthanize mice as per your IACUC protocol. We utilize CO_2 asphyxiation followed by cervical dislocation as secondary method of euthanasia.
3. Inside the biosafety hood, spray the mouse with ethanol and cut open the abdomen just below the rib cage to snip the portal vein and collect the blood in Microtainer tubes (as explained in Subheading 3.4).
4. Make an incision of the skin in the mid-abdomen and remove the skin from the distal part of the mouse including the skin covering the lower limbs.
5. Cut off the muscles from the lower extremities using scissors and carefully cut at the hip joint, while avoiding breaking the femur head.
6. Remove the remaining muscles from the femur and tibia using a scalpel and scissors and separate the femur from the tibia at the knee joint. Be careful not break the bone ends. Place the bones in a petri dish containing ice-cold RP-10.

   From now till **step 15** all procedure must be done on ice/4 °C.
7. In a petri dish, quickly rinse each bone with ice-cold 70% ethanol followed by three washes in ice-cold sterile PBS to wash off the ethanol.
8. Inside a new petri dish, snip off the epiphyses at both ends of the bones. Remove more tissue until it becomes easy to see bones. Make sure enough is cut off from the tip to allow insertion of a needle for flushing out the marrow.
9. Using a 25-G needle and a 10 ml syringe prefilled with RP-10 and 2 mM EDTA, flush the bone marrow cells from both ends of the bone shafts into a 50 ml screw top sterile Falcon tube. Use 2 ml for each side until bones look clear and not reddish (*see* **Note 12**).
10. Use pipette to gently mix the cells and disrupt clumps.
11. Filter the cell suspension through a 100 μm filter strainer into a 50 ml tube placed on ice.
12. Centrifuge at 328 rcf for 5 min at 4 °C.
13. Lyse the red blood cells by resuspending the cell pellet in 5 ml of Lysis Buffer and mixing up and down gently using a pipette. Wait 1–2 min on ice and then neutralize with 5 ml of HBSS− (*see* **Note 13**).
14. Centrifuge for 5 min at 328 rcf at 4 °C. Aspirate out the supernatant and resuspend the pellet in 5 ml of RPMI supplemented with 10% FBS and 2 mM EDTA.
15. Centrifuge again for 5 min at 328 rcf at 4 °C.

16. Discard the supernatant and resuspend the pellet in 1 ml of sterile PBS (room temperature).
17. In a sterile 15 ml conical tube, add 3 ml of Histopaque 1119 (density, 1.119 g/ml). Very slowly overlay 3 ml of Histopaque 1077 (density, 1.077 g/ml) on the 3 ml of Histopaque 1119 (*see* Notes **14**–**16**).
18. Slowly overlay the bone marrow cells (from **step 16**) on top of the Histopaque 1077.
19. Centrifuge for 30 min at 913 rcf at 25 °C without brake (OFF) (*see* **Note 17**).
20. Collect the PMNs at the interface of the Histopaque 1119 and Histopaque 1077 layers. Collect the lower layer only as it is the one containing mature PMNs ($Ly6G^{hi}$). Make sure to place your pipette tip on top of the layer and as you draw the liquid up, rotate your pipette to collect all the cells. Transfer the cells into a new 15-ml conical tube (*see* **Note 18**).
21. Add 5 ml RP-10 and centrifuge at 328 rcf for 5 min at 4 °C.
22. Remove supernatant and resuspend the cell pellet with 5 mL RP-10. At this point, take 10 μl out and mix with 10 μl of trypan blue for counting the cell number.
23. Centrifuge the remaining volume at 328 rcf for 5 min at 4 °C.

    During, count the neutrophils using a Hemocytometer and determine their viability by trypan blue exclusion. Bright cells are alive, while cells that appear blue are cells that have lost their membrane integrity. Count under the microscope (the 4 × 5 squares filled with small squares surrounded by double lines).

    Cell numbers: Average count × 2 (df) × 10^4 × 5 (volume of cell suspension in **step 22**).

    – df is the dilution factor (10 μl of the cell suspension + 10 μl of Trypan blue dye).
    – 10^4 is to convert the cell count to cells per ml as each square has volume of 10^{-4} ml.
24. Resuspend the cells in −−+ buffer at the desired cell number. Keep the cells on ice until use. At this point, PMNs can stay on ice for 2–3 h without significant effect on their viability (*see* **Note 19**). Cell purity can be determined by staining with anti-Ly6G and CD11b antibodies [10, 16].

3.7 Opsonophagocytic Killing Assay (OPH) Using Primary Neutrophils

1. The day of the assay prepare +++ buffer (Hanks (HBSS+) with calcium and magnesium and 0.1% gelatin (*see* **Note 20**). We recommend testing each condition at least in quadruplicates (*see* **Note 21**).

2. Label the reaction plate (non–tissue culture treated round bottom 96-well plate) (*see* **Note 22**) and add +++ buffer as follows:
   (a) Add 50 µl of +++ buffer to ALL wells.
   (b) Add 40 µl of +++ buffer to No PMN wells.
   (c) Leave at room temp until PMNs are ready to be added.

   The abovementioned volumes can be adjusted as needed to scale up or down. It is crucial to keep the final volumes the same in +PMNs and No PMNs wells and across different groups.
3. Dilute mid-logarithmic phase grown *S. pneumoniae* to 10^5/ml in +++ buffer (*see* **Notes 23–25**).
4. For opsonization of *S. pneumoniae*, mix bacteria with the target mouse sera. To achieve 3% sera in the final reaction, mix 3 µl sera with 10 µl (10^3) bacteria. A master mix should be prepared to use in replicate wells taking into account +PMN and No PMN controls. Always prepare for three extra wells. For example, if you have 12 experimental wells total, prepare for 15 wells by mixing 45 µl sera with 150 µl of diluted bacteria in a microcentrifuge tube.
5. Incubate the reactions at 37° C for 30 min in a shaking incubator with the speed set at 78 rpm (*see* **Note 26**). The ratio of bacteria to immune sera in the final reaction should be adjusted as needed (*see* **Note 27**).
6. During the last 5 min of above incubation, add 10^5 PMNs (40 µl) to the "PMN wells" of your reaction plate containing +++ buffer. Leave the plate at room temperature till the bacteria are ready to be added.
7. Once *S. pneumoniae* are opsonized, add 13 µl of bacteria/sera mix to all the wells (PMNs and No PMNs). Use of multichannel pipette is advised for speed and to avoid technical error in dispensing equal number of bacteria.
8. Incubate the plate at 37 °C shaking (78 rpm) for 40 min. During this incubation, titer the bacteria/sera mix by plating serial dilutions on blood agar plates to estimate the bacterial "Input."
9. Once the 40 min incubation is over (**step 8**), place the reaction plate on ice for ~2 min to stop the reaction. Immediately afterward, plate 10 µl volumes of the reaction mix on blood agar plates using the drip method. Plating the zero dilution (directly from the plate) should allow for countable colonies. The reaction mix can also be diluted and then plated if more than 10^3 bacteria per reaction are being used (*see* **Note 28**).
10. Incubate the plates overnight at 37 °C + 5% CO_2. Next day, count the number of colonies (*see* **Notes 29–31**) (Fig. 3).

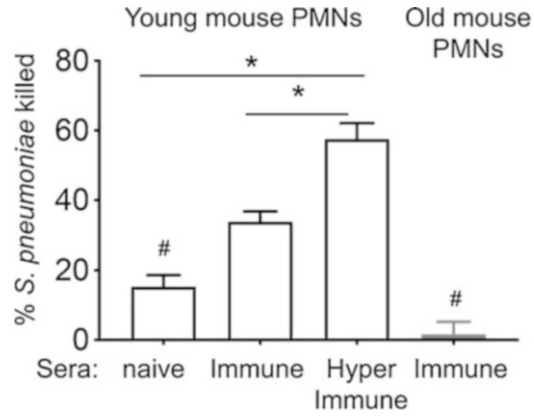

Fig. 3 Immune sera significantly boost the killing of *S. pneumoniae* by PMNs. PMNs were isolated from the bone marrow of young (10–12 weeks) or old (18 months) C57BL/6 mice. The ability of PMNs to kill *S. pneumoniae* TIGR4 preopsonized with the indicated sera was determined. Percent bacterial killing was determined with respect to a no PMN control. Data shown are pooled from at least three separate experiments where each condition was tested in triplicate ($n = 3$ technical replicates) per group. Bars represent the mean ± SD. # indicate significant differences from immune sera + young PMNs and * indicate significant differences between indicated groups by Student's *t*-test

$$\text{Plot\%Bacterial killing as} : 100 - \text{\%bacterial survival}$$

$$\text{\%Bacterial survival} = \frac{\text{Average no.of bacterial CFU in} + \text{PMNs condition}}{\text{Average no.of bacterial CFU in No PMNs condition}} \times 100$$

4 Notes

1. Wherever possible, prepare the syringes just prior to vaccination. The vaccine needs to be shaken well prior to injection and works best if prepared fresh. Do not allow the polysaccharides to precipitate and settle in the syringe. This happens if large volumes are preloaded in a single syringe or there is a delay in administering the vaccine. If precipitates form, give a gentle tap to the syringe to dissipate these.
2. Regardless of the method used for intramuscular injections, care must be taken to avoid hitting and injuring the sciatic nerve which runs along the length of the femur. The mouse can be anaesthetized or manually restrained by another person. Aim to direct the needle caudally rather than cranially, into the caudal thigh muscles. The mouse needs to be properly restrained for this injection as any loose hold could cause injury to the muscles or the nerve. A restrainer can also be used for *i*.

m. injections. Gently pull out the leg to be injected through the restrainer and locate the caudal thigh muscle. While holding the foot firmly, swab the site with 70% ethanol, aspirate and inject the bacterial suspension with the needle bevel up.

3. The same protocol can be used for immunizing mice with Pneumovax®. *S. pneumoniae* TIGR4 used here, is a serotype 4 strain covered by both vaccines.
4. For intranasal inoculation, restrain the mouse without anesthesia in a way and position similar to during intraperitoneal (*i.p.*) injections. Make sure their head is held steady and wait for normal breathing. Pipet 10 μl of inoculum. Make a drop (with approximately half the volume of the inoculum) on the tip of pipette and deliver into a nostril. Do not touch the nose with tip of pipette since it will induce sneezing. Approach the mouse from the front, opposite to the eyes so that the mouse does not see you (otherwise the mice will try to eat the drop). Deliver the rest to the other nostril the same way. Alternatively, the whole inoculum can be delivered in a single drop between the two nostrils.
5. For timing consideration, hyperimmune sera is achieved by intranasally vaccinating the mice with live bacteria (once weekly for 3 consecutive weeks), followed by immunizing with Prevnar 13®. The procedure requires 4 weeks for the successive treatments followed by 4 weeks until sera collection and produces very high titers of anti-pneumococcal IgG and IgM [17]. If higher antibody titers are desired, heat-killed bacteria can also be injected (*i.p.*) 4 weeks following Prevnar 13® and mice can be bled a week after the injection. We typically prepare hyperimmune sera once a year using 40 mice which yields 28 ml of sera. The antibody titer in the hyperimmune sera can be determined by ELISA as previously described [17].
6. While euthanizing mice for immune sera, it is advised to handle one animal at a time. Once the blood has been transferred to Microtainer tube, proceed with the next mouse. Euthanizing multiple mice at one time could cause delay and coagulation of blood. Do not allow the blood or the sera to sit for a long time in the Microtainer tubes. Once spun, immediately transfer to a 15-ml conical tube or a 1.5 ml microfuge tube.
7. Make sure the sera are saved at −80 °C. We have noticed that if the sera are saved at higher temperatures (e.g., −20 °C), then with time they become toxic to the bacteria and their ability to induce opsonophagocytic killing decreases.
8. This assay can be used with *S. pneumoniae* strain not expressing GFP (or other fluorescent) tags. However, fluorescent tagging can help with gating on the bacteria during flow cytometry analysis. For pneumococcal strains lacking fluorescent proteins,

it is possible to use cell-permeable amine-binding dyes. Bacterial cells are considerably smaller than mammalian cells and therefore the side scatter and forward scatter on the flow cytometer have to be adjusted accordingly. Operation of flow cytometers is beyond the scope of this chapter.

9. This procedure is adapted from a method originally published by Moor et al. [18] and tailored to quantify the amount of antibodies bound on the surface of *S. pneumoniae* TIGR4 strain opsonized with different sera. We recommend using 1%, 3% or 10% of immune-sera (of the total reaction volume; i.e., 0.5, 1.5, or 5 μl correspondingly per well). These dilutions provide a dose-dependent response and can be used to parse out differences between immune and naïve sera (Fig. 2a, b). No increase in the amounts of surface-bound antibodies is seen above 10% using immune sera indicating the signal is saturated. Sera from naïve-unimmunized mice should be included as a control for the baseline amounts of cross-reactive antibodies found in the sera. Hyperimmune sera (Subheading 3.3) can be used as an internal control for interassay variation.
10. The use of necessary controls to exclude the possibility of nonspecific binding is advised. To ensure there is no cross-reactivity between the secondary antibody and bacterial surface, stain unopsonized bacteria (bacteria not incubated with any sera) with the secondary antibody alone. To determine the specificity of the Prevnar 13® vaccine response, we also used an *S. pneumoniae* capsule deletion mutant (Δcps) and detected no fluorescent signal compared to the wild-type TIGR4 strain indicating the specificity of the antibodies against surface polysaccharide (Fig. 2c).
11. To isolate bone-marrow derived PMNs we use a Histopaque density based centrifugation method using a protocol established by Swamydas and Lionakis [16] with very few modifications. Make sure to cite the Swamydas and Lionakis reference in publications using the Histopaque PMN isolation protocol. Other density based centrifugation methods using Percoll [19] or negative/positive selection methods using commercial kits may also be used.
12. Blanching of bones indicates that the bone marrow cells have been flushed out. Use approximately 10 ml of media to flush a set of femur–tibia pairs. Adding EDTA to the medium is important to prevent cell clumping.
13. Following isolation of cells from bone marrow, avoid using any buffer or media containing calcium (Ca^{++}). This is to avoid premature neutrophil activation prior to bacterial challenge.
14. Histopaque 1119 and Histopaque 1077 should be warmed to 18–26 °C before use.

15. Prepare gradients immediately before use as preparing the gradient in advance will result in diffusion between the two layers and suboptimal neutrophil purity and recovery.
16. Overlaying the bone marrow cell suspension over Histopaque 1077 needs to be done slowly in order to avoid disturbing the interface between the cells and Histopaque 1077. To do so, dispense drop by drop in order to avoid mixing the two densities, which will preclude cell separation during centrifugation.
17. Centrifugation of the gradient at room temperature is critical and essential for effective separation of the neutrophils.
18. Neutrophils are typically >95% viable and >90% pure as determined by FACS analysis. The typical yield of neutrophils from the bone marrow (i.e., two femur and two tibia bones) of an uninfected 8- to 12-week-old C57BL/6 mouse is ~4–6 million cells. In a situation when two distinct layers of cells are not seen, the researcher is advised to collect the entire cell layer in a new 15 ml conical tube and wash the cells once in RPMI + 10% FBS and resuspend in 1.0 ml of PBS and repeat the Histopaque step.
19. We usually resuspend the PMNs in −−+ media (HBSS Ca/Mg-free with 0.1% gelatin) as the glucose in the HBSS− maintains PMN viability while the gelatin prevents cell clumping and the buffer is Ca/Mg-free to prevent activation.
20. For OPH assay, prepare fresh 2% gelatin and all the buffers on the day of experiment. Any possible contamination in previously prepared buffers could prematurely activate PMNs.
21. This assay can be technically challenging as PMNs can be activated easily which can lead to variability. We recommend to test each condition in at least quadruplicates, particularly for beginners.
22. Avoid using any tissue culture treated plastic, as this may cause PMNs to adhere and get activated.
23. A standard number of opsonized bacteria are added to the reaction mix containing PMNs and the survival percentage is calculated at the end of the reaction to estimate the killing efficiency. In these assays, we typically aim for 10^3 *S. pneumoniae* per 10^5 PMNs and 3% sera in a 100 μl reaction. These conditions have been optimized for the following *S. pneumoniae* strains: TIGR4, 19F, 23F, and D39.
24. *S. pneumoniae* aliquots frozen down at midlogarithmic phase or grown the day of the assay till mid-logarithmic phase can both be used for this assay.

25. The MOI can be increased to 1.0 and efficient killing will still occur. However, high MOI with virulent *S. pneumoniae* strains like TIGR4 should be avoided because of the risk of neutrophil death due to the release of the pore-forming toxin pneumolysin by the bacteria [20]. We recommend that the MOI be adjusted depending upon the bacterial strain used.
26. Do not exceed the opsonization incubation more than 30 min. If kept longer, the bacteria could start replicating and this could affect the MOI. Alternatively, you can skip the preopsonization over time step and mix the sera and bacteria, then immediately proceed to adding the mix to the PMNs.
27. An alternative approach when comparing OPH efficacy of the sera is to test a range of percentages as for the bacterial binding assay. We found a dose dependent increase in bacterial killing by PMNs when the sera were increased from 1% to 3% to 10%. However, we found that adding more sera did not increase bacterial killing and in fact using >10% sera triggered bacterial growth and impaired their killing by PMNs.
28. Add equal volume of 40% glycerol in THY broth to each well and store the plate at −20 °C. This is helpful in case there is a need to replate the reaction conditions for any reason (e.g., bacterial CFU too high to be counted accurately).
29. These assay parameters allow for the differentiation in opsonophagocytic capacity of different immune vs. naive sera (Fig. 3). This assay, when using the same opsonic conditions, further allows the testing of intrinsic PMN function. For example, PMNs from old mice have an intrinsic decline in antibacterial function as they fail to kill *S. pneumoniae* as efficiently as PMNs from young counterparts, even when the bacteria are opsonized with same sera (Fig. 3).
30. Hyperimmune sera can be used as "in-house" standard reference sera for the OPH assays as well as the antibody bacterial binding assays. Both bacterial killing and antibody binding obtained by using hyperimmune sera can be set to 1% (or 100%) and can then be used to compare the opsonophagocytic and bacterial binding efficiency of the test-sera.
31. The same assay can be used with PMNs isolated from any other source such as tissue or circulating PMNs [21].

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References

1. Kadioglu A, Weiser JN, Paton JC, Andrew PW (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 6:288–301
2. Chong CP, Street PR (2008) Pneumonia in the elderly: a review of the epidemiology, pathogenesis, microbiology, and clinical features. South Med J 101:1141–1145; quiz 1132, 1179
3. Chen WH, Kozlovsky BF, Effros RB, Grubeck-Loebenstein B, Edelman R, Sztein MB (2009) Vaccination in the elderly: an immunological perspective. Trends Immunol 30:351–359
4. Adderson EE (2001) Antibody repertoires in infants and adults: effects of T-independent and T-dependent immunizations. Springer Semin Immunopathol 23:387–403
5. Simell B, Vuorela A, Ekstrom N, Palmu A, Reunanen A, Meri S, Kayhty H, Vakevainen M (2011) Aging reduces the functionality of anti-pneumococcal antibodies and the killing of *Streptococcus pneumoniae* by neutrophil phagocytosis. Vaccine 29:1929–1934
6. Song JY, Moseley MA, Burton RL, Nahm MH (2013) Pneumococcal vaccine and opsonic pneumococcal antibody. J Infect Chemother 19:412–425
7. Romero-Steiner S, Musher DM, Cetron MS, Pais LB, Groover JE, Fiore AE, Plikaytis BD, Carlone GM (1999) Reduction in functional antibody activity against *Streptococcus pneumoniae* in vaccinated elderly individuals highly correlates with decreased IgG antibody avidity. Clin Infect Dis 29:281–288
8. Usinger WR, Lucas AH (1999) Avidity as a determinant of the protective efficacy of human antibodies to pneumococcal capsular polysaccharides. Infect Immun 67:2366–2370
9. Romero-Steiner S, Frasch CE, Carlone G, Fleck RA, Goldblatt D, Nahm MH (2006) Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. Clin Vaccine Immunol 13:165–169
10. Bou Ghanem EN, Clark S, Roggensack SE, McIver SR, Alcaide P, Haydon PG, Leong JM (2015) Extracellular adenosine protects against *Streptococcus pneumoniae* lung infection by regulating pulmonary neutrophil recruitment. PLoS Pathog 11:e1005126
11. Garvy BA, Harmsen AG (1996) The importance of neutrophils in resistance to pneumococcal pneumonia in adult and neonatal mice. Inflammation 20:499–512
12. Hahn I, Klaus A, Janze AK, Steinwede K, Ding N, Bohling J, Brumshagen C, Serrano H, Gauthier F, Paton JC, Welte T, Maus UA (2011) Cathepsin G and neutrophil elastase play critical and nonredundant roles in lung-protective immunity against *Streptococcus pneumoniae* in mice. Infect Immun 79:4893–4901
13. Rolston KV (2001) The spectrum of pulmonary infections in cancer patients. Curr Opin Oncol 13:218–223
14. Alonso De Velasco E, Dekker BA, Verheul AF, Feldman RG, Verhoef J, Snippe H (1995) Anti-polysaccharide immunoglobulin isotype levels and opsonic activity of antisera: relationships with protection against *Streptococcus pneumoniae* infection in mice. J Infect Dis 172:562–565
15. Gerdts V, Littel-van den Hurk S, Griebel PJ, Babiuk LA (2007) Use of animal models in the development of human vaccines. Future Microbiol 2:667–675
16. Swamydas M, Lionakis MS (2013) Isolation, purification and labeling of mouse bone marrow neutrophils for functional studies and adoptive transfer experiments. J Vis Exp (77): e50586. https://doi.org/10.3791/50586
17. Bou Ghanem EN, Maung NHT, Siwapornchai N, Goodwin AE, Clark S, Munoz-Elias EJ, Camilli A, Gerstein RM, Leong JM (2018) Nasopharyngeal exposure to *Streptococcus pneumoniae* induces extended age-dependent protection against pulmonary infection mediated by antibodies and CD138 (+) cells. J Immunol 200:3739–3751
18. Moor K, Fadlallah J, Toska A, Sterlin D, Balmer ML, Macpherson AJ, Gorochov G, Larsen M, Slack E (2016) Analysis of bacterial-surface-specific antibodies in body fluids using bacterial flow cytometry. Nat Protoc 11:1531–1553
19. Dalia AB, Standish AJ, Weiser JN (2010) Three surface exoglycosidases from *Streptococcus pneumoniae*, NanA, BgaA, and StrH, promote resistance to opsonophagocytic killing by human neutrophils. Infect Immun 78:2108–2116
20. Zysk G, Bejo L, Schneider-Wald BK, Nau R, Heinz H (2000) Induction of necrosis and apoptosis of neutrophil granulocytes by *Streptococcus pneumoniae*. Clin Exp Immunol 122:61–66
21. Bou Ghanem EN, Clark S, Du X, Wu D, Camilli A, Leong JM, Meydani SN (2015) The alpha-tocopherol form of vitamin E reverses age-associated susceptibility to *Streptococcus pneumoniae* lung infection by modulating pulmonary neutrophil recruitment. J Immunol 194:1090–1099

Chapter 34

Viral Infectivity Quantification and Neutralization Assays Using Laser Force Cytology

Colin G. Hebert, Keya L. Rodrigues, Nicole DiNardo, and Anna-Barbara Hachmann

Abstract

Despite the widespread need to assess cell-based viral infectivity during vaccine development and production, as well as viral clearance monitoring and adventitious agent testing for viral safety, traditional methods, including the end-point dilution assay (TCID50) and viral plaque assay, are slow, labor-intensive, and can vary depending upon the skill and experience of the user. LumaCyte's Radiance® instrument uses Laser Force Cytology™ (LFC), a combination of advanced optics and microfluidics, to rapidly analyze the viral infectivity of cell populations in a quantitative fashion. LFC applies optical and fluidic forces to single cells in order to measure their intrinsic biophysical and biochemical properties without the use of stains, antibodies or fluorescent labels. These properties, including refractive index, change with a wide variety of biological phenomena, including viral infection, cell differentiation, activation, size, and cytoskeletal stiffness. Here, we present the experimental design and methods to use LFC data to facilitate rapid and robust infectivity measurements for a variety of applications including initial titer measurement (TCID50 replacement), in-process infectivity (e.g., bioreactor monitoring), and viral neutralization (PRNT replacement).

Key words Viral infectivity, Viral titration, TCID50, Plaque assay, Laser force cytology, Vaccines, Neutralization assay

1 Introduction

The quantification of infectious virus is an important analytical need across the biopharmaceutical industry, whether it be for vaccine research and development, vaccine production [1, 2] or the detection of adventitious viruses to ensure the safety of biologics, (e.g., monoclonal antibodies) [3]. Widely accepted methods include the plaque and TCID50 assays as well as antibody-based methods [4–6]. However, plaque and TCID50 assays are labor intensive, lengthy, and subjective as they often rely on individual interpretation of results [7, 8]. As such, a large number of dilutions and replicates are needed in order to obtain statistically relevant data [9, 10].

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Laser Force Cytology (LFC) is a unique combination of fluidic and optical forces to analyze single cells without the use of labels [11]. Cells are delivered through a microfluidic channel and exposed to optical forces, which arise when a sufficient number of photons from a laser reflect or refract through a cell or particle, imparting a fraction of their momentum as they do so. The magnitude of these forces is correlated with the properties or changes in the intrinsic biochemical and biophysical properties of the cells. Specifically, subtle cellular changes in membranes, cytoplasmic organelles, and nuclear features manifest themselves as changes in the velocity (optical force), size, shape, position of cells, and dozens of other optical and hydrodynamic force related parameters, as they pass through a region of laser photon pressure. Differences in cell biochemistry, morphology, and deformability (cytoskeletal changes), which are often associated with viral infection, cancer, sepsis, and other diseases, give rise to detectable differences in optical force and deformability which are quantitated by Radiance. The ability to measure so many biologically relevant parameters without the use of labels is incredibly powerful and provides opportunities for multivariate data analysis and machine learning for advanced detection and characterization [11, 12]. Here we present the application of LFC to the quantification of infectious virus in two variations, as well as an LFC-based viral neutralization assay.

The first section describes a rapid replacement for a standard infectivity assay in which a correlation is created between LFC data and a viral titer measured in a traditional way such as a TCID50. Once established, the LFC based assay reduces the time, labor, and variability when compared to a traditional assay, while at the same time removing subjectivity and automating the infectivity calculation. The second assay provides the capability of analyzing cells with LFC from an ongoing process and calculating the infectivity directly, once a correlation has been established between the LFC measurements and infectivity assays [11]. The second case represents often hundreds of fold reduction in time, allowing for near real-time infectivity determination. This drastic reduction in the time needed for in-process infectivity measurements has numerous benefits, including harvest time optimization in order to improve yield, purification tuning in order to maximize efficiency, and rapid process development in order to reduce the time needed to bring vaccines and other viral based products to market. The third section describes an LFC based neutralization assay that can provide results similar to the plaque reduction neutralization test (PRNT) but with reduced time and labor and without the need to optimize an overlay medium. In addition, a labeling antibody is not needed, reducing the assay development time for new and emerging threats, for which antibodies have not yet been developed, and improving the speed at which valuable vaccines can be developed for emerging threats. Schematics for each of the three methods are shown in Fig. 1.

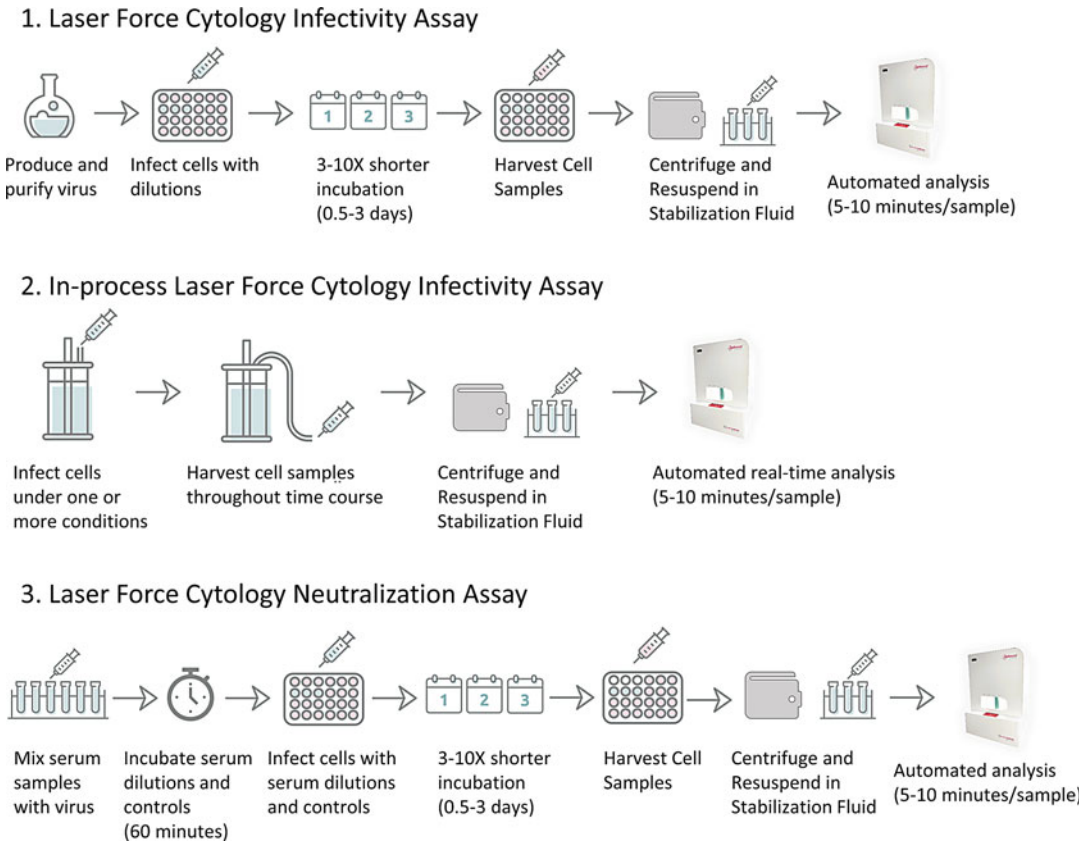

Fig. 1 Schematic outlining the experimental steps for each of the three laser force cytology assays described in this study. The laser force cytology infectivity assay (1) measures the viral titer of a stock and serves as a replacement for traditional methods such as the plaque assay or TCID50. The in-process laser force cytology infectivity assay (2) provides the capability of analyzing cells with LFC from an ongoing process and calculating the infectivity directly based on a known correlation. Finally, the laser force cytology neutralization assay (3) can be used to measure the ability of serum or other agents to neutralize a virus, similar to the more traditional plaque reduction neutralization test

2 Materials

2.1 Infectivity Assay Replacement with Adherent HEK-293 Cells (Initial Titer)

1. Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS; 1×).
2. Cell dissociation reagent such as TrypLE™ or Accutase® (*see* **Note 1**).
3. Cell culture medium (*see* **Note 2**).
4. Viral stock of known titer (*see* **Note 3**).
5. LumaCyte Stabilization Fluid (LumaCyte SF1800).
6. Paraformaldehyde solution (optional—*see* **Note 4**): 4% in 0.1 M phosphate buffered saline, pH 7.4.

7. 24-Well tissue culture treated plates.
8. LumaCyte 96-well plate (LumaCyte WP3110).
9. LumaCyte Radiance instrument.

2.2 In-Process Infectivity Assay with Sf9 Cells (Real-Time Titer)

1. Cell culture medium (*see* **Note 2**).
2. Viral stock of known titer (*see* **Note 3**).
3. LumaCyte Stabilization Fluid (LumaCyte SF1800).
4. Paraformaldehyde solution (optional—*see* **Note 4**): 4% in 0.1 M phosphate buffered saline, pH 7.4.
5. 125 mL shake flasks.
6. 6-Well plates.
7. LumaCyte 96-well plate (LumaCyte WP3110).
8. LumaCyte Radiance instrument.

2.3 Viral Neutralization Assay with Vero Cells

1. Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS; 1×).
2. Cell dissociation reagent such as TrypLE or Accutase (*see* **Note 1**).
3. Cell culture medium (*see* **Note 2**).
4. Viral stock of known titer (*see* **Note 3**).
5. LumaCyte Stabilization Fluid (LumaCyte SF1800).
6. Paraformaldehyde solution (optional—*see* **Note 4**): 4% in 0.1 M phosphate buffered saline, pH 7.4.
7. 24-Well tissue culture treated plates.
8. LumaCyte 96-well plate (LumaCyte WP3110).
9. LumaCyte Radiance instrument.

3 Methods

The following sections provide example protocols for developing an infectivity assay with HEK-293 cells, an in-process or real-time infectivity assay with Sf9 cells, and a viral neutralization assay with Vero cells. Each of the three assays can be used with different cell types, viruses, and growth regimes (i.e., adherent vs. suspension), by adapting these protocols as needed.

3.1 Infectivity Assay Replacement with Adherent HEK-293 Cells (Initial Titer)

1. Maintain a working stock of cells using standard cell culture techniques. It is important to monitor the growth and passage number in order to ensure consistent results.
2. On the day of seeding, detach cells from their growth vessel using standard techniques for that particular cell line and the manufacturer's instructions for the dissociation reagent. For

example, in the case of adherent HEK-293 cells (ATCC® CRL-1573) and TrypLE cultured in a T-75 flask:

(a) Aspirate the cell culture medium and then gently wash the cell monolayer with 5 mL of DPBS.

(b) Remove the DPBS and add 3 mL of TrypLE.

(c) Incubate for 5 min at 37 °C or until the cells have visibly detached.

(d) If needed, gently tap the vessel to dislodge the cells.

(e) Add an appropriate volume of cell culture medium to resuspend the cells, pipetting up and down to break up any clumps that are present.

3. Once the cells are resuspended, perform a count in order to determine the dilution required.
4. Seed cells at 150,000 cells/well in a 24-well plate (*see* **Note 5**).
5. Incubate overnight (*see* **Note 6**), and then infect cells the following day using the standard infection procedure for that virus (*see* **Note 7**). Using a virus stock with known titer, infect cells with a range of MOI values (*see* **Note 8**). Start with an MOI as high as practical (typically between 1 and 10) and infect with an additional 5–6 MOI dilutions. For example, infect with MOI values of 1.0, 0.1, 0.01, 0.001, 0.0001, and 0.00001. If infecting with an initially unknown stock, it is recommended to start with a dilution of 10^{-2} and 6 additional dilutions. Sacrificing one or two wells in order to get an accurate cell count prior to infection is recommended to improve the accuracy of the calibration curve.
6. Each day following infection, observe the cells. Once there are visible cytopathic effects at the highest virus concentration, harvest the cells at all MOIs as detailed below and analyze using Radiance.
7. On the day of harvest, detach cells using TrypLE or Accutase per the manufacturer's protocol (e.g., with 200 μL of dissociation solution per well of a 24-well plate, incubating for 5–7 min). In some cases, it is beneficial to save the cell supernatant rather than discarding it as it may contain floating infected cells. The supernatant can be combined with the dissociated cells to create a complete picture of the infected cell population.
8. If the cell concentration is expected to be >1,000,000 cells/mL, it is recommended to count the cells after they have been harvested to determine the volume of LumaCyte Stabilization Fluid required to dilute the cells to an optimal concentration of ~600,000 cells/mL (*see* **Note 9**).

9. If large cell aggregates or clumps are expected, filter cells through a 50–70 μm filter or cell strainer.
10. Centrifuge the cells for 5 min at a speed of 150–200 × *g* (*see* **Note 10**).
11. Aspirate the supernatant and resuspend the cells in an appropriate volume of Stabilization Fluid at ~600,000 cells/mL (*see* **Note 11**). If fixation will be necessary, add paraformaldehyde to a final concentration of 0.5%. Prepare the mixture of paraformaldehyde and Stabilization Fluid in a conical tube before adding to cells (*see* **Note 12**).
12. Add 200 μL of each sample to the LumaCyte 96-well plate. Place the plate in the Radiance instrument for 15 min before initiating analysis. Please refer to the Radiance +Autosampler Quick Start Guide or User Manual for additional details on operating the Radiance instrument and data analysis. The Radiance measurements will be correlated with the initial amount of virus added to each condition (i.e., the MOI).
13. Store cells at 4 °C if not analyzing immediately. Do not freeze.
14. Once an initial virus titer correlation has been established between the Radiance and traditional methods, the Radiance software can be programmed to provide virus titer measurements in subsequent assays.

3.2 In-Process Infectivity Assay with Sf9 Cells (Real-Time Titer)

1. Seed cells following standard cell culture techniques. For Sf9 suspension cells, seed cells in a 125 mL shake flask at 800,000 cells/mL in 30 mL growth medium at 26–28 °C and 125–150 rpm.
2. Culture cells until they have reached a density between 1,000,000–2,000,000 cells/mL and transfer cells to 6-well plates at 500,000 cells/mL in 3 mL. After 2 h, infect at a range of MOI values using a virus stock of known titer. Start with an MOI as high as practical (typically between 1 and 10) and infect with an additional 5–6 MOI dilutions. For example, infect with MOI values of 1.0, 0.1, 0.01, 0.001, 0.0001, and 0.00001. If infecting with an initially unknown stock, it is recommended to start with a dilution of 10^{-2} and 6 additional dilutions (*see* **Notes 5** and **8**).
3. At the desired time points (generally 1–3 days post infection), determine the cell density of 2–3 different conditions and harvest a sufficient volume to achieve a concentration of ~600,000 cells/mL (*see* **Note 9**).
4. Centrifuge cells at a speed of 200 × *g* for 5 min.
5. For each condition, save a separate sample to determine the titer using the comparative method. If the virus is secreted, save a supernatant sample. If the virus is cell-associated, save a

sufficiently large cell sample in order to determine the titer (*see* **Note 13**).

6. Resuspend the cells in an appropriate volume of Stabilization Fluid in order to achieve the optimal cell count of ~600,000 cells/mL.
7. Add 200 μL of each sample to the LumaCyte 96-well plate. Place the plate in the Radiance instrument for 15 min before initiating analysis. Please refer to the Radiance +Autosampler Quick Start Guide or User Manual for additional details on operating the Radiance instrument and data analysis. The Radiance measurements can be correlated with the titer of the supernatant or cell samples taken at the same time after they have been quantified using comparative methods.
8. The Radiance software can be programmed to provide virus titer measurements in subsequent assays.

3.3 LFC Viral Neutralization Assay with Vero Cells

1. Prior to developing a neutralization assay with LFC, it is necessary to develop an infectivity assay following the procedure described in Subheading 3.1. This will allow for the optimization of MOI and harvest time. These parameters should be selected to maximize the dynamic range of the assay in order to quantify the largest possible range of serum neutralizing concentrations.
2. Maintain a working stock of cells using standard cell culture techniques. It is important to monitor the growth and passage number in order to ensure consistent results.
3. On the day of seeding, detach cells from their growth vessel using standard techniques for that particular cell line and the manufacturer's instructions for the dissociation reagent. For example, in the case of Vero cells (ATCC CCL-81) and TrypLE cultured in a T-75 flask:
   (a) Aspirate the cell culture medium and gently wash the cell monolayer with 5 mL of DPBS.
   (b) Remove the DPBS and add 3 mL of TrypLE.
   (c) Incubate for 5 min at 37 °C or until the cells have visibly detached.
   (d) If needed, gently tap the vessel to dislodge the cells.
   (e) Add an appropriate volume of cell culture medium to resuspend the cells, pipetting up and down to break up any clumps that are present.
4. Once the cells are resuspended, perform a count in order to determine the dilution required.
5. Seed cells at 125,000 cells/well in a 24-well plate (*see* **Note 14**).

6. Incubate overnight.
7. Prior to infection, count 2–3 wells to determine the amount of virus that should be added in order to achieve the desired MOI.
8. For each condition of virus and serum samples (not including the media-only controls, serum-only controls, and virus-only controls), prepare several serum dilutions in a volume of 100 μL (*see* **Note 15**) to be added to a volume of 100 μL containing the appropriate amount of virus to achieve the desired MOI.
9. Add each of the 100 μL serum dilutions to a 100 μL virus sample and incubate for 60 min at 37 °C (*see* **Note 16**). Prepare an appropriate 200 μL volume for each control: media only for the media-only control, serum in media (no virus) for the serum-only control, and virus in media (no serum) for the virus-only control.
10. After the neutralization incubation period, remove 200 μL medium from each well of the 24-well plate(s) prepared in **step 5** and replace it with the 200 μL volume of the control or test sample prepared in **step 9**.
11. Incubate the cells at 37 °C for the previously determined amount of time.
12. On the day of harvest, detach cells using TrypLE or Accutase per the manufacturer's protocol (e.g., with 200 μL of dissociation solution per well of a 24-well plate, incubating for 5–7 min). In some cases, it is beneficial to save the cell supernatant rather than discarding it as it may contain floating infected cells. The supernatant can be combined with the dissociated cells to create a complete picture of the infected cell population.
13. If the cell concentration is expected to be >1,000,000 cells/mL, it is recommended to count the cells after they have been harvested to determine the volume of LumaCyte Stabilization Fluid required to dilute the cells to an optimal concentration of ~600,000 cells/mL (*see* **Note 9**).
14. If large cell aggregates or clumps are expected, filter cells through a 50–70 μm filter or cell strainer.
15. Centrifuge the cells for 5 min at a speed of 150–200 × *g* (*see* **Note 10**).
16. Aspirate the supernatant and resuspend the cells in an appropriate volume of Stabilization Fluid at ~600,000 cells/mL (*see* **Note 11**). If fixation will be necessary, add Paraformaldehyde to a final concentration of 0.5%. Prepare the mixture of Paraformaldehyde and Stabilization Fluid in a conical tube before adding to cells (*see* **Note 12**).

17. Add 200 μL of each sample to the LumaCyte 96-well plate. Place the plate in the Radiance instrument for 15 min before initiating analysis. Please refer to the Radiance +Autosampler Quick Start Guide or User Manual for additional details on operating the Radiance instrument and data analysis. Either the media-only or serum-only controls will be considered 100% neutralization, while the virus-only controls will be considered 0% neutralization. The neutralizing values of the test samples containing virus and serum will be determined based on where they fall between these two bounding values.
18. Store cells at 4 °C if not analyzing immediately. Do not freeze.

4 Notes

1. If working with adherent cultures, cells will need to be detached using an appropriate dissociation buffer. In general, a gentler enzyme-based preparation such as TrypLE or Accutase yields better results than standard Trypsin.
2. A strong correlation can be obtained with various types of cell culture media, including serum-containing and serum-free. It is recommended to be consistent with the medium that is used for growth or infection whenever possible as this can help minimize changes as a result of media composition rather than viral infection.
3. A viral stock of known titer is needed to create a correlation between Radiance measurements and a traditional viral infectivity assay. In general, a correlation can be made between various types of assays, including TCID50, plaque assay, or fluorescent foci assays.
4. Fixation of cells with paraformaldehyde can in some cases improve the consistency of results. It is best to determine this empirically for a given cell and virus combination.
5. The precise cell density can be optimized based on the cell type and virus. The value in this example represents a cell density that can be used for next-day infection. Seed sufficient wells for three replicates per condition, including MOI 0 as a negative control, for each time point, as well as three wells to count prior to infection. When developing an initial LFC-based assay, use the incubation time of the plaque or TCID assay as a guide. In general, the time post infection can be shortened 3–5 times. Thus, a 6–8-day plaque assay can generally be shortened to 2 days with an LFC-based assay.
6. If desired, cells can sometimes be seeded and infected on the same day. In this case, the cell density should be increased and the cells allowed to attach for an appropriate period of time,

generally 1–3 h. For same-day infection, the cell count at infection can be assumed to be the same as the seeding density.

7. Various methods can be used for infection. In some cases, a medium exchange is required in which case the virus can be mixed into the replacement media. At other times, the volume of virus required is sufficiently small that it can be added directly to the culture. A third variation includes the reduction of culture medium volume, addition of the viral inoculum for a set period of time (e.g., 1 h), removal of the virus and addition of fresh medium.
8. If the precise titer is not known, dilutions can instead be used and the Multiplicity of Infection (MOI) calculated once the titer has been determined.
9. Counting the negative control, the highest virus concentration, and a dilution somewhere in the middle will help you estimate the volumes of stabilization fluid needed for the other conditions. If the cell count is below the desired concentration of 600,000 cells/mL, and further concentration through centrifugation is not possible, cells can be resuspended at a lower concentration keeping in mind the 200 μL volume required to run on Radiance.
10. The centrifugation speed can be adjusted as needed depending on the cell line.
11. Stabilization fluid should be at room temperature before adding to cells.
12. It is recommended to prepare a fresh stock every time.
13. In order to develop a method for real-time infectivity determination, a titer must be determined for each sample. This allows for the correlation between the Radiance data and the titer of each sample. If desired, correlations can also be made between other quantities, for example virally driven protein production.
14. The precise cell density can be optimized based on the cell type and virus. The value in this example represents a cell density that can be used for next-day infection. Seed sufficient wells for three replicates per serum condition, including MOI 0 as a negative control, a serum-only control, and a virus-only control, as well as three wells to count prior to infection.
15. The serum dilutions should be selected based on the particular assay, but in general an LFC based assay has a larger dynamic range than a PRNT and thus needs fewer dilutions. For example, where a PRNT might need two- or threefold dilutions, an LFC based assay can be performed with tenfold serum dilutions.
16. The neutralization time and temperature can be adjusted as needed for each particular virus.

References

1. World Health Organization (1997) Manual of laboratory methods for testing of vaccines used in the WHO Expanded Programme on Immunization
2. Guidance for Industry (2010) Characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications. U.S.D.o.H.a.H. Services, editor. Office of Communication, Outreach and Development (OCOD) (HFM-40)
3. Gombold J et al (2014) Systematic evaluation of in vitro and in vivo adventitious virus assays for the detection of viral contamination of cell banks and biological products. Vaccine 32 (24):2916–2926
4. Gates IV et al (2009) Quantitative measurement of varicella-zoster virus infection by semi-automated flow cytometry. Appl Environ Microbiol 75(7):2027–2036
5. Donis RO et al (2014) Performance characteristics of qualified cell lines for isolation and propagation of influenza viruses for vaccine manufacturing. Vaccine 32(48):6583–6590
6. Shurtleff AC et al (2012) Standardization of the filovirus plaque assay for use in preclinical studies. Viruses 4(12):3511–3530
7. Chou SW, Scott KM (1988) Rapid quantitation of cytomegalovirus and assay of neutralizing antibody by using monoclonal antibody to the major immediate-early viral protein. J Clin Microbiol 26(3):504–507
8. Thomas JA et al (2016) Development and application of a simple plaque assay for the human malaria parasite *Plasmodium falciparum*. PLoS One 11(6):e0157873
9. Heppner DG Jr et al (2017) Safety and immunogenicity of the rVSVG-ZEBOV-GP Ebola virus vaccine candidate in healthy adults: a phase 1b randomised, multicentre, double-blind, placebo-controlled, dose-response study. Lancet Infect Dis 17(8):854–866
10. Salje H et al (2014) Variability in dengue titer estimates from plaque reduction neutralization tests poses a challenge to epidemiological studies and vaccine development. PLoS Negl Trop Dis 8(6):e2952
11. Hebert CG et al (2018) Rapid quantification of vesicular stomatitis virus in Vero cells using Laser Force Cytology. Vaccine 36 (41):6061–6069
12. Hebert CG, Hart SJ, Terray A (2014) Label free detection of pseudorabies virus infection in Vero cells using laser force analysis. Analyst 139 (6):1472–1481

INDEX

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