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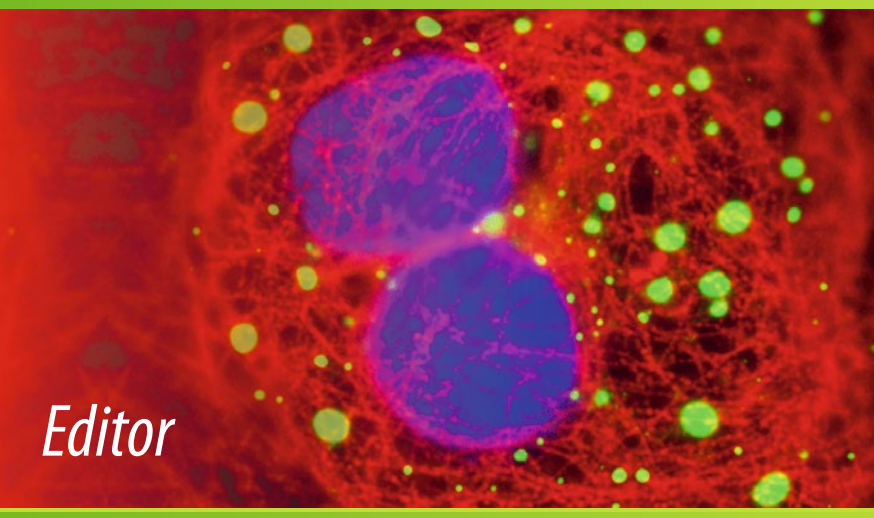

Alejandro Brun *Editor*

Vaccine Technologies for Veterinary Viral Diseases

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

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Preface

A great amount of knowledge has been generated in recent years on veterinary vaccine technologies, so presently many different antigen delivery systems are available for vaccine research, in particular against viral diseases. This book was intended to review most popular antigen production and delivery strategies that have been tested in veterinary species. Viral vectors as well as genetic and protein subunit vaccines or large-scale protein production systems are considered among the different chapters of the book. Our aim has been to facilitate the access to real and well-established protocols to those beginning in this interesting and laborious field as well as to provide basic knowledge when attempting a novel vaccine design or platform. This book, in addition, will provide an updated view of most options available when vaccine development is considered. In spite of the number of alternatives available for vaccine design, few of them often reach market and commercialization, but, nonetheless, the data obtained along experimental trials contribute to the exploration and understanding of the immune mechanisms and immune correlates relevant in protection among different animal species. Therefore this book also could be of help to those interested in basic and applied immunology, since these technologies represent interesting tools to induce or modulate different host immune mechanisms.

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Chapter 1

Vaccines and Vaccination for Veterinary Viral Diseases: A General Overview

Alejandro Brun

Abstract

A high number of infectious diseases affecting livestock and companion animals are caused by pathogens of viral etiology. Ensuring the maximum standards of quality and welfare in animal production requires developing effective tools to halt and prevent the spread of those infectious diseases affecting animal husbandry. To date, one of the best strategies is to implement vaccination policies whenever possible. However many of the currently manufactured vaccines relies in classical vaccine technologies (killed or attenuated vaccines) which, under some circumstances, may not be optimal in terms of safety or adequate for widespread application in disease-free countries at risk of disease introduction. One step ahead is needed to improve and adapt vaccine manufacturing to the use of new generation vaccine technologies already tested in experimental settings. Here we present in the context of animal viral diseases of veterinary interest, an overview of some current vaccine technologies that can be approached for virus pathogens with a brief insight in the type of immunity elicited.

Key words Virus vaccines, Attenuated vaccines, Viral vectors, DNA vaccines, Subunit vaccines, Innate immunity, Adaptive immunity, Vaccine technologies

1 Viral Diseases of Animals and the Need for Vaccination

One of the biggest transformations in history occurred when mankind shifted from a hunter-gatherer to an agricultural lifestyle. During millenniums livestock and companion species (ruminants, swine, poultry, cats and dogs) were domesticated and raised first for survival (in this sense the word "livestock" is meaningful) then for profit and commerce. Since then, animal husbandry evolved as one of the most important activities for civilization and development. The importance of such activity is obvious since a proper management of land use and animal resources is always required to avoid malnutrition and famine in developing countries or in countries where intensive farming is essential for subsistence. The explosive

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growth rate of the world's human population complicates this picture (particularly in developing countries) so other sources of dietary consumption, such as farmed fish, will be more demanded in the near future. Inevitably, the intensive farming of animal species leads to the onset of diseases mainly caused by propagation of infectious pathogens, affecting animal welfare, reducing productivity, and in the worst cases, seriously undermining the economy of nations. In some cases, livestock or animal pathogens can also cause disease in humans, so means to control and eradicate them have to be implemented.

Among the plethora of infectious diseases in animals, those of viral etiology account for a high burden of cases and are among the most relevant from a veterinary perspective. In fact, approximately half of the most important animal diseases are caused by viruses, according to the OIE's classification for terrestrial and aquatic notifiable animal diseases (*see* Table 1). The listed viral diseases comprise virus from 22 different viral families and four families of virus (herpesvirus, rhabdovirus, poxvirus and paramyxovirus) concentrate a high number of diseases (Fig. 1). Several of the listed animal virus diseases can be also transmitted to humans (zoonotic diseases) either by direct contact with infected animals, infected animal tissues and fluids or by means of arthropod vectors, impacting both public health and food security. Thus, preventing transmission of infectious diseases at the animal–human interface is important for protecting the world population from both epizootics and pandemics, constituting the basis for the "One Health" concept [1, 2]. Prevention by vaccination remains as one of the most cost-effective intervention strategies against infectious diseases. For most of the listed diseases there are "licensed" or available vaccines, eventually obtained by "classical" production methodologies. An important exception is that of diseases caused by retroviruses, for which classical vaccine technologies have not been successful, and that of aquatic diseases, for which only vaccines for fish have been so far developed. In some cases the efficacy of vaccination against viral diseases of animals has been very successful, as it can be illustrated by the eradication of rinderpest [3] (probably the most deadly disease of cattle and ruminants, caused by a morbillivirus) by the use of an attenuated/avirulent strain of the causative virus. Recent evidences advice to support efforts to control emerging viral pathogens where they primarily occur, in order to avoid uncontrolled spread of deadly viruses [4]. Within this perspective, some technologies for vaccine design may constitute powerful platforms to rapidly generate new experimental vaccines based on previous knowledge about the immune responses generated in the host.

Table 1
The OIE's notifiable viral diseases and infections of terrestrial and aquatic animals

Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
Bluetongue	BTV[a]	Reoviridae	Orbivirus	Live attenuated
Crimean Congo hemorrhagic fever[b]	CCHFV[a]	Bunyaviridae	Nairovirus	Not available
Equine encephalomyelitis (Eastern)[b]	EEEV[a]	Togaviridae	Alphavirus	Inactivated
Foot and mouth disease[b]	FMDV	Picornaviridae	Aphtovirus	Inactivated (BEI)
Infection with Aujeszky's disease virus (Pseudorabies)	SHV-1	Herpesviridae (α-herpesvirinae)	Suid Herpesvirus	Attenuated (deletion of glycoproteins gE, gC, gG)
Infection with rabies virus[b]	RABV	Rhabdoviridae	Lyssavirus	Inactivated/Attenuated/Recombinant poxvirus; Adenovirus
Infection with rinderpest virus	RPV	Paramyxoviridae	Morbillivirus	Attenuated
Japanese encephalitis[b]	JEV[b]	Flaviviridae	Flavivirus	Inactivated/Attenuated
Rift Valley fever[b]	RVFV[b]	Bunyaviridae	Phlebovirus	Attenuated
Vesicular stomatitis[b]	VSV[b]	Rhabdoviridae	Vesiculovirus	Inactivated/Attenuated
West Nile fever[b]	WNV[b]	Flaviviridae	Flavivirus	Inactivated/Attenuated/Recombinant canarypox/DNA vaccine (USA)
Epizootic hemorrhagic disease	EHDV[b]	Reoviridae	Orbivirus	Inactivated/Attenuated (licensed USA, Japan)
Cattle diseases				
Bovine viral diarrhea	BVDV	Flaviviridae	Pestivirus	Inactivated/Attenuated

(continued)

Table 1
(continued)

Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
Enzootic bovine leukosis	BLV	Retroviridae	Lentivirus	Not available
Infectious bovine rhinotracheitis/ Infectious pustular vulvovaginitis	BoHV-1	Herpesviridae (α-herpesvirinae)	Varicellovirus	Deleted glycoprotein gE inactivated or attenuated
Lumpy skin disease	LSDV	Poxviridae	Orthopoxvirus	Attenuated
Sheep and goat diseases				
Caprine arthritis/encephalitis	CAEV	Retroviridae	Lentivirus	Not available
Infection with peste des petits ruminants virus	PPRV	Paramyxoviridae	Morbillivirus	Attenuated/Recombinant capripoxvirus
Maedi-visna	MVV	Retroviridae	Lentivirus	Not available
Sheep pox and goat pox	SPV	Poxviridae	Orthopoxvirus	Inactivated/Attenuated
Equine diseases				
Equine encephalomyelitis (Western)	WEEV[a]	Togaviridae	Alphavirus	Inactivated
Equine infectious anemia	EIAV	Retroviridae	Lentivirus	Not available
Equine influenza[b]	EIV	Orthomyxoviridae	Influenzavirus	Inactivated/Recombinant canarypox
Infection with equid herpesvirus-1	EHV-1	Herpesviridae		Inactivated/Attenuated
Infection with equine arteritis virus	EAV	Arteriviridae	Arterivirus	Inactivated/Attenuated
Venezuelan equine encephalomyelitis[b]	VEEV[a]	Togaviridae	Alphavirus	Inactivated/Attenuated

Infection with African horse sickness virus	AHSV[a]	Reoviridae	Orbivirus	Live attenuated
Swine diseases				
African swine fever	ASFV[a]	Asfiviridae	Asfivirus	Not available
Infection with classical swine fever virus	CSFV	Flaviviridae	Pestivirus	Attenuated/Subunit (E2)
Porcine reproductive and respiratory syndrome	PRRSV	Arteriviridae	Arterivirus	Live attenuated
Swine vesicular disease	SVDV	Picornaviridae	Enterovirus	Not available
Transmissible gastroenteritis	TGEV	Coronaviridae	Alphacoronavirus	Not available
Avian diseases				
Avian infectious bronchitis	IBV	Coronaviridae	Gammacoronaviridae	Inactivated/Attenuated/Inactivated multivalent
Avian infectious laryngotracheitis	ILTV	Herpesviridae (α-herpesvirinae)	Gallid herpesvirus-1	Attenuated/Recombinant herpesvirus/Recombinant fowlpox
Duck virus hepatitis	DHV-1	Picornaviridae	Avihepatovirus	Inactivated/Attenuated
Duck virus enteritis	DEV-1	Herpesviridae (α-herpesvirinae)	Anatid herpesvirus-1	Attenuated
Infection with avian influenza viruses and infection with influenza A viruses of high pathogenicity in birds other than poultry including wild birds[b]	AIV	Orthomyxoviridae	Influenzavirus A	LPAI inactivated/Recombinant fowlpox (HPAI vaccination banned or discouraged)

(continued)

Table 1
(continued)

Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
Fowl pox	FPV	Poxviridae	Avipoxvirus	Modified live attenuated
Infectious bursal disease (Gumboro disease)	IBDV	Birnaviridae	Avibirnavirus	Inactivated/Attenuated/Recombinant herpesvirus-VP2
Newcastle disease[b]	NDV	Paramyxoviridae	Avulavirus	Inactivated/Attenuated (lentogenic and mesogenic). Recombinant avian herpesvirus and avipoxvirus
Marek's disease	MDV (GaHV-2)	Herpesviridae (α-herpesvirinae)	Gallid herpevirus-1	Live attenuated
Turkey rhinotracheitis	aMPV	Paramyxoviridae	Metapneumovirus	Live attenuated/Inactivated
Lagomorph diseases				
Myxomatosis	MV	Poxviridae		Live attenuated
Rabbit hemorrhagic disease	RHDV	Caliciviridae		Recombinant poxvirus
Other infections				
Camelpox		Poxviridae		Inactivated/Attenuated
Bunyaviral infections (*Akabane, Cache Valley, Schmallenberg, and Nairobi sheep disease*)	AKAV[b] CVV[b] SBV[b] NSDV[b]	Bunyaviridae	Orthobunyavirus Orthobunyavirus Orthobunyavirus Nairovirus	Inactivated
Hendra and Nipah virus diseases[b]	HeV NiV	Paramyxoviridae	Henipaviruses	Not available

Fish diseases				
Infection with HPR-deleted or HPR0 infectious salmon anemia virus	ISAV	Orhtomyxoviridae	Isavirus	Inactivated
Infection with salmonid alphavirus	SAV	Togaviridae	Alphavirus	Inactivated
Epizootic hematopoietic necrosis	EHNV	Iridoviridae	Ranavirus	Not available
Infectious hematopoietic disease	IHNV	Rhabdoviridae	Novirhabdovirus	Inactivated/DNA
Koi herpesvirus disease	KHV	Alloherpesviridae	Cyprinivirus	Live attenuated
Red sea bream iridoviral disease	RSIDV	Iridoviridae		Formalin inactivated
Spring viraemia of carp	SVCV	Rhabdoviridae	Vesiculovirus	Not available
Viral hemorrhagic septicemia	VHSV	Rhabdoviridae	Novirhabdovirus	Not available
Mollusc diseases				
Infection with Ostreid Herpesvirus 1 microvariants	OsHV-1	Herpesviridae		Not applicable
Infection with abalone herpesvirus	AbHV	Herpesviridae (Malacoherpesviridae)		Not available
Crustacean diseases				
Infectious hypodermal and hematopoietic necrosis	IHHNV	Parvoviridae	Brevidensovirus	Not developed
Infectious myonecrosis	IMNV	Totiviridae	Totivirus	Not developed
Taura syndrome	TSV	Dicistroviridae	Aparavirus in the Family	Not developed

(continued)

Table 1
(continued)

Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
White spot disease	WSSV	Nimaviridae	Whispovirus	Not developed
White tail disease (Infection by Macrobrachium rosenbergii nodavirus)	MrNV and XSV associate virus	Nodaviridae	Nodavirus	Not developed
Yellowhead disease	YHV	Roniviridae (O. Nidovirales)	Okavirus	Not developed
Amphibian diseases				
Infection with ranavirus	FV3	Iridoviridae	Ranavirus	Not available

[a]Arthropod-borne virus (arbovirus)

[b]Zoonotic disease

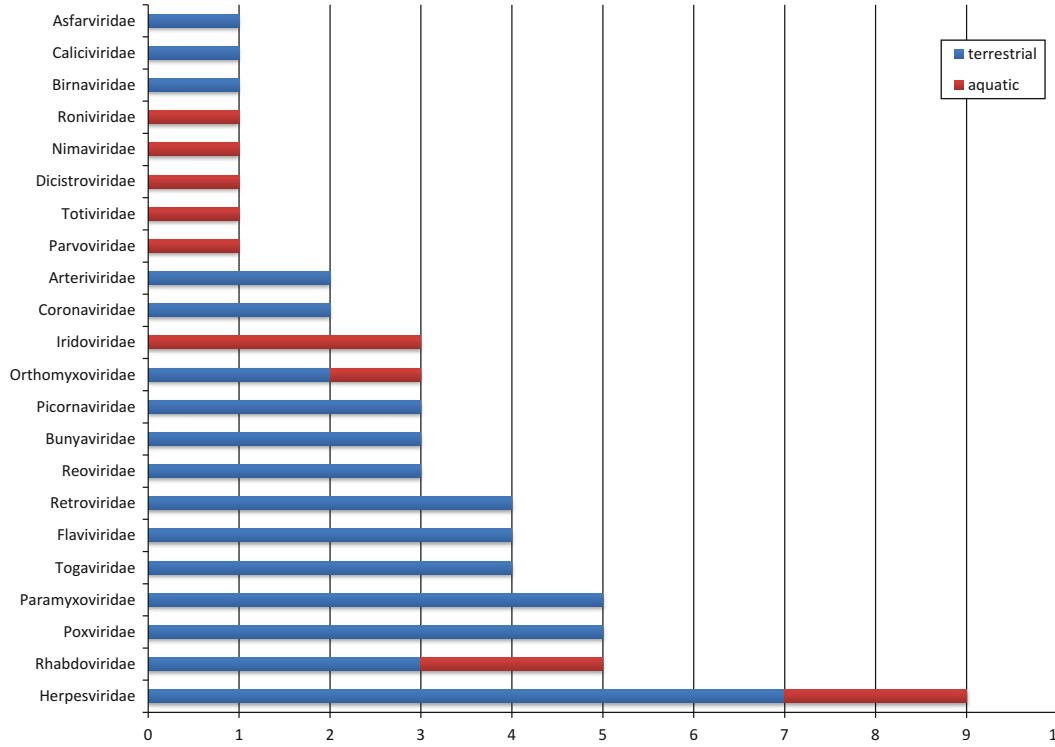

Fig. 1 Virus families including members causing notifiable animal diseases. The figure depicts the number of pathogenic members from each virus family causing important diseases in terrestrial and aquatic animals

2 Immunology Matters

The objective of vaccination is to achieve a specific stimulation of the immune system enabling the host to mount an efficient (and desirably long-lived) memory immune response that can recognize the pathogen and eventually eliminate it once is present in the organism upon infection. This can be achieved providing the appropriate antigenic stimulus (the vaccine) to activate cellular mechanisms involved in recognition of the nonself. Thus, an efficient vaccine needs to be recognized as a nonself entity and, ideally, be able to stimulate innate immune responses that further "instruct" subsequent adaptive and memory responses. The first step is carried out either by infected or by specialized phagocytic cells (antigen presenting cells or APCs, including macrophages and dendritic cells) able to present antigenic determinants to näive (B and T) lymphocytes (Fig. 2).

Though the innate immune response is broadly reactive and unspecific it strongly conditions the magnitude and the composition of the specific (adaptive or acquired) immune responses. Cellular pathogen recognition receptors (cPRRs) either membrane

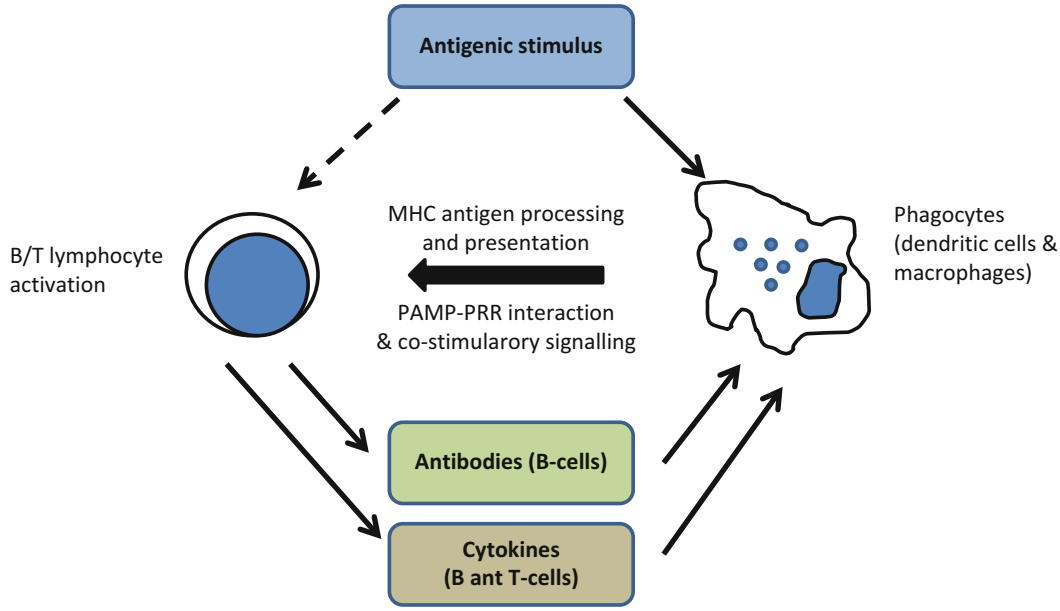

Fig. 2 The cellular cooperation in the immune response. After vaccination, specialized phagocytes present the processed antigens to näive B or T-cells that may become activated only if proper co-stimulatory signals are produced (derived from the interaction of PAMPs with cellular PRRs). Activation drives lymphocytes to secrete soluble mediators and antibodies initiating inflammatory responses (adapted from [33])

bound (Toll-like-, C-type lectin- and scavenger receptors) or cytosolic (NOD-like and RIG-like receptors) of phagocytes eventually bind to pathogen associated molecular patterns (PAMPs) carried on infecting microbes [5]. In particular, encountering of pathogenic virus ligands (such as single or double stranded RNA) to intracellular PRRs activates the phagocytic cells from a normal quiescent state by inducing NFκB-mediated gene transcription of a number of co-stimulatory molecules, proinflammatory cytokines and chemokines as well as IRF-mediated transcription of type I-interferons (IFNs) and other cytokines such as IL-1β and TNF-α [6]. Other immune cell types such as the natural killer (NK) cells express functional TLRs specifically for detecting viral PAMPs and can be also activated by type-I interferons [7]. NK cells can eliminate cells in which expression of MHC molecules is reduced upon viral infection. NK cells secrete IFN-γ which in turn can enhance the phagocytic activity of macrophages and antigen presentation by mature dendritic cells (DCs), a key player in the bridging of innate and adaptive immunity. DCs signaling to naïve lymphocytes will determine whether these cells should be eventually involved in fighting against the viral infection. This fact is exploited by those vaccines based in attenuated viruses or in replicating live virus vectors where the initiation of innate immune responses greatly aug-

ments the quality and magnitude of the adaptive response in contrast to that elicited by vaccines based on inert antigens (inactivated virus or subunit vaccines). Recently, the central role of dendritic cells or APCs in regulating the immune response has made antigen targeting to these cells a major subject for specific immune stimulation aimed to improve vaccine efficacy as well as other forms of immunotherapy [8, 9].

Upon näive lymphocyte activation by interaction with DCs the specificity of the immune response is granted and clonal expansion of B and T-cells capable of recognizing the specific antigen will take place. A pool of specific lymphocytes containing memory and effector cells will be expanded as a primary response to the vaccine stimulus. Upon infection and virus antigen encounter the secondary response will be greatly increased, potentially leading to protection and long-lived immunity by specific effector and memory cells (Fig. 3). Therefore, the two main principles to be exploited by vaccines are necessarily specificity and memory. When designing vaccines the issue of specificity is crucial for the success of a vaccine and can be approached by an adequate selection of the antigen fraction, whole antigen or antigens of choice, being able to recall

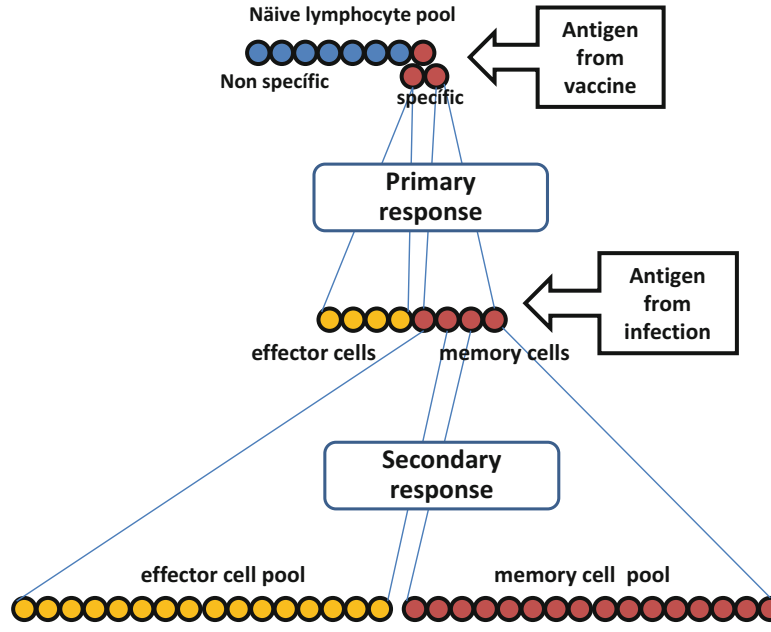

Fig. 3 Vaccination exploits the induction of specificity and immunological memory. A primary clonal expansion of lymphocytes is produced upon activation of naïve T-cells by phagocytes primed with specific vaccine antigens/stimulus. Both effector and memory cell pools are generated that upon encounter with pathogen (infection) will undergo a massive secondary expansion of both cell pools (adapted from [33])

the memory lymphocyte pools produced in the primary responses after vaccination.

Previous identification of the correlates of protective immunity upon infection is one of the logical approaches for vaccine design, for example including relevant epitopes that induce neutralizing antibodies made upon infection and/or the key T-cell epitopes responsible for helper or cytotoxic functions [10]. Ideally, this knowledge should be derived from the pathogenesis of viral infection in the target species to which the vaccine is intended for but unfortunately these type of studies are often more difficult to perform than in laboratory animals (mainly due to genetic diversity of the outbred species, the lack of reagents and markers for cell phenotype characterization, and the limitation in the number of animals used for experimentation). Nonetheless, in some cases the pathogenesis of other animal models of disease (mainly rodents), correlates well enough with that of the target species and valuable information can be obtained about the immune mechanisms of protection. After the knowledge gathered in the past decades of virus research it becomes evident that, in a general sense, for virus with less complex pathogenesis a successful immunoprophylaxis could be obtained by the generation of an immune response against surface antigens displayed on virions and/or virus infected cells. For other virus (for example poxvirus, herpesvirus, asfivirus, respiratory viruses, and lentivirus) that have developed more complex pathogenesis (i.e., induction of persistence, replication in immune privileged tissues, using immune evasion strategies, induction of harmful host immune responses) the effective vaccine should elicit, in addition to neutralizing antibodies, specific T-cell responses [11].

3 Vaccine Technologies

A first classification of vaccines has been outlined above with respect to the level of immunogenicity elicited (inactivated/killed non-infectious versus live attenuated vaccines). Therefore two broad categories of antiviral vaccines can be considered with respect to the nature of the virus used (live or death) or the relevant antigen (whole or fractionated); in fact all licensed vaccines against viral diseases available to date (both for the medical and veterinary use) could fall in either one of these categories. This dichotomy helps to categorize vaccines into four general types (Table 2).

In this categorization type I vaccines include those produced by means of inactivating methodologies while type II vaccines include all attenuated virus used as vaccines, including those generated by reverse genetics. Type III and IV vaccines include those in which only components or a fraction of the pathogen is used as the vaccine antigen. Thus, type III vaccines would include subunit vaccines, including carrier micro/nanoparticle and virus-like particles

Table 2
A proposed classification for the current vaccine technologies

VIRUS	Whole antigen	Fraction/component
Death	*Type I* (inactivated, killed)	*Type III* (subunit, VLPs, genetic DNA or RNA, killed recombinant vectors)
Live	*Type II* (modified live attenuated, reverse genetics modified)	*Type IV* (recombinant viral vectors expressing antigens)

(VLPs) vaccines produced by recombinant technologies in both prokaryotic and eukaryotic cells and inactivated recombinant vectors expressing heterologous vaccine antigens. In this category, both nucleic acid vaccines as well as peptide based vaccines could be also included. Finally, the type IV vaccines are those delivered by a live viral vector that codes and expresses particular (selected) heterologous vaccine antigens. Obviously, from all these categories further classifications can be made, depending on the formulation of the vaccine (for example, inactivated vaccines can be subdivided into those composed of whole inactivated infected cultures or purified virus fractions), and the type of adjuvant used to augment the immune responses. Attenuated vaccines could include those natural virus isolates with reduced virulence, or attenuated virus generated by serial passages, or virus rescued by means of a reverse genetics approach. Nucleic acid vaccines can be based on DNA plasmids or self-replicating RNA molecules launched by a DNA plasmid encoding a viral replicon. For each technology several methodologies for production or antigen expression can be used and further modifications and formulations applied, therefore the potential combinations that can be tested experimentally are many. The choice of one or another may depend on the experimental (preclinical) data obtained in models of infection if available. Further classification of vaccine technologies could be done on the basis of the main type of immunity provided (mucosal, systemic, humoral or cellular), preferred delivery method (oral, parenteral) or prime-boost combination (*see* Table 3).

4 Type I Vaccine Technologies

Inactivated (killed) antiviral vaccines have being used for long and are based on the disruption of the ability of a virus to replicate by generally chemical or physical methods. Among chemical methods used, formaldehyde and organic compounds such as cyclic esters (β-propiolactone) or binary ethylenimine (BEI) have been most widely used. Other cross-linking agents such as glutaraldehyde can be an option for the inactivation but its use has not been as wide as formaldehyde. Two main caveats of the use of cross-linking agents

Table 3
General features of laboratory (experimental) vaccine technologies

Type	Type of modification	Production platform	Delivery method	Adjuvants	Dosage	Immunity provided	Safety
I. Inactivated	Physical, Chemical	Eukaryotic cell culture	Parenteral	Chemical	Repeated	Humoral and Th responses	+++
II. Live Attenuated	Physical Chemical mutagens, Reverse genetics, Tissue propagation (in vitro in vivo)	Cell culture	Parenteral	none	Single Repeated	Humoral, and cellular including CTL responses	+
IIIa. Subunit & carrier technologies, glycoconjugate and peptide vaccines, microparticle and nanoparticle formulations, virus-like particles		Prokaryotic cell culture, Eukaryotic cell culture, Plant based. Chemical synthesis	Parenteral/mucosal	Chemical/ Molecular	Repeated	Humoral and Th	++++
IIIb. Nucleic acid	VpG, delivery, liposome	Prokaryotic cell culture	Parenteral	Molecular	Repeated	Humoral and cellular	+++
IV. Viral Vector based		Mammalian, Insect, Plant cell culture	Parenteral	None/molecular	Single Repeated	Humoral and	+++

for vaccine preparation can be cited; the first one is the potential for aggregation leading to disruption or modification of antigenic epitopes possibly accounting for the reduced immunogenicity of these vaccines, usually requiring two or three booster doses to maintain adequate and lasting levels of protective immunity. Another problem is the risk for incomplete inactivation leading to exacerbation of disease if the partially (or suboptimal) induced immunity cooperates with infectivity by mechanisms such as antibody dependent enhancement (ADE). In this case, monocytes or macrophages (Fc-receptors bearing cells) can be infected by virus complexed to non-neutralizing antibodies, a process described in dengue virus infections [12]. Finally, another issue with inactivated vaccines is overcoming the differentiation of infected and vaccinated animals not to interfere with the surveillance diagnostics. While formaldehyde reacts primarily with proteins, β-propiolactone (BPL) and binary ethylenimine (BEI) modify mainly DNA or RNA so BPL is expected to maintain a high immunogenicity during the inactivation of viruses. However it has been reported that BPL may also and react to some amino acids including cysteine, methionine, and histidine so certain modification of proteins may also affect the immunogenicity of BPL vaccines. Similarly BEI has been also shown to react with proteins [13]. This compound is used widely for the inactivation of foot and mouth disease virus (FMDV) in the preparation of vaccines. Nonetheless, inactivated vaccines remain as a leading methodology for vaccine production (both for human and veterinary use) in part due to the effectiveness of adjuvants (mainly aluminum salts) in the vaccine formulations overcoming the main issue of limited immunity. In fact, this technology may benefit from other inactivation approaches such as the use of hydrogen peroxide or protonating compounds, such as diethylpyrocarbonate (DEPC). Hydrogen peroxide could inactivate both DNA and RNA viruses (vaccinia virus, LCMV, WNV and YFV) with little damage to the antigenic structure, thus minimizing the effect on immunogenicity. More interestingly, this inactivation approach rendered vaccines able to induce both humoral (neutralizing antibodies) and cellular immune responses including WNV and LCMV specific CD8+ cytotoxic T-cells [14, 15]. Using a histidine-protonating agent such as DEPC it was reported the abolishment of vesicular stomatitis virus (VSV) infectivity and pathogenicity in mice. These animals survived a further lethal challenge and this protection was associated to the induction of neutralizing antibodies [16] although no further reports have arose since the first description. In spite of the advances made in different technologies for stimulating the immune responses the classic inactivation methodology is still broadly used to manufacture many vaccines for veterinary use, in part since manufacturers need to balance carefully the investment needed to adapt their traditional manufacturing processes to the new technologies and the

expected profitability. Other classical inactivation techniques by physical methods have been exposure to several types of radiation: thermal, electromagnetic or ionizing. UV radiation has been one of the most used in human vaccine manufacturing.

5 Type II Vaccine Technologies

Live attenuated virus vaccines are among the most successful forms of vaccines particularly with regards to immunogenic character. The ability to replicate makes these vaccines stronger inducers of innate responses, a feature that critically may influence the outcome of the acquired immune responses as discussed above. Several veterinary and companion animal vaccines are based on attenuated viruses and these types of vaccines are used in the human side as well. The common feature shared by attenuated virus vaccines is the lost of virulence factors while the immunogenicity is maintained. Traditional methods for development of attenuated vaccines were the serial passage or propagation of the virus in heterologous cell cultures or in brain tissue from rodents, suckling mice, rabbits or goats, in particular for veterinary use. Propagation in different tissue usually ends up with a change of tropism. For example, hepatotropic viruses passaged in brain tissues were unable to replicate in liver though acquired neurovirulence. A different approach is to induce mutations with mutagenic compounds such us nucleoside analogous. Temperature sensitive mutants grown at lower temperatures were then unable to replicate at normal temperatures in the hosts. The main advantages of attenuated vaccines over inactivated or killed vaccines or subunit vaccines can be related with a wider presentation of epitopes since, obviously, more proteins will be expressed as a consequence of virus replication into the infected host cell (in the infected cell protein fragments will be presented through MHC-I), and also with the possibility of administration by similar or natural routes of infection (i.e., nasal/mucosal route for influenza vaccines). The immune responses elicited are also similar to that of infections, including triggering of innate immune responses, as well as humoral and/or cellular responses. Importantly, the costs of generation and manufacturing these types of vaccines are usually affordable for the veterinary vaccine markets. On the other side, possible disadvantages of attenuated virus vaccines are the genetic instability, allowing reversion to virulence or lost of replicating phenotype, problems related with immunosuppressed individuals, or deleterious effects of some attenuated vaccines when used in gestating animals. This usually accounts for those vaccines obtained by methods in which the inactivation process is not fully controllable or understood (i.e., serial propagation in tissue culture). Table 4 summarizes advantages and disadvantages between killed and attenuated vaccines. For diseases affecting

several species a vaccine that is safe for a specific ruminant host might not be safe for swine. In general terms it is generally accepted that inactivated vaccines offer less safety problems than attenuated vaccines. Advances in the knowledge of pathogen biology, immunology and molecular biology allowed to carry out more rational vaccine designs so novel alternatives to the attenuated type of vaccines have been developed. Particularly for RNA viruses, the generation of reverse genetic systems (i.e., the ability to rescue fully infectious virus from cloned viral genomes and transcripts) [17] has allowed to develop novel attenuated vaccines with enhanced safety features. For DNA viruses defining virulence and/or immunomodulatory genes allowed its deletion by homologous recombination techniques [18]

In most of the cases the modification of these genomes allowed the introduction to these vaccines of an important characteristic for veterinary vaccines: the possibility of differentiate infected from

Table 4
Most recognizable pros and cons of inactivated and attenuated vaccines

Inactivated vaccines		Attenuated vaccines	
PROS	**CONS**	**PROS**	**CONS**
No risk of infection	May potentiate disease (paramyxovirus, lentivirus, coronavirus vaccines)	Systemic and local immune activation. Humoral and cellular immune responses	Presence of adventitious agents
No residual adventitious agents	Parenteral administration (No mucosal immunity)	Durable immunity	May cause illness
	Low rate of CTL responses	Effective immunity	May loose attenuation
	Low immunity	Low cost of production	Spread to contacts
	Need boosting doses	Easy administration	May loose infectivity
	Expensive manufacturing	Herd immunity (most if vaccine spreads)	Storage limited Risk for pregnancy
		Single dose administration	Interference with live virus (preexisting immunity). Presence of defective interfering particles
			Discrimination of vaccinates and infected animals more difficult
			Immunosuppression

vaccinated animals [19]. This is particularly important when surveillance diagnostic is implemented for example to maintain the condition of a disease-free country.

6 Type III Vaccine Technologies

Once identified, protective antigen fractions or components from whole pathogens can be isolated and/or produced by cloning and expression in heterologous systems (bacterial, yeast, plant, eukaryotic cell). We include in this category both subunit particulate and nucleic acid vaccines. With this approach the specificity of the immune response generated is maximized but the magnitude of the immune response tends to be lower than that of attenuated vaccines. Thus, immune adjuvants, targeting strategies or prime boost regimens might be considered to enhance the immune responses.

Subunit vaccines have several advantages over conventional attenuated vaccines in particular regarding safety and production. Most used systems to produces subunit vaccines are based on bacteria, yeast, insect or mammalian cells. More recently other systems based on non-fermentative approaches such as live organisms have been developed, particularly plants or insects. In plants two main alternatives have been developed, either genetically modified or expressing transiently antigens encoded by plant virus or bacterial vectors. In live insects (Lepidoptera) recombinant baculoviruses can be used to infect insect larvae and transgenic silkworms can be also generated (reviewed in [20]). A particular feature of subunit vaccines is the possibility of generation of virus like particles (VLPs) by co-expression of capsid proteins constituent of virions, but devoid of ribonucleoproteins. Like the viral capsids, the VLPs are composed of a geometrically arranged array of proteins, forming repetitive structures against which soluble antibodies and/or B-cell receptors can interact with high avidity. These structures are thus good inductors of T-cell independent responses. In addition the VLPs can be also internalized and processed by APCs to induce both Th and CTL responses, therefore having the potential to stimulating broader immune responses than monomeric forms of protein subunits. Another advantage of VLPs is that they can be produced in a variety of expression systems (baculovirus, poxvirus, alphavirus replicons, plants, Salmonella, E. coli, yeasts, and so on) and can be engineered in order to even express foreign epitopes or immune-stimulatory molecules in the form of chimeric-VLPs, or by covalent linking of immunomodulators (either linear or cyclic peptides, haptens, glycans). VLPs can be obtained from enveloped viruses by budding from cells expressing the VLP components (such in the case of influenza virus). A more specialized technique

is the reconstitution of viral envelopes in unilamelar liposomes, termed virosomes. These synthetic structures can be also complemented with immune-stimulatory conjugates or even heterologous molecules such as DNA, siRNA, antibody fragments (reviewed in [20]). Perhaps from the veterinary vaccine perspective, the generation of VLP subunit vaccines and derivatives is being hampered by the higher costs for production precluding a more generalized use as a vaccine production technology.

Instead of using whole proteins as antigens, immunogenic epitopes previously identified allows to design synthetic peptides to direct more specifically the immune response. Known B and T-cell peptides and combinations can be included in a peptide vaccine design [21]. One of the advantages of peptides over subunit protein vaccines is the simple production, storage and distribution, as well as the flexibility to introduce modifications or mutations (for those highly changing viruses). In spite of these advantages, peptide vaccination is not yet a generalized approach since it needs a deeper knowledge of the protective immune responses in the host species and the intrinsic lower immunogenicity of peptides over whole proteins. However immunogenicity can be enhanced by multimerization strategies [22] or by the use of micro/nano particulate delivery of covalently attached peptides including or not targeting signals to facilitate interaction with immune cell receptors.

Genetic vaccines were discovered upon gene therapy experiments by Wolff and Felgner when intended to deliver DNA into muscle cells by using cationic lipids containing DNA [23]. In fact DNA uptake was produced even in the absence of lipids and expressed the encoded protein. Thus transcriptional units encoding HA antigens were placed under control of a viral promoter (CMV) so a DNA vaccine against influenza was first described in 1993 [24]. Usually DNA vaccines are delivered by intramuscular or intradermal injections. In the first case muscle cells can be directly transfected and express the protein. Dendritic cells present in the interstitial spaces could uptake the soluble antigen, or take up cells killed by the vaccine, or even being transfected directly. On the other hand, the cytosolic expression of the protein enables its MHC-I processing in either muscle or dendritic cells. MHC upregulation is one of the consequences of innate immunity stimulation by unmethylated CpG motifs upon TLR-9 receptor engagement. The main advantages of DNA vaccines are the ease to design and produce, allows differentiation of vaccinated and infected animals (DIVA), antigen is processed naturally, mimicking the immune response induced by virus replication thus stimulating the development of both cellular and humoral immune response. Finally, as with other vaccine strategies, DNA allows combining several antigens, targeting signals, or immunostimulatory molecules (cytokines and

chemokines) to improve the immune response elicited. DNA vaccination has been so far successful in mice models of disease. The only DNA vaccines licensed to date have been against WNV in horses and VHS in salmonids [20]. However, experimental DNA vaccination in large animals against livestock viral diseases still needs further optimization in order to achieve stronger immune responses (the amount of plasmid needed for immunization may represent a serious disadvantage). This handicap could be addressed by the use of stronger promoters, replicon based plasmids (Alphavirus), increasing plasmid uptake efficiency or by co-delivery of immune-stimulatory molecules. Nonetheless, it remains a very attractive way for a rationale design of vaccines, combining the simplicity of production and the potential use in combined vaccine approaches such as prime boost.

7 Type IV Vaccine Technologies

Recombinant viral vectors constitute a very important platform for vaccine design and experimental vaccination approaches. Virtually any infectious, non-pathogenic, virus can be used to express foreign genes, provided a system for recombinant incorporation and expression has been developed. This has been achieved for different RNA viruses that were previously attenuated by using reverse genetics systems or in DNA virus by means of homologous recombination techniques. Among the DNA viruses used to deliver vaccine antigens Poxvirus (from both orthopoxvirus and parapoxvirus genus), Herpesvirus, Adenovirus and Baculovirus have been the most widely used in experimental vaccine trials. The main advantage of DNA viruses over RNA viruses is related with the higher stability of DNA genomes, greater insertional sites and availability of BAC-DNA clones available making engineering and rescuing of recombinant virus a conventional laboratory task. Additional features include the cytoplasmic replication (with the exception of herpesviruses) and the induction of long lived humoral and cellular immune responses, with emphasis on the strong CD8-T-cell activation that is mediated by attenuated poxvirus and adenovirus infections. On the RNA virus side, several viruses from different families have been used as foreign gene carriers: Alphavirus, Bunyavirus, Coronavirus, Flavivirus, Paramyxovirus, Retroviruses, Rhabdovirus [25]. This has been possible by the establishment of reverse genetics technologies allowing the rescue of infectious virus from a copy of its genome. Paramyxoviruses are very potent inducers of humoral and cellular immune responses conferring complete long-life protection when used as attenuated vaccines. They allow interchange of nucleoproteins or envelope glycoproteins between related family

members giving rise to chimeric viruses for use as bivalent marker attenuated vaccines. In addition they can accommodate additional genetic information for expression of foreign antigens maintaining stability during propagation in cell culture, therefore they can be used also to immunize against pathogenic paramyxovirus and other infectious agents [25]. Attenuated rhabdoviruses (generated by manipulation of the viral glycoprotein and phosphoprotein and/or genome order rearrangement) offer similar characteristics for use as a vector for delivery of foreign genes, including the induction of innate and adaptive immune responses. One additional advantage of this type of vectors is the absence of seropositivity in both human and animal populations [25]. Replication deficient alphavirus have been also modified to express foreign antigens for use as vaccines, and in cancer and gene therapy studies. An interesting characteristic of alphaviruses is the induction of mucosal protective immune responses [26, 27]. For some bunyavirus the identification of virulence genes nonessential for growth in vitro allowed replacement for reporter genes or other viral antigens [28, 29]. As attenuated viruses they are capable of sustain limited replication in the host's enabling the initiation of innate immune responses against the transgene. All this examples outline the number of strategies than can be selected when designing attenuated vector vaccines as well as the possibility to design marker vaccines to elicit protection against several virus pathogens simultaneously (multivalent vaccines).

8 New Approaches for Vaccine Design

The conventional approaches for vaccine design are often not sufficient to provide immunity against highly variable pathogens or when T-cell immunity is crucial for protection. Tools from molecular biology integrating systems biology (genomics, proteomics, structural biology) approaches allow researchers to identify ways to improve the quality of vaccines or identify repertories of potentially protective antigens. For example, high throughput sequencing can identify the presence of adventitious viral pathogens in commercial vaccines, or defective genomes in cell culture lines used for vaccine production. Structural modeling of the interaction of neutralizing antibodies and/or antibody fragments with antigen can uncover the molecular signatures defining protective epitopes (cryptic (hidden) epitopes or involving quaternary structures) being another approach for vaccine antigen (or antiviral compounds) design. Additionally, novel flow and mass cytometry technologies [30] may help to gain deeper knowledge of specific cell types involved in protective immune responses for each viral disease. Finally,

integrating data of vaccine trials, including vaccine antigens, adjuvant usage or in silico epitope prediction algorithms, may allow development of platforms for experimental vaccine antigen candidates [31]. Though these approaches are far from being generalized they hold promise on the future of rationale vaccine design for some relevant viral diseases [32].

9 Concluding Remarks

Transition of successful experimental vaccines to industrial production and manufacturing may become a bottle neck in vaccinology since veterinary vaccines need to fulfill several important requisites, among them environmental and safety issues, manufacturing costs and marketability prospects. Considering that most novel vaccine technologies (other than killed or attenuated vaccines) need to adapt the current production processes, many vaccines will never develop further enough to reach market. Nonetheless, animal vaccine research is a very attractive research field with many advantages and complexity over human vaccine field. Firstly, due the larger number of target animal species or segments (ruminant livestock, poultry, porcine, equine, companion animals, aquaculture, and other animal vaccines), secondly, the lack of deep knowledge in the immune mechanisms and lack of reagents adds more difficulties if immune response characterization is needed. The possibility to test the efficacy of the vaccine prototypes in the target species and study the immune response evoked is one important difference that can speed the process of vaccine development over that of human vaccines. Another important advantage is the possibility of testing more innovative approaches that can be further tested for human vaccine development.

The following chapters illustrate a number of different techniques to provide antigen delivery in order to develop vaccines against viral diseases. Though the number of techniques is not exhaustive, the ones showed can be considered most currently used by laboratory researchers in the field of animal health. The reader will find useful examples for application to a particular viral disease since most of the techniques can be virtually applied to any virus pathogen. Among them, representative protocols for each of the broad categories for vaccine technologies discussed above. More discursive chapters are also included related to different techniques and protocols for analyses of the immune responses, the use of adjuvants as an essential part of vaccines based on non-live organisms, and experiences on the use of DNA vaccination in large animals.

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Chapter 2

Using IC-Tagging Methodology for Production and Purification of Epitope-Loaded Protein Microspheres for Vaccination

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Abstract

Particulate material is more efficient in eliciting immune responses. Here we describe the production of microspheres formed by protein muNS-Mi from avian reoviruses, loaded with foreign epitopes by means of IC-Tagging, for their use as vaccines.

Key words IC-Tagging, Microspheres, Vaccines, Particulate material, Avian reovirus, Adjuvants

1 Introduction

We have devised a molecular tagging system that has many applications [1–4]. One such application is to generate particulate material that potentiates the host immune response for vaccination purposes [5]. Our method encompasses two components: (1) Protein muNS-Mi: this is a truncated version of avian reovirus (ARV) non-structural protein muNS, that forms the matrix of the globular viroplasms produced by ARV in the infected avian cells [6]. Protein muNS-Mi forms close-to-spherical, ordered inclusions when being expressed in insect cells with the baculovirus protein expression system ([2, 5], *see* also Fig. 1b). The size of such inclusions range between 1 and 4 μm, so we named them "microspheres" or MS [5]. (2) The second component of our method is a 66 residues long domain of muNS-Mi, called Intercoil or IC that we use to tag the N- or the C-terminus of a protein of interest. The presence of the IC tag has no effect whatsoever in the distribution or activity of the tagged protein by itself, but efficiently relocates the tagged protein to muNS-Mi microspheres. Thus, we can load

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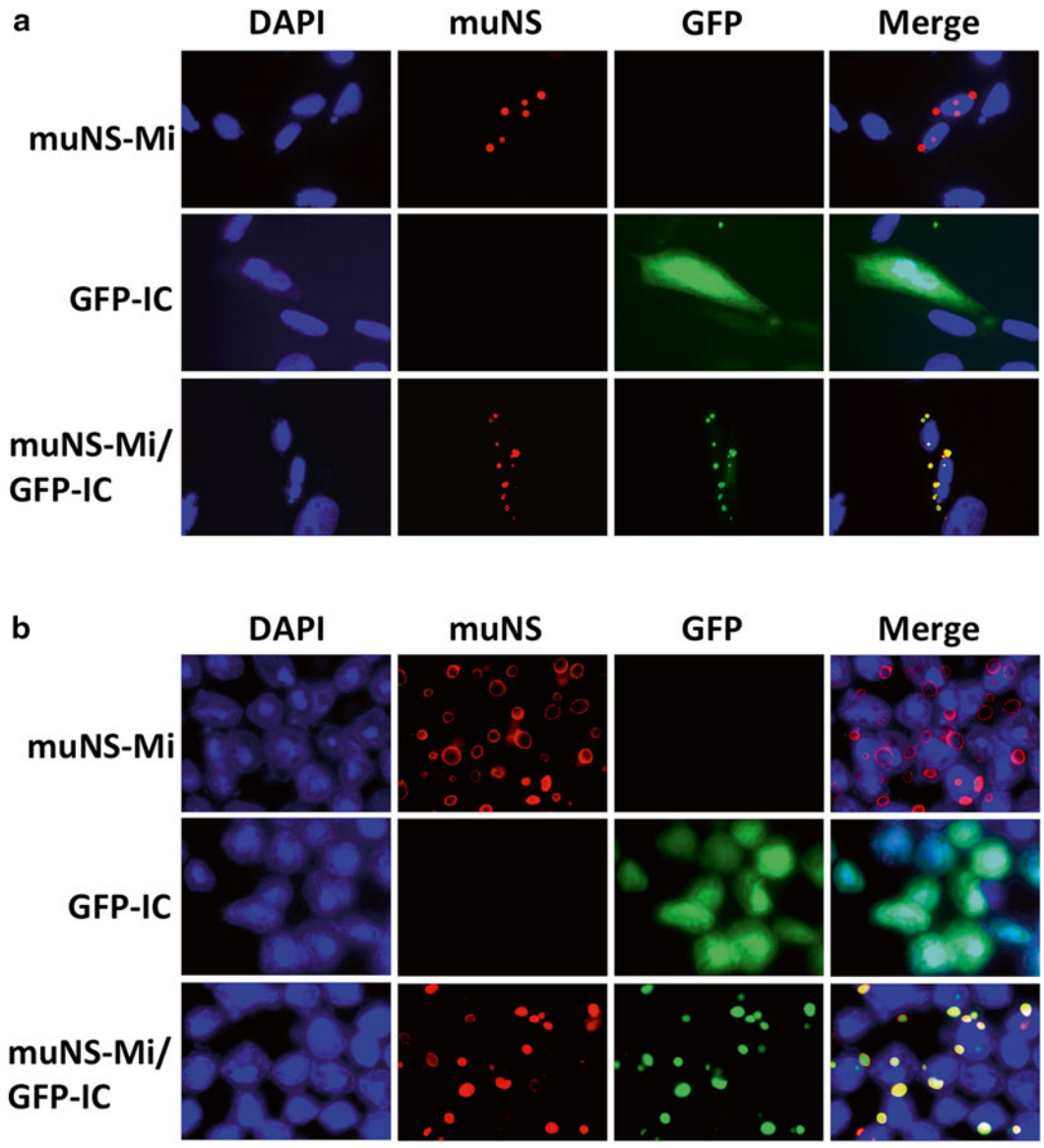

Fig. 1 Capture of IC-tagged proteins in muNS-Mi microspheres. (**a**) The figure shows CHO-K1 cells that express the proteins indicated at the *left* of the figure, after transfection with the corresponding expression plasmids (see text). In *green* is shown the autofluorescence of the GFP protein (GFP column). muNS-Mi microspheres are shown in *red* as detected by indirect immunofluorescence using primary antibodies directed against ARV protein muNS (column muNS), and Alexa Fluor 594 anti-rabbit secondary antibody. Nuclei were counter-stained *blue* with DAPI. (**b**) As in *a*, but the detected proteins were expressed by recombinant baculoviruses in insect Sf9 cells. All images were obtained with an Olympus DP-71 digital camera mounted on an Olympus BX51 fluorescence microscope

muNS-Mi MS with any foreign epitope and use them for immunization purposes. Microspheres will carry many copies of the tagged epitopes and can be loaded with either individual epitopes to be combined with other MS carrying different epitopes to generate multivalent vaccines, or the different epitopes can be simultaneously loaded into the same MSs with the same purpose. The latter is specially suited for complex epitopes formed by the interaction of several different proteins. We have previously shown that presentation of epitopes in muNS-Mi MSs have adjuvant effect, as animals immunized with three bluetongue virus (BTV) epitopes loaded in MSs were fully protected against a lethal challenge with BTV, while the same was not true when immunizing the animals with the BTV proteins alone [5]. Here we describe how to generate and purify muNS-Mi microspheres loaded with foreign IC-tagged antigens to use them for immunization. The protocol starts after the recombinant baculoviruses expressing the IC-tagged epitope(s) have been generated.

2 Materials

2.1 Cell Culture

1. Sf9 cells (Life Technologies).
2. Chinese hamster ovary cells (CHO-K1, ATCC).
3. Fetal bovine serum (FBS) and 100× penicillin–streptomycin–glutamine solutions.
4. SF-900II SFM medium (Life Technologies), supplemented with 10 % of FBS and 1 % of 100× penicillin–streptomycin–glutamine solution.
5. Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % FBS and 1 % of 100× penicillin–streptomycin–glutamine solution.
6. Medium SF-900 1.3× (Life Technologies).
7. Low melting point agarose. Prepare at 4 % in Milli-Q water and sterilize by autoclaving.
8. Neutral Red Solution (0.33 %). Prepare a 1:9 dilution in PBS as Staining Solution.
9. Trypan Blue. Prepare a 0.25 % solution in PBS and sterilize by filtration.
10. Lipofectamine® 2000 (Life Technologies).
11. Incubator (29 °C) for monolayer cultures of Sf9 cells.
12. Shaking incubator (29 °C) for suspension cultures of Sf9 cells.
13. Incubator at 37 °C with 5 % CO_2 atmosphere and humidity for culturing CHO-K1 cells.

14. Membrane filters (pore diameter 0.22 μm) for sterilization of autoclave-sensitive solutions.
15. Glass Erlenmeyer flasks (250 ml to 1 l) for suspension cultures.
16. Plastic tissue-culture flasks (75 and 150 cm^2) for monolayer cultures.
17. Aluminum foil.

2.2 Fluorescence Microscopy

1. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4, 1.5 mM KH_2PO_4. Adjust pH to 7.3 with NaOH and sterilize by autoclaving.
2. Rabbit antiserum against ARV muNS protein (raised in our laboratory [6]).
3. Alexa Fluor® 594 goat anti-rabbit IgG (H + L) antibody (Life Technologies).
4. Fixing solution: 4 % PFA in PBS. To prepare, add 10 g of paraformaldehyde to 250 ml PBS, completely dissolve by heating at 60 °C. Allow to cool at room temperature, adjust pH to 7.2 with NaOH, filter through 0.22 μm membrane filter and store at 4 °C.
5. Blocking solution: 2 % bovine serum albumin (BSA) in PBS.
6. 1000× DAPI staining solution: 0.1 mg/ml DAPI solution in water or PBS. Mix until completely dissolved and store at −20 °C. Keep a working aliquot in the dark at 4 °C.
7. Mowiol/DABCO solution—Mix 2.4 g of Mowiol, 6 g of glycerol, and 6 ml of water. Incubate on a shaker at room temperature from 3 to 6 h, add 12 ml of 0.2 M Tris–HCl (pH 8.5) and incubate at 50 °C for 10 min with occasional mixing. Centrifuge at 5000 × *g* for 15 min to pellet insoluble material. Add DABCO to a final concentration of 0.1 % (w/v) to the solution and store in 500 μl aliquots at −20 °C.
8. Microscope slides, round coverslips 15 mm in diameter, and coverslips of 22 × 60 mm.

2.3 Microsphere Purification and Analysis

1. RB-Buffer: 10 mM HEPES pH 7.9, 10 mM KCl.
2. RB-T Buffer: 10 mM HEPES pH 7.9, 10 mM KCl, 0.5 % Triton X-100.
3. Protease inhibitor cocktail: we use a Protease Inhibitor Cocktail, containing 23 mM AEBSF, 100 mM EDTA, 2 mM Bestatin, 0.3 mM Pepstatin A, and 0.3 mM E-64 (Sigma-Aldrich).
4. Refrigerated centrifuge with adaptor for 15 and 50 ml conical centrifuge tubes.
5. Ultrasonic liquid processor or sonicator, with a small probe/tip able to be inserted in a 15 ml conic centrifuge tube.
6. 10 % sodium dodecyl sulfate (SDS) in water.

3 Methods

3.1 Growing Sf9 Cells and Baculoviruses

1. Put 50 ml of Sf9 cells in SF-900 II SFM medium supplemented with 10 % FBS (*see* **Note 1**) and 1 % of glutamine–antibiotics solution in a 250 ml sterile Erlenmeyer flask (*see* **Note 2**) at a concentration of $2–5 \times 10^5$ cells/ml. Place the flask in a shaking incubator and incubate at 29 °C with moderate shaking (120 rpm).
2. Three days later, take a small aliquot of the culture, mix with Trypan Blue solution and count cells by placing a small amount of the mixture on a Neubauer chamber (*see* **Note 3**).
3. When cell concentration rises over 2×10^6 cells/ml (usually 3 days), dilute the culture 1/10 in fresh medium and repeat the incubation.
4. For amplifying baculovirus stocks, take 2×10^7 Sf9 cells from the suspension culture and place them on a 175 cm^2 T-flask. Wait until they get attached to the flask (at least 1 h), remove used medium and replace with 50 ml of fresh medium.
5. Add 0.1 plaque forming units (pfu) of baculovirus stock per cell, distribute well and incubate for 5–7 days at 29 °C (*see* **Note 4**).
6. Decant the incubation medium into a 50 ml centrifuge tube and use it as stock virus. Store it in the dark at 4 °C.
7. Sterilize all used materials in the autoclave before discarding.

3.2 Titration of Baculovirus Stocks

1. In 6-well plates, plate 1.2×10^6 cells/well and allow to settle at least 1 h.
2. While the cells are settling, prepare serial dilutions of the baculovirus stocks in medium SF-900 II SFM without supplements. Start with the 10^{-2} dilution: add 10 µl of the viral stock in an eppendorf tube containing 990 µl of medium SF-900 II. From that, prepare serial 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} dilutions by adding 100 µl from the previous dilution to 900 µl of SF-900 II medium on an eppendorf tube to get the next dilution (*see* **Note 5**).
3. Replace medium from cells with 800 µl of SF-900II without supplements.
4. To each well, add 200 µl of each virus dilution: make duplicate samples for each dilution and label the plates accordingly.
5. Incubate at 29 °C 1 or 2 h with occasional rocking.
6. Remove the inoculum and cover the cells with 3 ml of melted solid titration medium (*see* **Note 6**). Allow the medium to cool and solidify.

7. Add 2 ml of medium SF-900 II supplemented with 10 % FBS and 1 % glutamine–antibiotics on top of the solid medium and incubate at 29 °C for 4 days.
8. Replace the overlaying liquid medium with neutral red staining solution and incubate in the dark for at least 4 h, but no longer than 12 h (*see* **Note 7**).
9. Remove the staining solution and count plaques under a transilluminator by turning the 6-well plates upside-down.
10. Calculate viral titer: "medium number of plaques in the duplicate samples" × "dilution order" × "inoculum dilution" (*see* **Note 8**).

3.3 Validation of the IC-Tagging Method for Your Particular Epitope

1. In a laminar flow hood, immerse round 15 mm diameter coverslips in methanol, sterilize them by flaming and put them in sterile, tissue-culture 12 well plates.
2. Trypsinize CHO-K1 monolayers (or the cell of your choice) (*see* **Note 9**), count them as above in the Neubauer chamber and add 6×10^5 cells/well to the coverslip-containing 12-well tissue-culture plates.
3. Transfect CHO-K1 cells (*see* **Note 10**) with eukaryotic expression plasmids for muNS-Mi (A), the epitope of interest (B), and the epitope of interest carrying the IC-tag (C), either individually, or using the following combinations: A + B and A + C.
4. Incubate to allow expression for 24–48 h.
5. Remove medium, wash three times with PBS and add 0.5 ml/well of 4 % PFA fixing solution and incubate at 37 °C for at least 15 min.
6. Wash once with PBS, and permeabilize by incubating 5 min at room temperature with PBS containing 0.5 % of Triton X-100.
7. Wash once with PBS and incubate for 1 h with blocking solution on a rocking platform at room temperature.
8. Incubate with the corresponding primary antibodies. Incubate wells expressing the individual proteins with their corresponding antibodies, and the combinations (A + B; A + C) with the antibodies against the corresponding epitopes. For most antibodies, a dilution of 1:1000 in PBS or blocking solution will work well.
9. Wash three times with PBS and add the secondary antibody of your choice following the manufacturer's instructions (*see* **Note 11**).
10. Remove the coverslips with forceps and place them on a slide on top of a drop of mounting medium, with the cells facing down (*see* **Note 12**).

11. Let them dry for at least 1 day and observe them on a fluorescence microscope: check if the distribution of the IC-tagged epitopes change upon co-expression with muNS-Mi as can be seen for GFP in Fig. 1 (*see* **Note 13**).

3.4 Microsphere Production and Purification

1. Dilute Sf9 cells grown in suspension at least ten times with fresh medium to a final concentration of 1.5×10^6 cells/ml.
2. Add 100 ml of the cell suspension to a sterile 500 ml Erlenmeyer flask (*see* **Note 2**) and infect them with 0.5 pfu/cell of each baculovirus (expressing muNS-Mi and the IC-tagged epitope of interest).
3. Incubate at 29 °C for 6–7 days: after the fourth day, take aliquots every day to check at the microscope for the presence of microspheres. Stop the incubation when most cells are dead (*see* **Note 14**).
4. Spin down the cells for 7 min at 1500 × *g* and 4 °C (*see* **Note 15**).
5. Wash the cells twice with 10 ml PBS containing protease inhibitor cocktail in a 15 ml centrifuge tube, spinning as in **step 4** in each wash.
6. Resuspend the pellet in RB-T buffer containing the protease inhibitor cocktail and leave 5 min on ice.
7. Introduce the sonicator tip in the cell suspension keeping the tube in on ice and sonicate giving two pulses of 1 min each, separated by a 30 s rest (*see* **Note 16**).
8. Centrifuge at 500 × *g*, 5 min at 4 °C.
9. Wash the pellet four times with 5 ml of RB-T buffer and resuspend finally in 1 ml of RB-buffer.
10. Check the appearance of the MS at the microscope: if cell debris or intact nuclei are still visible in the sample, repeat the procedure from **step 7** (*see* **Note 17**).
11. Check the incorporation of your epitope in the purified microspheres by SDS-PAGE, followed by Coomassie blue staining and/or Western-blot analysis (*see* **Note 18**).

4 Notes

1. We use SF-900 II medium because cells reach higher densities in this medium, but we add serum to avoid proteolysis when cells break after long periods of incubation for microsphere formation.
2. For being used in suspension cultures of Sf9 cells, we put two layers of aluminum foil as a lid for Erlenmeyer flasks. Put the inner layer (that should be double) and fit it to the flask's shape. Then add the outer layer (could be simple or double)

and sterilize by autoclaving. The aluminum foil cover should reach at least half the height of the erlenmeyer flask (ideally 3/4) so the outer layer (dirty) lid, is removed when the culture is taken into the laminar flow hood, where the inner double layer will serve as clean lid once conveniently loosened.

3. We dilute the cell suspension five times with Trypan blue solution (400 µl Trypan Blue solution 0.25 % in PBS plus 100 µl of cell suspension), before loading the cell suspension into the Neubauer chamber. Thus, we multiply five times the result of cell counting in the chamber to obtain the cell concentration.
4. We use to get higher baculovirus titers when allowing them to replicate until most cells die. The serum in the culture medium protects the released baculovirus from damage by proteolysis.
5. Always change the micropipette tip between dilutions.
6. To prepare the titration medium, melt the 4 % LMP agarose solution in the microwave. When completely melted, mix with cold medium SF-900 1.3×: use 9 ml of agarose per 30 ml of medium. Use immediately.
7. Always check if the Neutral Red solution presents precipitates and discard if that is the case. Buy the solution fresh and in small volumes.
8. Example: Taking that you followed the given protocol and inoculated with 200 µl of the corresponding dilution, plus 800 µl of medium, that makes a inoculum dilution of five times. Thus, if the 10^{-6} dilution shows 15 plaques in one duplicate and 17 in the other, $15 + 17 = 32$; $32{:}2 = 16$; Titer $= 16 \times 10^6 \times 5 = 8 \times 10^7$ pfu/ml.
9. It is advisable to check first if the method works with the protein of your choice in a mammalian cell line like CHO-K1. Sometimes is difficult to decide if the IC-Tagging is working when observing baculovirus-infected Sf9 cells. This is because some proteins produce precipitates when overexpressed with the baculovirus system, and also because the insect cells are spherical and the nucleus increases size upon baculovirus infection. Thus, testing the system in a typical flat mammalian cell line makes easier the observation of the possible capture of the IC-tagged protein by muNS-Mi MSs.
10. There are many commercial transfection reagents that produce good results, and all of them use their own protocols. In our laboratory we use as standard Lipofectamine® 2000 from Life Technologies and follow the manufacturer's instructions, using between 0.5 and 1 µg of each plasmid per transfection.
11. At this point the DNA stain DAPI can be included and co-incubated with the secondary antibody for nuclear staining. For this, dilute the 1000× DAPI solution to 1×.

12. In order to obtain cleaner preparations, we use to place the coverslips on the slides with the cells facing up, put a drop of mounting medium on top and cover them with clean coverslips. In this way we use to put two or three round coverslips (15 mm diameter) per slide, and cover them with a single, rectangular, 22 × 60 mm coverslip.
13. In Fig. 1 we included for comparison the same example performed in Sf9 cells. In this particular case, the recruiting of IC-tagged GFP to muNS-Mi MSs is perfectly seen in both cell lines.
14. MSs can be obtained after 3 days of infection [2] with the recombinant baculoviruses, but we observed that the method is more reproducible, consistent and requires less baculovirus input when the infections are allowed to proceed for 6–7 days [5]. The drawback of this method is the extensive cell lysis that could lead to protein damage by proteolysis. That is why we add serum to the culture medium.
15. The pelleted cells can be frozen at this point and the protocol continued in a different day. Thus, different pellets can be accumulated and simultaneously purified at convenience.
16. Different sonicators can produce different results. The one that we use in our laboratory is a DR. Hielscher UP200S, and we perform the sonication at maximum amplitude. Some other factors affecting the sonication step are the volumes used and the position of the sonicator tip. To be sure that the sonication was successful we use to check the cell suspension at the microscope before and after sonication to be sure that all nuclei were broken.
17. At this point, and when necessary, a single sonication pulse is usually enough.
18. In some particular preparations, the MSs are difficult to dismantle and variable amounts of protein are not able to enter the separating SDS-PAGE gel, accumulating in the stacking gel or the bottom of the wells. Thus, we dismantle the MSs by incubating them in 10 % SDS for 15 min before electrophoresis. Then, we mix them with SDS-PAGE loading buffer, boil them 5 min and proceed with the electrophoretic run.

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Chapter 3

Plant-Based Vaccine Antigen Production

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Abstract

The transient and stable expression of potentially therapeutic proteins in plants is a promising tool for the efficient production of vaccines and antibodies at low cost connected with a practically unlimited scale-up. To achieve these goals, two major challenges, inadequate production levels and non-scalable purification technologies, have to be overcome. Here we present and discuss protocols enabling to perform influenza vaccine production by transient expression in tobacco plants, to perform analytical experiments as Western blot, ELISA, and hemagglutination assays and to purify the antigens by classical affinity chromatography and scalable membrane-based Inverse Transition Cycling.

Key words Influenza vaccine, Membrane-based inverse transition cycling, ELPylation, Plant-derived hemagglutinin, Hemagglutinin trimer

1 Introduction

The biotechnological production of therapeutic proteins needs efficient expression and purification systems for recombinant eukaryotic proteins in native conformation. Transient or stable expression of such proteins in plants are promising tools to achieve unlimited scale-up potential and relatively low production cost [1, 2]. Nevertheless, the downstream processing steps are comparable in different production systems as mammalian cells, yeasts, bacteria or even plants. This cost could account for more of 80 % of the overall cost [1, 3]. Two major challenges have to be overcome to develop plant-made recombinant proteins as therapeutical tools: inadequate production levels and non-efficient and non-scalable purification methods. Here, the design and use of fusion proteins as elastin-like-polypeptide derivatives could help to overcome these limitations. ELPylation has been shown to enhance expression after transient and stable transformation in many cases [4, 5]. Furthermore, this tag could be used to purify the fusion proteins by a procedure called "Inverse Transition Cycling". This is

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especially useful for veterinary applications, where the need for low price production is a typical property of this market. This is essentially true for vaccine production, because ensuring animal health is a crucially important welfare goal for producers. This could be reasoned by animal care regulations or by the need to avoid contaminated food that causes important public health concerns. Zoonotic diseases as avian flu are of extraordinary interest in this respect. Outbreaks of avian flu and swine flu in the last years underlined the need to develop efficient and scalable vaccination methods [6]. The trimerization of the major flu antigen hemagglutinin (HA) seems to be an essential tool to achieve sufficient antigenicity [7]. This has also been shown for plant-derived hemagglutinins and hemagglutinin-ELP fusions [8]. ELPylation has been used as a tool to enhance expression as well as to develop a cheap and scalable purification method, also for trimers [8, 9].

In this chapter we provide and describe methods to produce ELPylated proteins in plants by transient expression and by ELP-dependent purification.

2 Materials

2.1 Plant Transient Expression Components

1. YEB medium: 5 g/l beef extract, 1 g/l yeast extract, 5 g/l peptone, 5 g/l sucrose, 2 mM $MgSO_4$, pH 7.0. The medium is sterilized by autoclaving.
2. Antibiotics: kanamycin (Kan), carbenicillin (Carb), and rifampicin (Rif). One gram of each antibiotic is added to a falcon tube. Water is added to 20 ml, resulting in the stocking solution 50 mg/ml. Antibiotic solutions are sterilized by passing through 0.2 μm cellulose acetate membrane.
3. 0.1 M MES. Dissolve 19.5 g of MES in 1 l water.
4. 1 M $MgSO_4$.
5. *Nicotiana benthamiana* plants: Plants are cultivated in the greenhouse at 21 °C, 16 h light per day. After 6–8 weeks old, they are ready for agro-infiltration.
6. Agrobacterial C58C1 strains harboring the shuttle vectors. These vectors are used for expression of monomeric hemagglutinin (H5) and ELPylated hemagglutinin (H5-ELP); trimeric hemagglutinin ((H5pII)3) and ELPylated hemagglutinin ((H5pII-ELP)3) (Fig. 1a, b).
7. Agrobacterial strain harboring the shuttle vector for expression of HcPro.

2.2 SDS PAGE and Immunoblotting Components

1. Hand casted SDS polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGEs are prepared by using the protocol described by Laemmli [10].

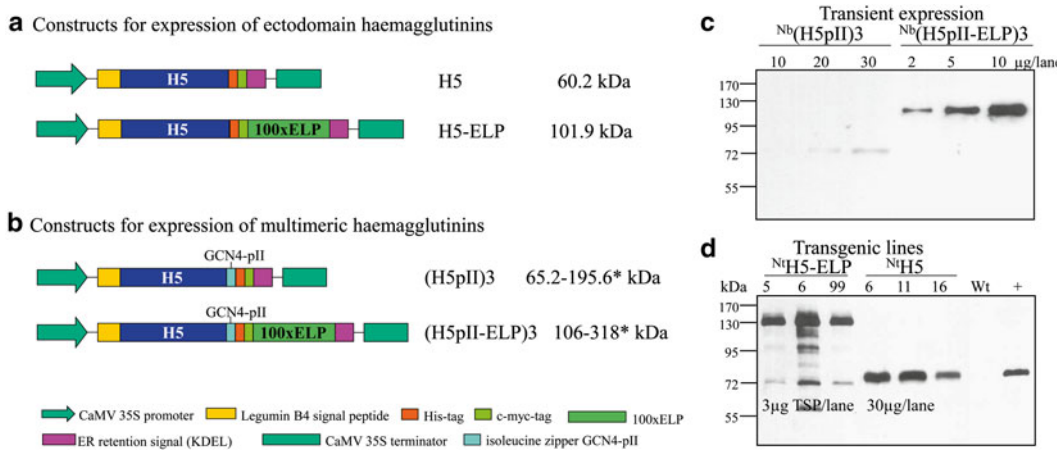

Fig. 1 Heterologous expression of influenza virus HAs in tobacco. The expression cassettes were (**a**) H5 (ectodomain of HA subtype 5), (**b**) multimeric H5 (H5 including the GCN4-PII trimerization motif). (*Asterisk*) The molecular weight range of the trimeric HAs. Western blotting analysis of leaf extracts from transgenic plant expressing HAs (**c**) transiently in *N. benthamiana* or (**d**) stably in *N. tabacum*. Signals were detected with either an anti-His antibody (**c**) or an anti-c-myc monoclonal antibody (**d**). +: 1 ng ^{Nt}anti-hTNFα-VHH-ELP, *Wt*: non-transgenic tobacco. The *numbers* refer to independent primary transgenics (**d**). *Nt, Nb* recombinant proteins expressed in stable transgenic *N. tabacum* and transiently transgenic *N. benthamiana*, respectively; *TSP* total soluble protein (reproduced from ref. [8] with permission from John Wiley & Sons)

2. SDS-PAGE running buffer pH 8.3: 125 mM Tris–HCl, 960 mM glycine, 0.5 % SDS.
3. 2× SDS Sample Buffer pH 8.3: 100 mM Tris–HCl, pH 6.8, 4 % SDS, 0.2 % (w/v), bromophenol blue, 20 % (v/v) glycerol.
4. PageRuler™ Prestained protein ladder (Thermo Scientific).
5. Whatman® nitrocellulose membranes.
6. Transfer buffer: 10 % methanol (v/v), 24 mM Tris, 194 mM Glycine.
7. Tris buffered saline pH 7.8 (TBS): 20 mM Tris–HCl, 180 mM NaCl.
8. Phosphate buffered saline, pH 7.4 (PBS buffer): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4).
9. Blocking solution: 5 % (w/v) fat-free milk in TBS.
10. ECL™ Western blotting detection reagents (GE Healthcare).
11. Antibodies: anti c-myc monoclonal antibody, monoclonal anti-polyhistidine antibody (Sigma), donkey anti-rabbit IgG horseradish peroxidase linked whole antibody, sheep anti-mouse IgG horseradish peroxidase linked whole antibody (GE healthcare), polyclonal rabbit anti-H5N1 virus antibody provided kindly by Dinh Duy Khang, Institute of Biotechnology, Hanoi, Vietnam.

2.3 ELISA Components

1. *p*-nitrophenyl phosphate (pNPP) in 0.1 M diethanolamine-HCl (pH 9.8).
2. Microtiter plates (ImmunoPlate Maxisorp, Nalgen Nunc International, Roskilde, Denmark).
3. Mouse polyclonal antibodies present in sera are prepared as described (*see* Subheading 3.6).
4. Secondary antibody: Rabbit anti-mouse IgG (whole molecule), alkaline phosphatase conjugate (GE healthcare).
5. Purified antigens: ^{Nt}H5, ^{Nt}H5-ELP, ^{Nb}(H5pII)3, ^{Nb}(H5pII-ELP)3.
6. PBST, 0.05 %: Tween 20 in PBS at 0.05 %.
7. Blocking solution, 3 % of bovine serum albumin (BSA): BSA in PBST at 3 %. Keep at 4 °C.
8. ELISA reader.

2.4 Protein Purification Components

1. 2 M NaCl: this solution is settled in a water bath at 25 °C.
2. Water bath: temperature is set up at 60 °C.
3. Water bath: temperature is set up at 25 °C.
4. Thermometer.
5. Vacuum pump.
6. 0.2 µm cellulose acetate membrane (Sartorius Stedim, Goettingen, Germany) with membrane diameter in 47 mm.
7. 0.22 µm polyethersulfone membrane (Corning, USA) with membrane dimensions: 63 × 63 mm.
8. 0.3 µm mixed cellulose ester membrane (Millipore, USA), membrane diameter 47 mm.

2.5 Hemagglutination Assay Components

1. Inactivated virus: rg A/swan/Germany/R65/2006(H5N1).
2. Purified hemagglutinins: H5, H5pII, H5-ELP, and H5pII-ELP.
3. PBS buffer, pH 7.4.
4. Plastic V-bottom microtiter plates.

3 Methods

3.1 Transient Expression of Recombinant Vaccine Antigen in Plants

1. A single colony of Agrobacteria harboring shuttle vectors for expression of recombinant proteins (Fig. 1a, b) and the plant vector for expression of HcPro are pre-cultivated separately in 40 ml of YEB medium with 50 µg/ml Kan, 50 µg/ml Carb and 50 µg/ml Rif. Agrobacterial cultures are grown overnight at 28 °C and 150 rpm resulting in the pre-cultures (*see* **Note 1**).

2. The single pre-culture is transferred to 300 ml of a new YEB medium containing appropriate antibiotics. Agrobacterial cultures are grown further 24 h at 28 °C and 150 rpm.
3. Bacteria harboring the shuttle vector for expression of a recombinant protein and the plant vector for expression of HcPro are combined with an equal volume, and concentrated by centrifugation at 4000 × *g*, 30 min, 4 °C.
4. Bacterial pellets are suspended in the fresh infiltration buffer (10 mM MES, 10 mM $MgCl_2$, pH 5.6). Agrobacterium suspensions are adjusted by the infiltration buffer to a final OD_{600} of 0.6–1.0.
5. A plastic beaker containing 2 l of the Agrobacterium suspension is placed inside a vacuum desiccator.
6. A whole plant is completely immersed in the Agrobacterium suspension. Vacuum is applied for 2 min and then rapidly released. Un-infiltrated and broken leaves are removed (*see* **Note 2**).
7. The plants are then placed in the greenhouse at 21 °C, 16 h light per day. Four days after infiltration, leaf samples are harvested and stored at −80 °C (*see* **Note 3**).

3.2 Western Blot Analyses

1. Frozen leaf discs are homogenized in a Mixer Mill MM 300 (Retsch, Haan, Germany) and the resulting powder is suspended in SDS sample buffer (*see* **Note 4**).
2. Samples are kept at 95 °C for 10 min.
3. Samples are then centrifuged at 19,000 × *g*, 30 min, 4 °C. Suspension is collected into a new 1.5 ml tube (*see* **Note 4**).
4. The concentration of total soluble protein (TSP) is determined using the Bradford assay (*see* **Note 5**).
5. Extracted plant proteins are separated by reducing SDS-PAGE (10 % polyacrylamide).
6. Proteins on the gel are electrotransferred to nitrocellulose membranes at 18 V, overnight.
7. The membrane is blocked with 5 % (w/v) fat-free milk powder dissolved in TBS for 2 h.
8. The membrane is then incubated for 2 h at room temperature with a primary antibody (monoclonal anti-polyhistidine antibody (Fig. 1c), monoclonal anti-c-myc antibody (Fig. 1d) or rabbit antibody against NIBRG-14 virus (Fig. 3b)) in TBS containing 5 % milk (*see* **Note 6**).
9. The membrane is washed five times with TBS containing 0.5 % milk.
10. The membrane is then incubated for 2 h at room temperature with a HRP-conjugated sheep anti-mouse IgG (Fig. 1) or HRP-conjugated donkey anti-rabbit IgG (Fig. 3) in TBS containing 5 % milk (*see* **Note 7**).

11. The membrane is washed three times with TBS containing 0.5 % w/v fat-free milk. TPS and PBS are used for the penultimate and final washes.
12. Peroxidase activity is detected by applying equal volume of Amersham ECL Western Blotting Detection Reagents.
13. The membranes are then exposed to X-ray film (*see* **Note 8**).

3.3 Immobilized Metal Affinity Chromatography (IMAC) Purification

The IMAC purification method is used to purify non ELPylated hemagglutinins tagged by polyhistidine at the C-terminal end from both stably transformed and transiently transformed leaves.

1. Leaf samples (100 g) are harvested, frozen in liquid nitrogen and homogenized using a commercial blender.
2. Plant proteins are extracted in 50 mM Tris buffer (pH 8.0). The extract is clarified by centrifugation (18,000 × *g*, 30 min, 4 °C) and then filtrated through paper filters.
3. The clear extract is mixed with Ni-NTA resin agarose (20 ml) washed twice with water and equilibrated with 50 mM Tris buffer (pH 8.0).
4. After mixing for 30 min at 4 °C, the mixture is applied on a chromatography column. Thereafter, the column is washed with 2 l of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM Imidazole, pH 8.0) (*see* **Note 9**).
5. Recombinant proteins are then eluted from the column by elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 125 mM imidazole, pH 8.0) (*see* **Note 10**).
6. Protein is concentrated by an iCON™ Concentrator with a molecular-weight cutoff of 9000 and stored at −20 °C (Fig. 3) (*see* **Note 11**).

3.4 Membrane-Based Inverse Transition Cycling Purification (mITC)

mITC is a temperature dependent purification method to enrich ELPylated hemagglutinins. Basically, when temperature of solution is raised above the transition temperature of the designed ELP [11, 12], ELPs can aggregate and form particles (about an average diameter of 357 nm [13]) which can be maintained on a membrane surface (Fig. 2a). ELP aggregates are then soluble in cold and low salt concentration buffer (Fig. 2). The protocol has been developed to purify avian ELPylated hemagglutinins both from stably transformed (Fig. 2b) and transiently transformed leaves (Fig. 2c).

3.4.1 mITC for Purification of ELPylated Hemagglutinins from Stably Transformed Leaves

1. Frozen *N. tabacum* leaves (150 g) are ground with mortar and pestle in liquid nitrogen and homogenized in 220 ml ice-cold 50 mM Tris–HCl (pH 8.0) (*see* **Note 12**).
2. Complete Protease Inhibitor Cocktail tablets (Roche, Germany) are added to the slurry, which is then cleared by centrifugation (75,600 × *g*, 30 min, 4 °C) before the addition of NaCl to a final concentration of 2 M.

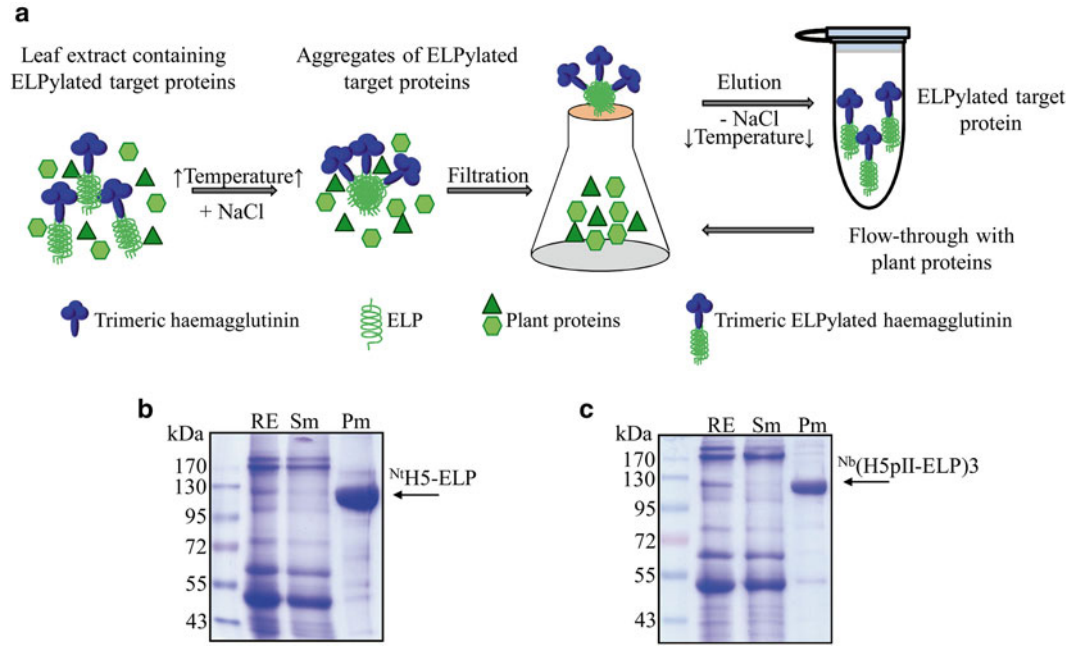

Fig. 2 Membrane-based inverse transition cycling purification method. (**a**) Plant proteins are extracted in a 50 mM Tris–HCl (pH 8.0). The extract is clarified by centrifugation. The resulting clear extract is supplemented with sodium chloride to a final concentration of 2 M, and temperature of the extract is raised to room temperature to trigger the aggregation of ELPylated proteins. The aggregated ELP fusion proteins are separated with nonELPylated plant proteins by filtration, solubilized and eluted from the membrane by low salt buffer. Proteins in the raw plant extract (RE), in the supernatant after passage through a 0.2 μm cellulose acetate membrane (Sm) and in the elution (Pm). SDS-PAGE-separated proteins detected (**b** and **c**) by Coomassie Brilliant Blue staining. *Nt, Nb* recombinant proteins purified from stable transgenic *N. tabacum* and transiently transgenic *Nicotiana benthamiana*, respectively (reproduced from ref. [8] with permission from John Wiley & Sons)

3. The cold extract with 2 M NaCl is centrifuged again at 75,600 × *g* for 30 min at 4 °C (*see* **Note 13**).
4. The solution is then passed through a 0.22 μm polyethersulfone membrane (Corning, USA) with the temperature maintained at 4 °C, to produce a pre-treated extract (*see* **Note 14**).
5. One hundred milliliter of the pre-treated extract is warmed to room temperature and passed through a 0.2 μm cellulose acetate membrane using a vacuum pump (Vacuubrand, Germany) (*see* **Note 15**) (Fig. 2).
6. The membrane is washed twice with 2 M NaCl to remove plant nonELPylated proteins (*see* **Note 16**) (Fig. 2).
7. Ice-cold Millipore-Q water is then passed through the filter to elute the protein-ELP fusions (*see* **Notes 17** and **18**) (Figs. 2 and 3).

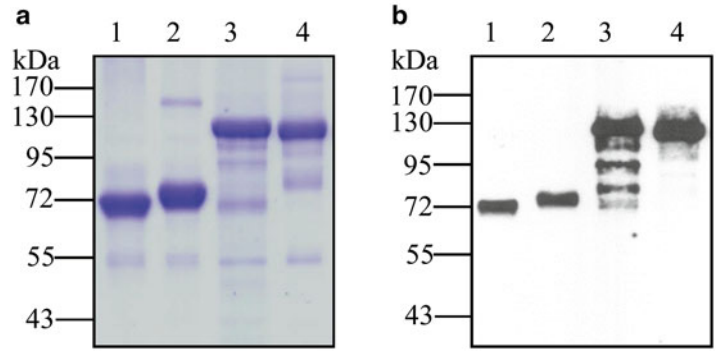

Fig. 3 Purity of heterologously expressed HAs used for immunization. (**a** and **b**) *Lane 1*, ^{Nt}H5, *lane 2*, ^{Nb}(H5pII)3, *lane 3*, ^{Nt}H5-ELP, *lane 4*, ^{Nb}(H5pII-ELP)3. Panel (**a**) represents a Coomassie Brilliant Blue stained gel, and panel (**b**) a Western blot in which the HAs were detected by probing with rabbit antibody recognizing A/Vietnam/1194/2004(H5N1). (Reproduced from ref. [8] with permission from John Wiley & Sons)

3.4.2 mITC for Purification of ELPylated Hemagglutinins from Transiently Transformed Leaves

When ELP fusion proteins are purified by mITC from the transiently transformed *N. benthamiana* leaves, the purification procedure is adapted.

1. Fifty grams of the transiently transformed *N. benthamiana* leaves are ground in liquid nitrogen. The resulting powder is homogenized in 170 ml ice-cold 50 mM Tris–HCl (pH 8.0) by a commercial blender (*see* **Note 19**).
2. The plant extract is cleared by centrifugation three times (75,600 × *g*, 45 min, 4 °C) before the addition of NaCl to a final concentration of 2 M.
3. The cold extract with 2 M NaCl is centrifuged again at 75,600 × *g* for 45 min at 4 °C (*see* **Note 20**).
4. The resulting extract with 2 M NaCl is passed through a 0.3 μm mixed cellulose ester membrane and then through a 0.22 μm polyethersulfone membrane. This filtrate is centrifuged again (75,600 × *g*, 30 min, 4 °C) resulting in the pre-treated extract (*see* **Note 21**).
5. Eighty milliliters of the pre-treated extract is warmed to room temperature and passed through a 0.2 μm cellulose acetate membrane using a vacuum pump (Vacuubrand, Germany) (*see* **Note 15**) (Fig. 2a, c).
6. The membrane is washed twice with 2 M NaCl to remove plant nonELPylated proteins (*see* **Note 16**) (Fig. 2a, c).
7. Ice-cold Millipore-Q water is then passed through the filter to elute the protein-ELP fusions (*see* **Notes 17** and **18**) (Figs. 2 and 3).

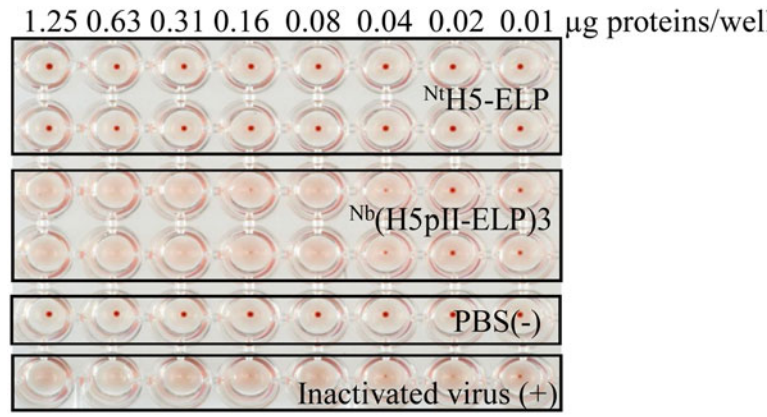

Fig. 4 Hemagglutination assay of purified ELPylated HAs. PBS was used as negative control, and an inactivated virus (strain rg A/swan/Germany/R65/2006(H5N1)) as positive control. (Reproduced from ref. [8] with permission from John Wiley & Sons)

3.5 Hemagglutination Assay

3.5.1 Collection and Preparation of Rooster Red Blood Cells

1. For hemagglutination test and hemagglutination inhibition test, blood (8 ml) from the wing vein of a rooster which is not vaccinated for Newcastle disease virus or other pathogens are collected in a sterile bottle containing 3.2 % sodium citrate, pH 5.1–5.3 (2 ml).
2. The bottle is rotated gently to mix thoroughly.
3. An equal volume of PBS, pH 7.4 is added and the suspension is centrifuged at 900 × *g* for 5 min two times and one time for 10 min to pellet erythrocytes.
4. The erythrocytes are washed twice with PBS, pH 7.4. 198 ml of PBS, pH 7.4 are added to 2 ml of packed red blood cells to get a final erythrocyte concentration of 1 %.

3.5.2 Hemagglutination Assay

1. 25 µl of PBS are added into all wells of a plastic V-bottom microtiter plate.
2. 25 µl of antigen are added into the first well of the plate containing.
3. Twofold serial dilution is made across the entire row.
4. 25 µl of 1 % red blood cells (RBC) were added.
5. Results are read after plates are incubated at 25 °C for 30 min. The endpoint dilution that causes a complete hemagglutination was defined as one hemagglutination unit (HAU) (Fig. 4).

3.6 Mouse Vaccination

1. 6–8 weeks old male BL6 (C57/Black6J) mice (ten per group, Charles River Laboratories, Research Models and Services, Germany GmbH) are immunized subcutaneously with ^{Nt}H5, ^{Nb}(H5pII)3, ^{Nt}H5-ELP, and ^{Nb}(H5pII-ELP)3 on day 0, 14, 21, and 35 with doses of 10 and 50 µg per immunization.

2. The antigens are formulated with 10 μg of Complete Freund's adjuvant in the first immunization and with 10 μg of Incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA) in the following immunization.
3. In control groups, mice received PBS plus adjuvants.
4. Mice are then retro-orbitally bled 1 week after the third and fourth immunization.
5. Blood samples are then centrifuged twice times at 16,200 x g, room temperature, 15 min.
6. Mouse sera are collected individually for ELISA tests.

3.7 ELISA

1. For testing mouse sera, microtiter plates (ImmunoPlate Maxisorp, Nalgen Nunc International, Roskilde, Denmark) are coated with 100 μl of 3 μg/ml recombinant antigen in PBS and incubated overnight at 4 °C.
2. After blocking with 3 % (w/v) bovine serum albumin (BSA), 0.05 % (v/v) Tween 20 in PBS (PBST) for 2 h, 100 μl of the specific dilution (2×10^{-4}) are applied and incubated at room temperature for 1.5 h.
3. Plates are washed five times with PBST, 100 μl of a rabbit anti-mouse IgG alkaline phosphatase conjugate diluted (2000 times) in 1 % (w/v) BSA in PBST are then added.
4. The enzymatic substrate, *p*-nitrophenyl phosphate (pNPP) in 0.1 M diethanolamine-HCl (pH 9.8), is added and the absorbance signal is measured at 405 nm after 1 h incubation at 37 °C (Fig. 5) (*see* **Note 22**).

4 Notes

1. The HcPro gene could also be cloned into the expression vector, but mixing of agrobacterial strains has been proven to be more comfortable.
2. It is important to remove the un-infiltrated leaves to obtain high recombinant protein concentration. The color of the un-filtrated leaves is unchanged, while filtrated leaves become darker because of penetration of bacterial suspension.
3. The transgenic plants (*N. tabacum*) expressing recombinant hemagglutinins (^{Nt}H5 and ^{Nt}H5-ELP) are germinated on 50 μg/ml kanamycin containing MS medium for 4 weeks. The healthy plants are grown at the greenhouse for further 4 weeks. Leaves are harvested and stored at –80 °C.
4. Purified hemagglutinins are diluted by SDS sample buffer and directly loaded on SDS-PAGE. Therefore, these steps are not necessary.

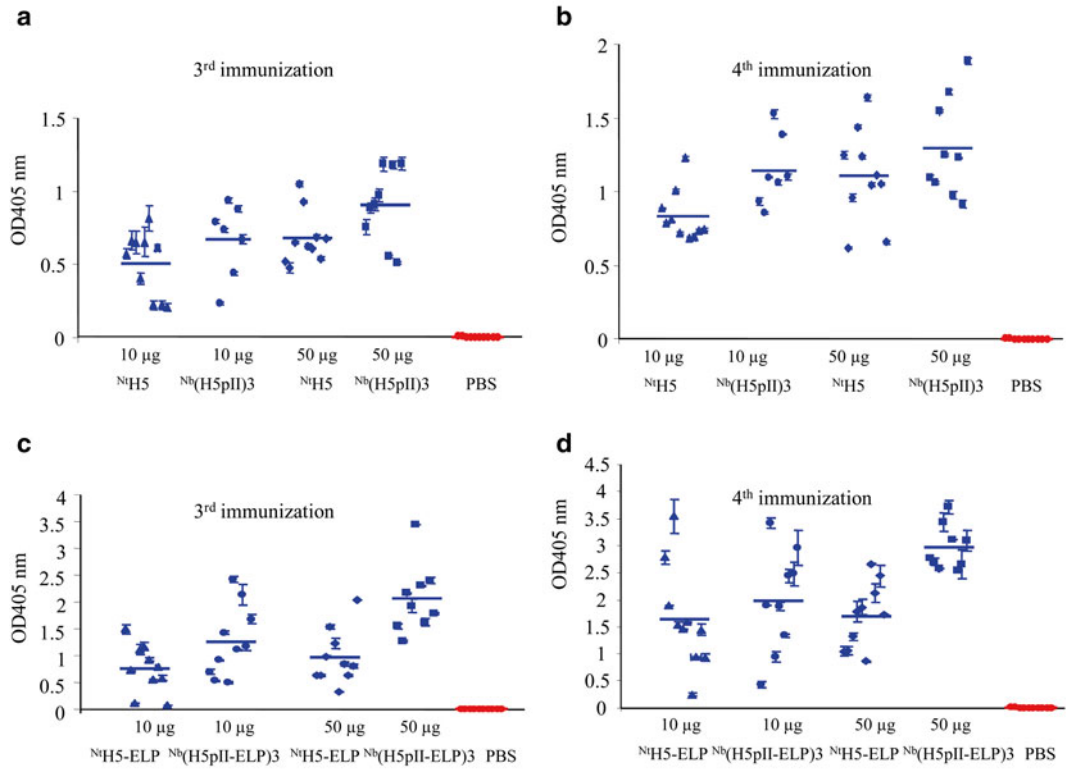

Fig. 5 ELISA-based assessment of antibody induction in immunized mice. Anti-^{Nt}H5 and ^{Nb}(H5pII)3 response after (**a**) the third and (**b**) the fourth immunization. Anti-^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 response after (**c**) the third and (**d**) the fourth immunization. A *single dot* represents the ELISA result from a single serum sample, and *bars* the mean of each test group. (Reproduced from ref. [8] with permission from John Wiley & Sons)

5. Other methods of protein concentration measurements could also be applied, but the concentration of SDS in the probe buffer and the amount of extract necessary for effective protein concentration measurements fit well if Bradford method is applied.
6. The dilution factors should be conducted following the specific manufacturer's recommendations. In this paper, a 1:2000, 1:50 and 1:3000 dilutions are applied for the monoclonal anti-polyhistidine antibody (Fig. 1c), the anti-c-myc monoclonal antibody (9E10) from hybridoma cell culture supernatant (Fig. 1d), rabbit antibody against NIBRG-14 virus (Fig. 3b), respectively.
7. The dilution factors should be conducted following the specific manufacturer's recommendations. In this paper, a 1:2000 dilution is applied for both the sheep anti-mouse IgG, HRP linked whole antibody and HRP-conjugated donkey anti-rabbit IgG from GE healthcare.

8. The incubation time depends on the sensitivity of the assay (mainly influenced by the quality of the primary antibody) and the background. Pre-experiments can be performed to optimize the whole procedure.
9. The incubation time could be prolonged, if necessary, until 12 h (overnight).
10. The imidazole concentrations for washing and elution depend on the individual proteins and should be optimized to achieve convincing results in terms of purity and effectivity.
11. The cutoff is critical and the concentration procedure should be controlled.
12. Other buffer systems which stimulate solubility of your ELPylated proteins could be used. However, buffers containing detergents like Triton are not recommended because the resulting extract will block the membrane.
13. When sodium chloride which lowers the transition temperature of ELP is added to the plant extract, temperature of the extract should be maintained at cold condition (about 4 °C) during the centrifugation steps. This condition reduces plant protease activities and avoids to trigger the transition of ELP fused proteins.
14. Temperature of the plant extract containing 2 M NaCl is adjusted to 4 °C to maintain the ELP fused hemagglutinins as soluble forms and because of filtration process also takes sometimes. This step can be performed at room temperature. However, if the pre-filter step takes long time, it should be performed at the cooling room to avoid aggregation of ELP fusion proteins caused by increasing temperature.
15. Temperature of the pre-treated extract is rapidly shifted from 4 to 25 °C by a water path at 60 °C. In this step, the ELPylated proteins are precipitated to form particles with an average diameter of 357 nm [13] which are retained on the surface of membrane with 0.2 μm.
16. 2 M NaCl solution should be kept constantly in the water bath at 25 °C. This step is performed at room temperature.
17. In this step, aggregates of the ELPylated proteins are solubilized by the ice cold-water. Other elution buffer systems could be used, however low salt buffers are recommended to use and they should be in an ice-cold condition.
18. If the ELPylated target proteins are still detected in Sm, next mITC can be performed from the **step 4** of the protocol.
19. Plant extracts from transiently transformed leaf materials pass difficultly through a 0.22 μm polyethersulfone membrane at 4 °C. Therefore, 50 g of the starting leaf materials are used in this step.

20. The high speed centrifugation is extensively prolonged to have enough clearance of the plant extract for mITC.
21. Passing of the plant extract through a 0.3 μm membrane is needed to clarify the plant extract, otherwise a 0.2 μm membrane is rapidly blocked.
22. In case of low activity the experiment could be "rescued" just by prolongation of the incubation time.

Acknowledgement

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Chapter 4

DNA Vaccines: Experiences in the Swine Model

Francesc Accensi, Fernando Rodríguez, and Paula L. Monteagudo

Abstract

DNA vaccination is one of the most fascinating vaccine-strategies currently in development. Two of the main advantages of DNA immunization rely on its simplicity and flexibility, being ideal to dissect both the immune mechanisms and the antigens involved in protection against a given pathogen. Here, we describe several strategies used to enhance the immune responses induced and the protection afforded by experimental DNA vaccines tested in swine and provide with very basic protocol describing the generation and in vivo application of a prototypic DNA vaccine. Only time will tell the last word regarding the definitive implementation of DNA vaccination in the field.

Key words DNA vaccine, Genetic adjuvant, Antigen presentation, Antibodies, Cytotoxic T-cell responses (CTL), ELI, APC, Electroporation, Swine, Veterinary virology

1 Introduction

Immunization by means of what we know today as nucleic acid vaccination was described in the early 90s [1], opening a new and promising way in the vaccination field. The principle behind DNA vaccines (the most common nucleic acid vaccines are based on the inoculation of DNA plasmids) is based on a very simple, albeit smart, principle: the capability of the cells to in vivo uptake the DNA, intracellularly express the encoded antigen and, finally, the induction of protective immune responses. In order to obtain a fine expression of the encoded proteins, the gene construct is cloned under the control of a mammalian promoter, usually, the promoter from human cytomegalovirus (CMVp). DNA vaccines can be administered in many different ways, being the two most common the intramuscular injection and the intradermal inoculation. In the first case, the plasmid is primarily taken up by muscular cells whereas in the second case, the cells that receive the plasmid are dermis cells, and, among them, the Langerhans cells (a type of Ag-presenting cells). Independently of the immunization route, the success of DNA immunization relies on the final uptake of

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DNA and/or the plasmid-encoded antigens by Professional Ag-presenting cells [2].

The advantages of DNA vaccines are many: chiefly, its safety. We do not have to worry at all about virulence, which is the main concern of attenuated vaccines. Also, depending on the DNA-construct used, we have the possibility of inducing humoral and/or cellular responses, being the lack of this latter possibility one of the main defaults of the inactivated vaccines, of importance while fighting intracellular pathogens. Last but not least, we have to keep in mind that DNA vaccines can be easily "à la carte"-designed (we can target the expressed antigens to induce different immune-responses) and, as other new generation subunit vaccines, they can behave as DIVA-vaccines (vaccines that allow us Differentiating Infected from Vaccinated Animals), an essential concept in veterinary medicine. DNA vaccination has been successfully developed in rodent-models, but results obtained in other animals showed to be contradictory. There are some commercial DNA vaccines available for fishes [3], for which DNA vaccines work extremely well, and for horses [4]. Albeit that, one of the major skepticisms generated by DNA vaccination in large animals relies in its low efficacy, in occasions attributed to the low efficiency of DNA transfection achieved in vivo. The most promising way to improve the efficiency of DNA delivery in vivo is the utilization of in vivo electroporation [5]. The use of other methods, such as biolistics or nanoparticles, to increase DNA-transfection efficacy have been also proposed. Besides the methodologies used to enhance the DNA uptake, research has provided many other strategies that have allowed enhancing the immune response induced and the protection afforded. Far from being able to present one "universal strategy" of vaccination, our experience have shown that vaccines should be tailored "à la carte", taking into account the target animal species and the pathogen to be fought.

2 Materials

1. DNA or RNA template containing the ORFs genes we want to immunize with.
2. Primers to amplify the aforementioned genes by means of PCR reaction (alternatively, synthetic ORFs can be used).
3. Plasmid DNA backbone (*see* **Note 1**).
4. Bacteria for transformation and large-scale production of plasmid DNA (*see* **Note 2**) and suitable media.
5. Mammalian Cell line for transfection, transfection reagent and suitable media.
6. Materials for Western-blotting.

7. Reagents and kits for plasmid ligation, purification of DNA (*see* **Note 3**).
8. The genetic adjuvant (*see* **Note 4**); if willing to use it.

3 Methods

As above mentioned, the first step prior to begin with the design of a DNA vaccine is to know what is relevant and what is not in protection against the virus we are working with. Which kind of immune response we are looking for? Shall we need production of antibodies or the induction of a cellular cytotoxic response? Maybe we should need both. Obviously, the answer to such questions lays in the nature of the pathogen antigens. For some viruses it may be simple, i.e., a DNA vaccine against E2 Ag from Classical Swine Fever Virus induced both humoral and cellular responses and conferred sterilizing immunity [6]. However, for many more complex pathogens, i.e., African Swine Fever Virus, is not that simple. Research from our group, based on DNA-vaccination strategy, found out that antibodies against certain proteins may even have deleterious effects [7].

The present chapter will review: (1) Methods used to enhance the efficacy of the DNA-delivery in the animal, focusing mainly on in vivo electroporation; (2) a discussion of strategies to enhance the immunogenicity of DNA vaccines (mainly focused in those approaches used in our laboratory that have been successfully used in swine); (3) a particular chapter dedicated to ELI immunization, an ideal protocol to search for protective antigens within complex pathogens; and (4) a final section dedicated to a simple protocol describing the steps involving the construction of a DNA vaccine and swine immunization and a brief consideration about prime-boost strategies.

3.1 Enhancing the DNA Delivery into Cells

One of the main criticisms of the commonly used methods of DNA immunization is the poor efficiency of in vivo transfection in animal cells. Some strategies, such as the use of electroporation, biolistics or, on the other hand, the delivery of the DNA-plasmid in formulations such as liposomes, have been proposed.

3.1.1 The Use of Liposomes

Liposomes are adjuvants of current use in traditional vaccines. Such compounds are able to entrap plasmid DNA and therefore facilitate the entrance of such DNA into the cell by penetrating the lipid bilayer of the cell membrane. Liposome-entrapped DNA has shown to enhance both humoral and cell-mediated immune response in a more effective way than naked-DNA. Such results could be explained by the ability of liposomes to protect their DNA content from local nucleases and direct it to APCs in the

lymph nodes draining the injected site [8]. Moreover, the use of liposomes to deliver DNA vaccines has opened the door to other administration routes for this type of vaccines, such as the oral route, playing the liposome a protecting role of the plasmid DNA against DNAse present in digestive tract. In mice model, the efficacy of a liposome-driven oral DNA vaccine has been successfully assayed [9] showing protection against Influenza challenge.

3.1.2 The Gene-Gun

The biolistics approach implies shooting the skin with plasmid-coated micron-sized particles (commonly made of gold) by means of ballistic devices such as the gene-gun. The particles are accelerated into skin tissue using the force of an electric discharge or compressed helium. Thus, DNA is delivered directly onto the cytoplasm of epidermal keratinocytes and thus, a very small amount of DNA is needed, compared to traditional DNA injection [10]. This particle-mediated DNA vaccine approach has shown to be effective in swine, inducing comparable CD8+ T-cell responses and superior antibody production with 100–1000 fold less DNA when compared to naked-DNA injection [11]. When epidermis cells are renewed, the transformed cells containing plasmid DNA will disappear, therefore stopping the production of Ag. Quite a different panorama of what happens with intramuscular DNA injection, where cells are able to produce the proteins for a longer period of time. Some authors [12] claim that, due to the limitations of the approach, it seems that the use of biolistics is slowing down, clearing a path to in vivo electroporation systems, the current most promising way to enhance DNA delivery into cells.

3.1.3 Electroporation

The principle behind electroporation is quite simple: to induce a temporary permeabilization of the cell membrane to allow the penetration of large molecules such as DNA. Briefly: Just after the injection of the DNA we will deliver in the plasmid-injection area a strong but short electric pulse followed by some other pulses a bit larger in duration but milder in voltage. The first pulse permeabilizes the cell membranes, whereas the following pulses induce a sort of in vivo electrophoresis, thus attracting the previously injected plasmid DNA into the temporary-permeabilized cells. Later on, the membranes recover its normal integrity. Optimal electroporation conditions result from a very subtle balance: If conditions are too aggressive, we will destroy the cells whereas if they are too mild, we will not induce the desired permeability. We can modify the following parameters: voltage (from 60 to several hundred Volts, depending on the tissue and type of electrode), pulse length (in milliseconds) and the number of pulses (ranging commonly from 2 to 12) [5] (*see* **Note 5**). On the other hand, some researchers suggest that mild tissue damage induced by the electric discharges may act as an adjuvant, inducing a release of danger signals (i.e., inflammatory mediators) in the affected zone,

enhancing the presence of APCs, as well as an increase of the release of the antigen proteins from injured cells, thereby improving antigen presentation [13].

There are various devices developed to perform in vivo electroporation in the market: TriGrid™ (Ichor medical systems), AgilePulse™ (BTX Harvard Apparatus), Cliniporator™ (IGEA), among others. Types of electrodes may vary from needle-free patch electrodes to multiple-needle array electrodes, depending logically on the chosen apparatus, but also on the tissue to be injected. The fact that animals must be anesthetized before the treatment, together with the bulky appearance of most devices, makes in vivo electroporation currently unfeasible for swine veterinary practice, remaining therefore to be employed in research or in small animal or human medicine. It is expected that a non too-distant future will bring us more portable devices, suitable to be used in massive vaccination as it happens in a regular swine farm.

3.2 Enhancing the Immunogenicity of the DNA Vaccine

Due to the complexity of the immune system, we should keep our experimental approach as simple as possible and therefore, we recommend choosing the adjuvant that better suit your interests taking into account both the animal species and the immune response willing to be induced. Here we summarize some of the most successful results described in the literature for DNA vaccines in swine.

3.2.1 The Use of Cytokines as Plasmid–Encoded Adjuvants

The use of plasmids encoding cytokines together with the DNA construct of interest has been proposed as one of the best adjuvant strategies for DNA immunization protocols. The main advantage of this strategy relies in the fact that after in vivo administration, the cytokines will act concomitantly and locally in the zone of Ag expression, therefore avoiding undesirable effects observed when they are systemically administrated and providing a more robust and long-term stimulation. The election of the cytokine to be included (IFN-γ, IL-18, IL-2, IL-12…) will depend on the type of response we want to elicit (*see* **Note 6**). Mostly of the reports of cytokines as DNA-adjuvant are carried out in mice models and, although there are not many reports on veterinary vaccination [14, 15], this strategy looks promising for the near future [12].

3.2.2 Targeting the Encoded Ag

From the many potential strategies to be used, this review will be focused in those successfully used for swine in our laboratory: (1) The employment of strategies aiming to drive the vaccine antigens to Antigen Presenting Cells (APCs); (2) to direct the vaccine-encoded antigens to the MHC-I pathway, in occasions avoiding the Ab production: and (3) the use of plasmid cocktails, an advantage of DNA immunization that allow even to immunize animals with thousands of plasmid-cocktails thus covering even large proteomes; an strategy first approached by Barry et al. [16] and named as ELI-vaccination.

Enhancing the Induction of CD4+ T-Cells and Antibodies: Directing the Viral Ag to APC

One of our favorite options is to target the viral Ag to the sites of the immune induction, a strategy first described in a mouse model with the use of CTLA-4 as a genetic adjuvant [17]. We have followed a similar approach, this time using as carrier the APCH1 molecule. APCH1 is a fragment of an Ab that recognizes an epitope of the Class II Swine Leukocyte Antigen (SLAII) molecule, highly expressed in swine APCs [18]. By fusing our DNA-construct to APCH1, the encoded fusion products were efficiently directed *in vitro* to SLAII positive cells and enhanced the *in vivo* induction of both specific antibodies and T-cell responses [7, 19]. Interestingly enough, the protection afforded by the vaccines totally varied depending on the antigens and pathogen used, going from the sterilizing protection observed in some pigs against Foot and Mouth Disease Virus (FMDV) challenge [19] to the viremia exacerbation observed in the case of the African swine fever virus (ASFV) challenge [7]. These results demonstrate once more that adjuvants are not universal and also that, in order to design a rational vaccine against a given disease, is absolutely required to have a deep knowledge about its pathogenesis.

The second choice we used in our laboratory was based on the so called sHA; extracellular domain of the ASFV Hemagglutinin, a molecule with important similarities to CD2 leukocyte molecule [20]. As described for the APCH1, fusion of antigens to the sHA, allowed the in vitro binding to APCs, most probably due to the expression on their surface to CD2 receptors. Also as described for APCH1, the in vivo reflection of this fusion allowed to exponentially enhance both the antibodies and the T-cell responses induced in pigs that again, did not result in any protection against ASFV lethal challenge [21].

Of course there are other strategies to target antigens to APC, but not many have been successfully used in swine [22]. In this way, the use of CD169 or CD163, two endocytic receptors mainly expressed on macrophages, resulted in a strong humoral response: either CD169 or CD163 could favor antigen uptake by subcapsular sinus macrophages, leading to the initiation and improvement of the humoral immunity [23]. The use of TLR-2, a member of the Toll-like receptors family, looks also promising in swine although the enhancement of antibody production was not as outstanding as the obtained with CD163 or CD169 [22].

Enhancing CTL Induction: Directing the Viral Ag to MHC I Pathway

The lack of success of our ASFV vaccines could be explained by either a failure in the induction of protective CTL responses, in view of the fact that specific CD8-T cell responses has been described as key players in ASFV protection [24], or simply due to the bad selection of the vaccine antigens (just 3 out of 150 antigens encoded by ASFV). In order to solve this “dilemma”, we decided to obtain a vaccine prototype encoding our favorite antigens as fusions with ubiquitin; a strategy successfully used in mice

to optimize the Class I antigen presentation of the encoded antigens, thus enhancing the CTL induced in vivo. Briefly, after the transcription, our DNA-construct results tagged with ubiquitin, which targets the protein to the proteasome. Hence, the protein is degraded by the proteasome and cleaved into short peptides that are carried via the "TAP" transporters to the endoplasmatic reticulum and there, such peptides are presented via MHC Class I to the specific cytotoxic CD8+ T-cells. Ten years later, also in our laboratory, we have been able to extend these studies to pigs by using DNA vaccines encoding the same previously mentioned ASFV antigens. Thus, the fusion of ubiquitin to the ASFV antigens not only enhanced the CTL induction, but also abrogated the antibody induction in vivo, as it was also described before for mice [25] and most importantly, allowed conferring partial protection ASFV against lethal challenge for the first time [21].

Once more, DNA vaccines provided with new lessons: One same antigen can induce from exacerbation to protection, depending on the immunological outcome that it provokes. Ubiquitin, as any other genetic adjuvant can fail to exhort its effects, as it happen in the case of the Aujeszky glycoproteins. Ubiquitination of such glycoproteins did not lead to an enhancement in the protection afforded, most probably due to the lack of efficient degradation in the proteasome as demonstrated in vitro [26] This result demonstrates once more the impossibility of designing universal vaccine strategies, depending always on the nature of the antigens to be used and also on the mechanisms involved in protection against the given pathogen.

Increasing the Number of Antigens in the DNA Vaccine: The Cocktail Option and the ELI-Strategy

As stated before, one of the major advantages of DNA vaccines is their flexibility, which allows us to blend specific cocktails for specific needs. I.e., we could design a DNA vaccine containing a mix of plasmids, each one specially designed following the different strategies explained so far. Such vaccine could contain, among others, a plasmid directing an Ag to MHC class II, thus inducing a CD4+ T-cell response and another plasmid containing the same Ag, but fused to ubiquitin, thus being directed to MHC class I pathway, enhancing a strong CD8+ CTL response. What is more: a single vaccine may include such blend of strategies, but repeated many times for different Ag. In addition, we have to keep in mind that plasmids used in DNA vaccines present less size limitation in comparison with other vectors used in vaccination field [27]. Nevertheless, we must take into account that the immune response is so complex and delicate that we should be really cautious when manipulating such response. Everything fits and works on paper, v we must always try on the animal to see if our idea works as expected: Immune system lays on a very fine-tuned balance with countless interactions and thus, some responses may inhibit others and vice versa.

Expression library immunization (ELI), a concept first described in mice [16] and later extended to many other target species, is a method for the systemic screening of any given genome to identify potential vaccine candidates. ELI strategy is in principle not specifically addressed to the generation of a vaccine including the whole genome, but a discriminating tool that will allow us to select antigens to build a rational plasmid cocktail to be used for vaccination purposes. The essence of this approach is that the entire genome of a pathogen can be cloned into genetic immunization vectors under the control of a eukaryotic promoter to create a library that would express all the open reading frames (ORFs) of a pathogen. We can associate such ELIs with APCH1, sHA, or Ub in order to target the Ag for obtaining the immune responses discussed before. Immunized animals can therefore be challenged with the viral pathogen to check which clones induced protective immunity. In our laboratory we have obtained partial protection against ASFV lethal challenge by means of ELI immunization [28].

3.3 A Prototypic Protocol to Obtain and Test a DNA Vaccine Specifically Designed for Swine

3.3.1 Construction of a DNA Vaccine

To build a typical plasmid-based DNA vaccine we need a plasmid backbone containing: (1) an origin of replication allowing for growth in bacteria, (2) an antibiotic resistance gene (it will allow to select plasmid-transformed bacteria); (3) a strong promoter for optimal expression in mammalian cells (most commonly, the promoter of human cytomegalovirus, CMVp); and (4) a polyadenylation signal sequence (that provides stability and effective translation) [29]. Moreover, some authors point out the fact that the plasmid itself has immunogenic properties due to its repetitive CpG motifs, which are able to induce strong B cell and T cell responses [30]. PCR products of the gene insert must be first cloned into the plasmid vector (Fig. 1), then used to transform bacteria and finally, the bacteria plated on medium containing the antibiotic for which resistance is encoded in the plasmid. Only bacteria with the plasmid incorporated will be able to grow. One bacterial colony containing the right insert will be large scale-growth and the obtained DNA-plasmid production must be purified in order to be free of bacterial toxins (if we are going to inoculate animals with such DNA).

DNA-Library Construction

The basic protocol to construct a DNA library will include the following steps:

1. Isolate the complete genome of the pathogen of interest.
2. Digest the genome with *Sau3A*I (New England Biolabs), a restriction enzyme that recognizes the $^{5'}$GATC$^{3'}$ sequence and cut commonly every 300–500 bp.
3. Clone fragments into the selected eukaryotic expression vector (*see* **Note 3**).

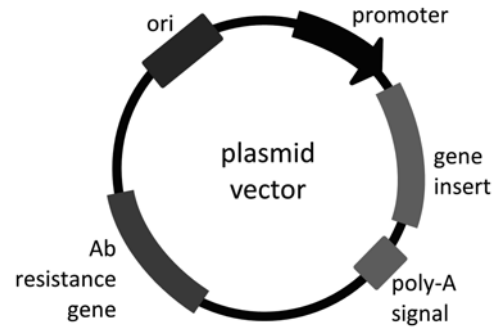

Fig. 1 Schematic representation showing essential components of a plasmid-DNA vaccine construct

4. Transform plasmids in selected bacteria (*see* **Note 2**) and afterwards plated in suitable medium in order to select the properly transformed bacteria.
5. Pick up a precise number of individual colonies for each restriction fragment and plasmid frame (*see* **Note 7**), to be individually inoculated into a 96-well culture plate.
6. Perform and store replicas of all plates at −70 °C with 15 % (v/v) of glycerol.
7. In order to obtain DNA material for vaccination purposes, a mixture of the generated plasmids must be carried-out (*see* **Note 8**) and therefore produce the DNA plasmid pool at large scale. Finally, the obtained DNA must be purified in order to be free from bacterial toxins.

3.3.2 In Vitro Assessing Ag-Expression

In order to prove that the obtained plasmids correctly express the inserted genes, it is recommendable to analyze its correct expression by Western blotting using specific polyclonal or monoclonal antibodies. Briefly, suitable cell lines shall be transfected with the vector, meanwhile control cells will be transfected with the void plasmid (or with the plasmid containing an irrelevant gene) using our favorite transfection protocol (electroporation, lipid based transfection, Calcium Phosphate based transfection…). Cells will be incubated for 24–72 h, and then harvested to evaluate its optimal in vitro expression kinetic. If everything is correct, the plasmid DNA is ready to be injected into the animal in order to elicit the desired immune response.

3.4 Swine Immunization

To finish with the present section dedicated to the production of DNA vaccines we include a simple and easy procedure to immunize swine currently used in our laboratory [21]. With this protocol we have achieved our best results: an immunization followed by a boost a fortnight after has given us a proper result and, in our experience working in ASFV, more boosts do not improve the

elicited immune reaction. Please remember, that since we will inject the produced DNA to animals it is compulsory to purify it from bacterial toxins.

1. Prepare a stock solution of 400 μg DNA/ml in commercial sterile saline-solution.
2. In an aseptic environment, put a sterile needle to a 2.5 ml sterile syringe. Charge one syringe per animal with 1.5 ml of the DNA stock solution. This is a DNA dose of 600 μg per pig (*see* **Note 9**).
3. Keep the charged syringes (with their cap on) in the fridge and transport them to the farm/animal facility into plastic bags inside a well protected polystyrene box filled with crushed ice (*see* **Note 10**).
4. Immobilize the animal according the animal welfare policies of your institution and clean and disinfect with absorbent paper imbibed with ethanol 70 % the injection points prior to the inoculation.
5. One third of the vaccine dose (0.5 ml) must be intramuscularly injected in the right rectus femoris quadriceps, one third must be injected in the right trapezius muscle of the neck and the last third must be subcutaneously injected in the right ear.
6. A fortnight after the first immunization, please repeat **step 5**, but performing the injections in the left side of the animal.

3.5 Prime Boost: The Hope for DNA Vaccines to Conquer the Market

DNA vaccination has gained a new impulse in the last years thanks to the exponential improvement of in vivo DNA transfection protocols both for human and veterinarian species (*see* Subheading 3.1.3): The maximal revolution seemed to arrive with the arrival of prime-boosting protocols. Even for the most skeptical, DNA electroporation clearly demonstrated to be the ideal protocol for immune priming, followed by boosting with recombinant viruses encoding same antigens or with recombinant proteins. Prime-boost strategies have improved the humoral immunity and also enhanced the DNA-primed CTL responses [31]. The most used viral vector platforms are, among others, the modified *Vaccinia* virus Ankara (MVA) and, specially, the adenoviral vectors [31]. The efficacy of both homologous and heterologous prime-boost strategies has been also tested in swine DNA vaccination with uneven results. Thus, optimal responses were obtained against Aujeszky disease by DNA priming—followed by a booster with an Orf virus recombinant-vaccine [32]. The efficacy of heterologous prime-boost regimes have lead to several human and nonhuman primates trials for important diseases such as HIV [33, 34], albeit in occasions homologous prime boost strategies have demonstrated to give optimal results [35]; insisting once more in the concept of individual vaccines for individual purposes. Independently

of the above mentioned results, DNA priming can greatly reduce the amount of booster vaccine needed as we have previously demonstrated for the Rift Valley Fever virus attenuated vaccine in sheep [36]. Similar concept was previously presented as an alternative to reduce the amount of booster vaccine needed at the time of influenza outbreaks, thus reducing costs and saving response time [37]. This concept could perfectly be extended to other diseases.

4 Notes

1. There are many options available in the market, having all in common the presence of a promoter capable to be recognized by the target species. In our case we used the pCMV plasmid from Clontech (Palo Alto, California), to express the vaccine-encoded product under the control of the immediate early promoter of human cytomegalovirus (CMVp). We also recommend the use of pVAX™200-DES (Invitrogen, California) which meets US Food and Drug Administration (FDA) guidelines for design of DNA vaccines.
2. We usually use electrocompetent *Escherichia coli* (ElectroMAX™ DH10B™ T1 Phage-Resistant Competent Cells, Invitrogen).
3. In order to clone the DNA insert inside the plasmid backbone we normally use the Quick Ligase Kit (New England Biolabs). To purify DNA products we commonly use Qiagen MinElute Reaction Cleanup Kit (Qiagen, The Netherlands). To purify from bacterial toxins the DNA-plasmid obtained by means of bacterial culture we usually use Endofree Plasmid Mega kit (Qiagen, The Netherlands).
4. The open reading frames (ORFs) encoding the antigens can be cloned into the plasmid backbone alone or as fusions with the ORFs encoding carrier molecules that act as genetic adjuvants. Some of the adjuvants (*see* Fig. 2) discussed in the present chapter are:
   - APCH1: the single chain of an antibody that recognizes the DR allele of the Class II Swine Leukocyte Antigen (SLAII) molecule.
   - sHA: the extracellular domain of the ASFV hemagglutinin (sHA), with homology to the CD2 leukocyte antigen.
   - Ub: a monomer of the mutated Ubiquitin (A76).
5. Please note that electroporation conditions must be optimized for each animal species.
6. Please remember that cytokines are species-specific to the host to be vaccinated.

7. The number of colonies to be picked up in order to ensure the representation of all *Sau*3AI fragments in the three possible frames was calculated following a formula that takes into account the length of each original viral DNA restriction fragment and the number of fragments generated by the complete *Sau*3AI digestion.

$$N = 2\left(\frac{\ln(1-P)}{\ln(1-f)}\right) \quad f = \frac{m}{L}$$

where:

N: Number of colonies to be picked up.
P: Probability (=0.9).
m: average length of fragments generated by *Sau3A*I.
L: full length of digested vector.

8. We prepare a pool by taking 0.5 μl from each individual clone. This pool is used as starter culture to inoculate 1 l of the proper

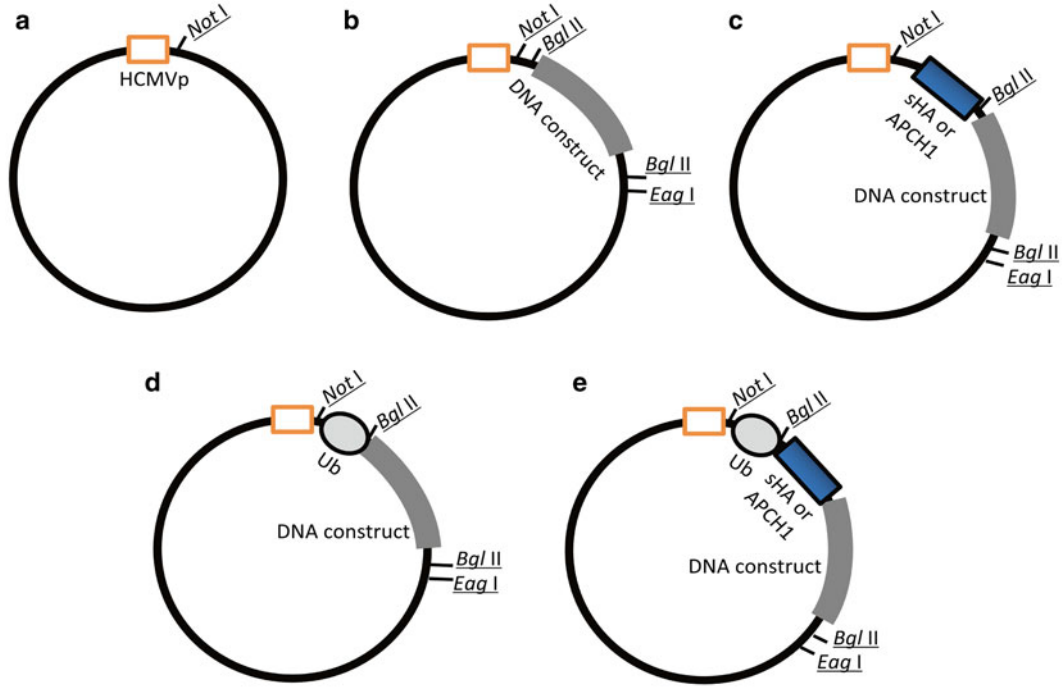

Fig. 2 Schematic representation of plasmids used for DNA-immunization. (**a**) Control plasmid, with no insert gene; (**b**) plasmid with a DNA insert; (**c**) plasmid containing the DNA construct fused to the ORF encoding for APCH1 or sHA; (**d**) plasmid containing the DNA construct fused to the ORF encoding for Ub; (**e**) plasmid containing the DNA construct fused to the ORF encoding for Ub and APCH1 or sHA. The plasmids contain the correspondent ORF within the unique *Not*I cloning site and contain their initiation AUG codon in a Kozak context for optimal transcription and with a *Bgl*II unique site in their 3′ for downstream in frame cloning of the target sequence

broth culture (we normally use LB medium), supplemented with the appropriate antibiotic.

9. We prefer to prepare the vaccine-doses the same day than the inoculation. If this is not possible, please keep the syringes under refrigeration (4 °C) until its use. If different DNA-constructs are going to be used, it is highly recommendable to mark the syringes with different color-tapes in order to avoid confusions during the immunization.
10. Please allow to reach room temperature the DNA doses. If the injection is to cold it may cause undesired additional pain to the animals.

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Chapter 5

Novel Adjuvants and Immunomodulators for Veterinary Vaccines

Peter M.H. Heegaard, Yongxiang Fang, and Gregers Jungersen

Abstract

Adjuvants are crucial for efficacy of vaccines, especially subunit and recombinant vaccines. Rational vaccine design, including knowledge-based and molecularly defined adjuvants tailored for directing and potentiating specific types of host immune responses towards the antigens included in the vaccine is becoming a reality with our increased understanding of innate and adaptive immune activation. This will allow future vaccines to induce immune reactivity having adequate specificity as well as protective and recallable immune effector mechanisms in appropriate body compartments, including mucosal surfaces. Here we describe these new developments and, when possible, relate new immunological knowledge to the many years of experience with traditional, empirical adjuvants. Finally, some protocols are given for production of emulsion (oil-based) and liposome-based adjuvant/antigen formulations.

Key words Adjuvant, Immunomodulator, Innate immune system, Vaccination, Protective immune responses

1 Introduction

An adjuvant can be defined as a substance or composition enhancing the quantity (i.e., augmenting and potentiating) and quality (i.e., inducing the optimal type) of an immune response to another (co-injected) substance. Often, adjuvants work by "organizing," e.g., aggregating or encapsulating an antigen, thereby enhancing its immunogenicity. In short, adjuvants increase the potency of vaccines.

One of the basic dogmas of immunology is that in order for a substance to induce an immune response, i.e., to be immunogenic, it has to be recognized as "foreign" (nonself) by the organism. However, nonself substances do not always induce an immune response and sometimes the immune system reacts against "self" molecules. Examples of nonself molecules with very little immunogenicity include small to medium-sized peptides which will induce no or very feeble immune responses even when comprising nonself

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sequences and even when administered subcutaneously or intravenously. For example, porcine insulin has been used for decades to treat human type 1 diabetics by frequent intramuscular injection over several years of a patient's life with only minor problems of immune activation and this observation extends to many other small to medium molecular weight "nonself" drugs. On the other hand, self-molecules may become perfectly immunogenic, provided they are of a certain molecular size and are introduced by an immunogenic route (e.g., by subcutaneous injection) in an optimal concentration window using a repetitive dosing scheme, and this "self-immunogenicity" is increased even more if the administration is accompanied by a co-injected adjuvant (see below). Even in the absence of an adjuvant it has been observed that certain peptide drugs based on self-sequences can cause unwanted immune stimulation especially if they self-aggregate.

The important factor here is that the immune system needs to perceive "danger" in order to step fully into action. This can happen in several ways, one being the immediate recognition by innate immune cells of exogenous signals in the shape of pathogen-associated molecular patterns (PAMPS) [1] and another being activation of the immune system by recognition of endogenous molecules released by damaged or perturbed tissue/cells (danger signals, tissue factors) [2, 3].

In adjuvants, such signals or means for their induction serve to enhance immune responses to a co-administered antigen dramatically. The promotion of antigen presentation, enhancement of uptake by antigen presenting cells and transport to lymph nodes together with providing these activation signals—either directly or by inducing them in host cells—enables an adjuvant to induce an optimal initial immune activation when administered with a vaccine, giving an efficient boost to the ensuing adaptive, antigen-specific immune response. Thus, the stimulatory activity of adjuvants is mediated by the innate immune system and is completely independent of antigen specificity. Figure 1 shows an example of the effect of adjuvant on the murine immune response to a 21 amino acid peptide. Inclusion of the adjuvant dramatically increased the serum antibody response reaching maximum levels after two injections while the peptide alone had negligible antibody inducing capacity.

The ideal adjuvant is potent, not toxic (or has low and acceptable toxicity), stable, well-defined, versatile, inexpensive and has a well-defined targeted action on the immune system helping a vaccine to reach its goal (Table 1). For most vaccines, the goal is to obtain fast, long lasting and complete protection against disease caused by a specific infectious agent. This means that a vaccine, in addition to inducing immunity having the right specificity and being able to induce immunological memory (which is the hallmark of vaccine) must induce a protective type of immunity.

However, it has to be borne in mind that host response correlates of protection vary widely from one type of infection to another [4]. This means that protective immunity against one type of infection, for example viral, as opposed to another type, for example bacterial, is accomplished by different effectors of the adaptive immune system. While some infections are efficiently counteracted by vaccination inducing strong humoral immune responses by eliciting long lived antibody producing plasma cells, chronic intracellular infections caused by for example *Mycobacteria* and others, including some viruses are not easily controlled by antibodies and require cell-mediated immune (CMI) responses to obtain protection. On the other hand, a strong CD8+ cytotoxic T cell response is irrelevant for protection against diseases caused by bacterial toxins.

Adjuvants also play a big role in controlling the type of immune response by selective boosting of immediate innate immune responses which activate the adaptive immune responses. This means that in addition to determining the magnitude of the adaptive immune response the adjuvant also has a major influence on the type (balance) of the ensuing adaptive response, being it dominated by antibodies or by cell-mediated responses, CD8+ versus CD4+ T cell responses etc. Thus, when estimating the potency of adjuvants in vaccines, the choice of cell-mediated and humoral immune correlates to be measured must reflect which type of immunological response will protect against the pathogen in question. In some specific cases even the short-lived immediate innate immune response itself may be beneficial, e.g., for post-exposure vaccination in which case immediate innate immune effects can contribute to protection. This is also of great interest with highly contagious virus diseases where vaccinations usually are employed in emergency situations to contain an immediate outbreak of the disease. Even with the most efficient vaccines it takes several days to develop specific protective immunity and therefore the immediate antiviral IFN-α production induced by the adjuvant system confers rapid protection, e.g., as demonstrated in mouse models of Foot and Mouth Disease virus (FMDV) infection [5, 6]. Adjuvants have a big role in inducing such efficient short-lived innate immune responses.

A number of basic physical–chemical features of antigens (unrelated to antigenic specificity) correlate with immunogenicity, understood both as the ability to induce an immune response as such and as the specific type of immune response being induced. A number of adjuvants simply work by influencing these antigen feavcontrolling features is the size of the antigen. Very few antigens below 5 kD are immunogenic by themselves. Above this threshold substances become more immunogenic and molecular or aggregate/particle size also influences the type of immune response

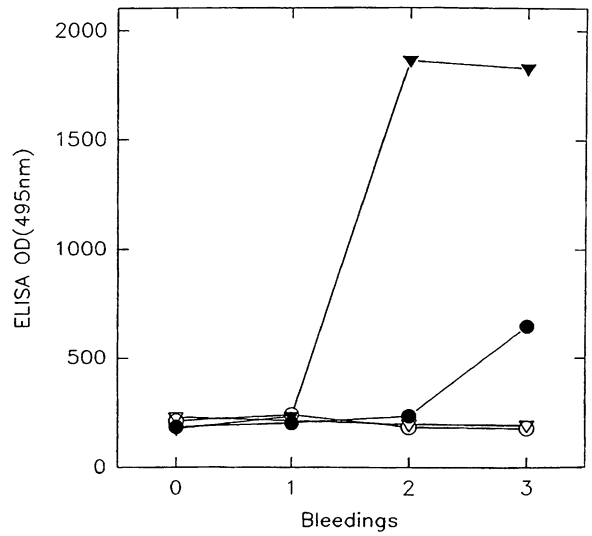

Fig. 1 Comparison between immunization with a peptide alone (administered intraperitoneally, i.p., black circle) and the same peptide administered together with the powerful adjuvant, Freund's incomplete adjuvant (FIA, see below) subcutaneously (s.c.) (black triangle). The peptide was a 21 amino acid synthetic peptide derived from the malarial erythrocyte binding protein-175 coupled to a small peptide carrier. Female 6–8-week-old C57Bl × Balb/c mice (four in each group) were immunized day 0, 21, and 49 with 16 μg of the peptide construct in 100 μl. The adjuvant was Freund's complete adjuvant for the first immunization and Freund's incomplete adjuvant for the following immunizations. The adjuvant was mixed 1 + 1 (v/v) with the peptide before immunization (*see* Protocols). Blood samples were collected from the tail vein on day −1, 12, 33, and 61. The antiserum was tested at 1/100 (in 0.5 M NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 1 % Triton X-100, 15 mM bovine serum albumin) in ELISA, using the peptide coupled to ovalbumin as the coating antigen (1 μg/ml in 0.1 M Na carbonate pH 9.6, Maxisorp ELISA plates from Nunc). Open symbols represent control groups immunized with irrelevant peptides

Table 1
Adjuvants: benefits and risks

Benefits	Risks
Accelerate immune response Prolong immune response	Increase reactogenicity of vaccine: Local adverse effects, systemic effects
Diversify immune response Focus immune response (Ab vs. CMI, T_H1/T_H2)	Nonspecific immune activation: Immune mediated diseases Inflammatory diseases Autoimmune diseases
Increase antibody affinity Improve long term memory Reduce antigen dose	Difficult to establish good biomarkers for evaluating adjuvants

being induced as the way in which the antigen is treated by the innate immune system differs according to antigen size; as a rule of thumb, below 25 nm (single proteins are below this size) antigens are directly trafficked to lymph nodes where presentation of antigens is done by B cells or by resident dendritic cells, while in the 40–80 nm size range (bigger protein aggregates and viruses), local dendritic cell take up the antigen, become activated, migrate to the lymph node and present the antigen to T cells [7]. Other types of antigen-presenting cells are engaged by particles in the 200 nm to 10 μm range (bacteria), and finally, particles above 10 μm will stay at the injection site and may release smaller particles or molecules for a repeated triggering of the immune system (slow release or depot effect). Aggregate formation, presentation in or on particles and polymerization or oligomerization will therefore often increase the immunogenicity of small molecules-they are simply more efficiently recognized and taken up by antigen presenting cells such as dendritic cells and macrophages when aggregated into particles above 25 nm. Also, aggregation of antigen will often result in a repetitive display of antigen epitopes and PAMPs ensuring an increased efficiency of uptake by antigen presenting cells such as B cells and dendritic cells, respectively.

Specifically, in peptides, immunogenicity is influenced by the amino acid sequence in at least three ways; first, T cell binding linear peptide motifs (T cell epitopes) will ensure T helper cell activation so that these cells can help B cells to become antibody producing plasma cells. Such motifs may be introduced as a fusion peptide to the antigen of interest or by coupling the antigen to a protein carrier sporting a range of T cell epitopes in its sequence. Second, as a general rule, antigens should be accessible to immune cells in order to be immunogenic, i.e., they should be hydrophilic. In addition, for peptides meant to induce an immune response towards a "mother" polypeptide sequence, hydrophilic peptides most often represent surface exposed parts of the polypeptide chain of natively folded polypeptide molecules, increasing the chances of obtaining an immune response recognizing the cognate polypeptide antigen. There are several algorithms for predicting peptidic B cell epitopes within a polypeptide sequence based on hydrophilicity parameters [8]. Third, repeated motifs will have increased immunogenicity (as mentioned above). The charge of the antigen is also important; negatively charged antigens are often less immunogenic than comparable non-charged or positively charged antigens, meaning that one may increase immunogenicity of antigens simply by removing negative charges through chemical, enzymatic or molecular biological means or by adding suitable ion-pairing agents.

In the following sections we will describe the application of these principles for increasing immunogenicity of antigens through the use of adjuvants.

2 Classical Adjuvants and the Lessons Learned

There are two classical adjuvant types, discovered and developed in the first part of the 1900s, namely the Freund's adjuvants (incomplete and complete, FIA and FCA, respectively) [9] and aluminum salt based adjuvants [10].

Freund's adjuvants are compositions containing mineral oil (paraffin oil), an emulsifier (mannide monooleate, a plant glycolipid) and either containing (FCA) or not (FIA) killed and dried mycobacteria. Freund's adjuvant is a so-called emulsion type adjuvant intended to be used with an aqueous solution of the vaccine antigen, forming stable water-in-oil emulsions after vigorous mixing of the aqueous solution and Freund's adjuvant (*see* Protocols). The resulting emulsions are quite viscous, have a whitish appearance and should be stable enough to pass the droplet test in which a drop of the emulsion is placed on the surface on an aqueous solution and observed for stability (*see* Protocols). The stability and viscosity of the emulsion are of central importance for the adjuvant activity of the formulation. In the stable emulsion the water soluble vaccine antigen will reside in the water phase microdroplets (1–10 μm) within the oil phase [11]. These adjuvant formulations are most often used for subcutaneous, intraperitoneal or intramuscular injection, and generally FCA may be used for the initial injection only, followed by booster shots with FIA-antigen emulsions. Killed mycobacterial material is able to attract and activate antigen presenting cells to the injection site (an effect of the PAMP structures present) and the emulsion forms a quite stable depot, residing at least a few days [12] at the injection site slowly releasing antigens from its water phase ("depot effect"). Because of its adverse effects, especially injection site reactions, Freund's adjuvant is not used in human vaccines, however is still widely used for laboratory immunizations (mainly for efficient production of antibodies in rodents), and many variations of the emulsion principle is used in veterinary vaccines, mainly for protecting animals against infectious diseases compromising the production (production animals) or animal welfare (companion animals).

Local site reactions caused by Freund's incomplete, and, in particular Freund's complete adjuvant include inflammation, granuloma formation and sometimes pain [12, 13]. On the other hand, these adjuvants are among the most powerful and broadly activating adjuvants known, inducing both Th1 (cell-mediated immunity) and Th2 (antibody production) types of immune responses

[12]. The oil used consists of a mixture of chain lengths and it has been shown that the shorter hydrocarbon chain (<14 C) molecules are more inflammatory than the longer chain molecules which consequently do not achieve the same immune stimulation as the shorter chains; alkyl lengths from 16 to 20 seem to be optimal for efficient immune induction with an acceptable level of local inflammation [14].

The nature of the surfactant(s) used in water/oil mixtures controls droplet formation; surfactants with a low hydrophilic/lipophilic balance tend to dissolve in the oil phase (forming reverse micelles) and therefore stabilize water droplet formation in the oil phase, i.e., water-in-oil emulsions, and vice versa. The properties of the emulsion, including the droplet size can be fine-tuned by including an additional surfactant such as is the case for the MF59 system, which is a more recent emulsion type adjuvant being an oil-in-water microfluidized emulsion [15]. In this adjuvant very small oil droplets (biodegradable squalene, <200 nm) are formed within the water phase with the help of Tween 80 (water soluble) and Span 85 (oil soluble) surfactants, resulting in a non-viscous milky white emulsion, in which the antigen molecules of the water phase do not associate with the oil phase. The main mechanism of oil-in-water adjuvants is thought to be recruitment and activation of antigen presenting cells by the oil phase. These types of emulsion adjuvants show significantly less injection-site adverse reactions than mineral oil based emulsion adjuvants, however oil-in-water emulsions are generally considered to be less immune stimulatory than water-in-oil and inducing shorter term immunity [11]. MF59 as well as other squalene-based oil-in-water emulsion adjuvants (such as AS03) are the only emulsion based adjuvants currently approved for human vaccines (for seasonal and pandemic flu, respectively).

In contrast, aluminum salt ("alum") is the dominating adjuvant in licensed human vaccines and is also used in a large number of veterinary vaccines. Aluminum based adjuvants are characterized by a superior safety profile compared to emulsion type adjuvants and have a long history of human use [16]. They may be based on aluminum hydroxide or aluminum phosphate. Alhydrogel® is a colloidal suspension of aluminumhydroxide in water and one of the most commonly used aluminum adjuvant types. In contrast to the strongly immune activating emulsion type adjuvants, aluminum salt adjuvants are not capable of inducing Th1 or cell-mediated immune activation to any significant degree however they are efficient Th2 inducers, giving rise to high antibody titers in the vaccinated individual. Slow release/depot effects as well as particulate formation promotion have been proposed as possible mechanisms, and these salts have also been proven to enhance phagocytic uptake in vitro. More recently evidence has been provided for the molecu-

lar basis of the adjuvant effect of aluminum salts implying the direct activation of the Nalp3 inflammasome of the antigen presenting cell by aluminum salt particles, initiating an inflammatory response, mediated by activated interleukin-1β. In addition, danger signals released from dying cells may contribute, as aluminum salts have been shown to induce cellular necrosis in vivo [17]. It is beyond this chapter to go into detail on the properties of aluminum based adjuvants, however an excellent text is found in Hem and HogenEsch [16].

The lessons learned from these classical (empirically based) adjuvants are that immunogenicity is dependent on a few, basic characteristics of antigen/adjuvant compositions:

- Immunogenicity is enhanced by particle, antigen droplet or molecular aggregate formation.
- Membrane active compounds (amphiphiles) often activate immune cells such as antigen presenting cells and also may promote antigen uptake.
- A "slow release" effect may be seen with some adjuvants, implicating that an antigen "depot" at the injection site enhances immune stimulation.

3 New Types of Particulate Carrier Systems/Adjuvants

Modern types of particle forming adjuvants include liposomes, ISCOMs, virus-like particles and synthetic (polymer based) microspheres [18]. In addition to enhancing immunogenicity by forming particles of immune relevant sizes, charge and repetitive antigen display (see above) these system can in many cases be modified to include co-stimulatory immunomodulator molecules, either molecules with adjuvant activity or molecules controlling immune activation directly (cytokines), as well as molecules with other functionalities, e.g., for targeting specific cells and tissues. Moreover they can be employed with many different types of antigens (generic adjuvants) just as the classical adjuvants. Immunomodulators are compounds having a separate, longer lasting effect on the immune system and while immunomodulators do not have adjuvant activity, adjuvants may have immunomodulatory activities.

Liposomes are lipid bilayer constrained lipid vesicles with the bilayer formed by any of a broad range of amphiphiles, including phospholipids and synthetic surfactants. They are very versatile platforms for particulate formulation of antigens and have been studied for many years, initially exclusively for drug delivery, and may take the form of unilamellar or multilamellar liposomes [19]. Very many designs yielding liposomes of different sizes, fluidity, membrane organization, and charge, carrying antigens either as

part of the aqueous phase inside the liposome or embedded in or attached to the liposome surface, and combined with additional immune stimulating molecules and/or targeting moieties exist. Basically, liposomes function as carriers of antigens until taken up by antigen presenting cells, however they may also themselves be immunostimulatory if constructed from or containing immunostimulatory lipids, and certain liposome constructs may promote specific antigen presentation pathways. Cationic liposomes are taken up and degraded by dendritic cells much more readily than anionic liposomes [20, 21]. As an example dimethyldioctadecylammonium salts (DDA), which are lipophilic quaternary ammonium compounds form cationic liposomes in water at temperatures above 40 °C and very efficiently enhances uptake of its antigen cargo into antigen presenting cells. Combinations of DDA and immunomodulating agents including TDB (trehalose-6,6′-dibehenate, see below) and MPL (monophosphoryl lipid A, see below) give rise to cell mediated responses higher than those seen with either component alone [22]. Illustrating the modular approach possible with such liposome systems DDA and another mycobacterial immunomodulator analog, MMG-1 (monomycoloyl glycerol analog) was combined with TLR3 targeting viral PAMP polyI:C (see below) into liposomes containing viral and other antigens and used for vaccination of mice. This resulted in superior stimulation of antigen-specific CD8+ cytotoxic T cell responses compared to both DDA/TDB or DDA/polyI:C liposomes [23]. Mucosal immunization, e.g., by intranasal administration, is readily accomplished by liposomes [24] and liposomes are generally biodegradable.

ISCOMs (immune-stimulating complexes) are formed by unassisted self-aggregation of the plant derived saponin mixture QuilA, cholesterol and phospholipid (e.g., phosphatidylcholine) together with an amphipathic, typically viral envelope-derived protein antigen [25]. This assembles into cage-like, virus-sized (approx. 40 nm diameter) particles, initially shown to have potent immune stimulating activities, including a much-looked for ability to induce CD8+ T cells (cytotoxic T cells) capable of destroying virus infected cells [26] and they have therefore been widely studied with viral vaccines. Quil A by itself can also stimulate CD8+ responses to a co-formulated antigen [27]. More recently, formulations allowing vaccine production just by mixing a composition containing preformed, antigen-free ISCOMs (ISCOMatrix) [28] with an antigen have emerged, as have charge-modified ISCOMs (PosIntro), incorporating a cationic cholesterol derivative allowing a more efficient interaction with antigen presenting cells which have net negatively charged surfaces [29]. As QuilA is strongly cytotoxic and hemolytic it represents a serious safety issue which has led to the identification and production of more pure as well as

synthetic versions of QuilA (QS21) claimed to have less cytotoxicity but still retaining adjuvanticity [30]. QuilA is used in a number of veterinary vaccines usually specified as "saponin" or Matrix M (in licensed rabies vaccine for cats and dogs).

Also aiming at inducing potent antiviral immunity, virus-like particles (VLP) can be defined as self-assembling virus proteins forming empty virus capsids devoid of genetic material [31]. They have been developed and are produced by molecular biology based methods and they are therefore easily modified to include one or more heterologous antigenic proteins in the structure (provided they do not adversely affect the VLP self-assembly) turning the VLP into a generally applicable particulate antigen carrier platform with enhanced immune stimulating activity due its size and due to the repetitive presentation of antigen at the surface [31, 32]. Moreover, sequence deletions can readily be introduced to make VLPs compatible with DIVA (differentiating infected from vaccinated animals) based serology. Also, VLPs may be mixed with other adjuvants and, evidently, are completely safe, highly ordered non-replicating protein aggregates.

Polymeric microparticles and nanoparticles can be manufactured by synthesis (e.g., poly lactide (glycolic) acid polymers, PLGA) or can be based on naturally occurring polymers such as chitosan (deacetylated chitin), starch and alginate [33]. All of these polymers are biodegradable and some of them have intrinsic adjuvant properties (chitosan and alginate). Just as liposomes polymeric microparticles and nanoparticles are remarkably good at stimulating mucosal immunity, and can be applied for, for example, intranasal administration; however, parenteral administration is also possible. For some classes of nanoparticles it is possible to produce them as monodisperse preparations. Antigens may be encapsulated by the particles, adsorbed to their surface through non-covalent bonds or covalently attached to their surface [33].

4 Knowledge-Based Adjuvant and Immunomodulator Design

With the increasing knowledge about the nature of the factors and interactions taking place during innate immune stimulation and the innate control of adaptive immune responses, it is potentially possible to tailor-make adjuvant systems directed towards inducing the specific types of immune effector responses needed to obtain protection. The most obvious way to achieve such targeted immune stimulation in a vaccine adjuvant composition is to include one or more of the innate immune factors known to control adaptive immune response types, i.e., endogenous factors like cytokines or host tissue derived danger signaling factors, or to include PAMPs or fragments thereof. This strategy has the added benefit of being

readily combinable with the "organizational" mode of action of traditional emulsion based and aluminum adjuvants. An example of the combination of the two strategies in a single adjuvant composition is the AS04 adjuvant (GlaxoSmithKline) consisting of aluminum salt and monophosphoryl lipid A (MPL) (more on this below).

Details about PAMPs and their innate immune cell receptors and effects will not be described here; instead, the reader is referred to recent excellent reviews by Guy [18] and De Veer and Meeusen [34] and we will limit the discussion to the most important classes of PAMPs.

One class of virus associated PAMPs are virus specific types of nucleic acids, either in the form of small molecule synthetic nucleobase analogs or in the form of synthetic mimics of virus RNA.

Synthetic nucleic acids include dsRNA analogs such as polyI:C, and ssRNA analogs such as poly U, binding to TLR3 and TLR7/8, respectively, and both inducing IL-12 and interferon type I, promoting cytotoxic T cell responses. In addition, imidazoquinolines such as R848, imiquimod and gardimiquimod (all binding to TLR7/8) as well as nucleobase analogs such as CL264 (9-benzyl-8 hydroxyadenine analog) and loxoribine (guanosine analog) (both binding TLR7 exclusively) also lead to IL-12/interferon type I responses. Thus for these types of PAMP mimics to be immune activating, they need to interact with intracellular (endosomal compartment) receptors (TLR3, TLR7, TLR8) just as is the case for CpG containing oligodeoxynucleotides (TLR9 binding, see below), and therefore formulation with amphiphilic nucleotide binding molecules (e.g., cationic phospholipids) will often greatly enhance their activity [35].

Bacterial cell surfaces are covered with immunostimulatory glycolipids, lipopeptides, and glycopeptides all of which are amphipathic structures which are readily incorporated into liposomes and other amphipathic delivery systems. The most prominent molecules include gram negative lipopolysaccharide (LPS), gram positive peptidoglycan (although also found in smaller amounts in gram negatives), and Pam_3Cys, mimicking bacterial lipopeptides. These molecules activate immune cells by binding TLR4 (LPS) and TLR2 (peptidoglycan and Pam_3Cys). Not surprisingly some bacterial cell wall PAMPs are cytotoxic, precluding their direct use as adjuvants. However, in some cases the adjuvant activity can be isolated from toxicity, as for example demonstrated with monophosphoryl lipid A (MPL) which is a truncated TLR4-binding Th1 inducing lipopolysaccharide (LPS) derived molecule with much less toxicity than the full molecule [36]. Another example is TDB which is an immunostimulating, nontoxic analog of the immunostimulatory but also highly cytotoxic mycobacterial cell wall component trehalose 6,6 dimycolate ("cord factor") [37]. As

mentioned above, MPL formulated with aluminum constitutes the GlaxoSmithKline adjuvant SA04 which has been approved for use in human vaccines directed against herpes and hepatitis B virus infections as the first licensed human adjuvant containing a TLR agonist. Yet another example of a modified bacterial PAMP is muramyl dipeptide (MDP) which is the minimal fragment of peptidoglycan having a (weak) immune stimulating activity, activating immune cells through interaction with the intracellular NOD2 receptor. Interestingly, it has been shown that the weak immunostimulatory activity of MDP can be increased to the level of intact peptidoglycan by oligomerizing the molecule on multivalent carriers (synthetic dendrimers [38]) offering a route to a fully synthetic, well-defined construct having high and well-defined immunostimulating activity.

The final major group of PAMPs is constituted by oligodeoxynucleotides carrying unmethylated CpG motifs being among the best Th1 inducing compounds known (see IL12p40 response in Fig. 2 below) and therefore potentially useful in vaccines against intracellular pathogens, including viruses. There are a number of intriguing differences between species with respect to the demands for the sequences flanking the CpG motif, leading to difficulties in reproducing the first, promising results in mice in other species including cattle [39] and pigs [40]. On top of this it appears that there are quite large individual-to-individual differences in responsiveness to unmethylated CpG oligonucleotides in outbred populations [41]. An attractive feature of CpG containing oligodeoxynucleotides is that they stimulate immune responses when applied to mucosal surfaces [42]; also, they are readily combinable with adjuvants containing cell membrane active surfactants, facilitating entry of oligonucleotides into cells where they can interact with their specific endosomal receptor TLR9. In a number of experiments the ability of CpG oligonucleotides to induce an immediate, short-lived increase in resistance to infection has been demonstrated including resistance to FMDV in a mouse model [6].

In Fig. 2 some examples on the ability of PAMPs to induce specific cytokines known to mediate fundamentally different types of immune response are shown (Fig. 2a, b). Various PAMPs and PAMP analogs were added to porcine peripheral blood mononuclear cell (PBMC) cultures and after 24 h of incubation cell culture supernatants were analyzed for porcine interferon-α (indicative of antiviral Th1 type response) and IL-6 (indicative of more Th2/Th17 like response) and IL-12p40 (indicative of a Th1 type response). It is clearly seen (Fig. 2a) that the viral PAMP mimics polyU/LyoVec™, poly I:C and CL264 predominantly induce interferon-α but very little IL-6, and that the bacterial PAMP lipopolysaccharide (LPS) in contrast induces only IL-6 and not

interferon-α. In the independent experiment in Fig. 2b, a type D CpG oligonucleotide is seen to induce IL-12p40 while the same oligonucleotide with its CpG sequence reversed to GpC loses all IL-12p40 inducing capacity; Il-6 is not induced by either type of oligonucleotide. The IL-12p40 induction by CpG was superior to that of both bacterial PAMPs LPS, peptidoglycan and $Pam_3CysSerLys_4$ as well as the virus-PAMP related polyU. In this particular experiment all bacterial PAMPs and polyU induced IL-6. Data such as these demonstrate that bacterial, viral and unmethylated CpG type PAMPs, respectively, induce non-similar cytokine responses in porcine mononuclear cells, with bacterial structures inducing IL-6 and to some extent IL-12 but no interferon-α, i.e., Th2/Th17 type of responses mixed with some Th1 induction, while viral PAMP mimics all induced the antiviral cytokine interferon-α and very little IL-6. Finally, unmethylated CpG induced IL-12p40 and interferon-α (the latter not shown here, *see* Sørensen et al. [43]) but not IL-6, i.e., a clean Th1 response. In these respects the pig behaves just as has been reported previously for lab rodents [44].

Figure 2c demonstrates an example of how the balance of a vaccine induced immune response can be skewed with selection of different adjuvants. Two groups of pigs were immunized with the same antigen extract of *Mycoplasma hyosynoviae* (a cause of lameness in pigs) formulated either with the POSINTRO adjuvant (positively charged ISCOM-like matrix) or with CAF01 adjuvant (liposome forming amphiphile DDA incorporating TDB, a mycobacterial PAMP mimic, see above). Although both adjuvants induce a mixed response, there is clearly a different balance between the antibody response on one side and the cell-mediated immunity (interferon-γ) response on the other side with the two different adjuvant types: Posintro leads to an antibody dominated response while the DDA/TDB adjuvant leads to an increased cell-mediated immunity response and a diminished antibody response. DDA/TDB has been shown previously in mice to induce a mixed Th1/Th17, cell-mediated immune response [20, 45], and the data shown in Fig. 2c confirm that, at least compared to Posintro, the TDB containing formulation leads to an increase in the cell-mediated immune response confirming that the same immune response type is obtained in pigs.

In summary, there is a large array of PAMP related molecules available for "targeted" immunostimulation, i.e., inducing specific immune response types. Natural PAMPs can be modified in order to decrease toxicity, they may be fragmented into minimal structures to increase their ease of synthesis and analyzability and they can in most cases be combined with conventional adjuvants (emulsions, aluminum salts and carrier systems) without problems. With the discovery of additional PAMPs and corresponding host recep-

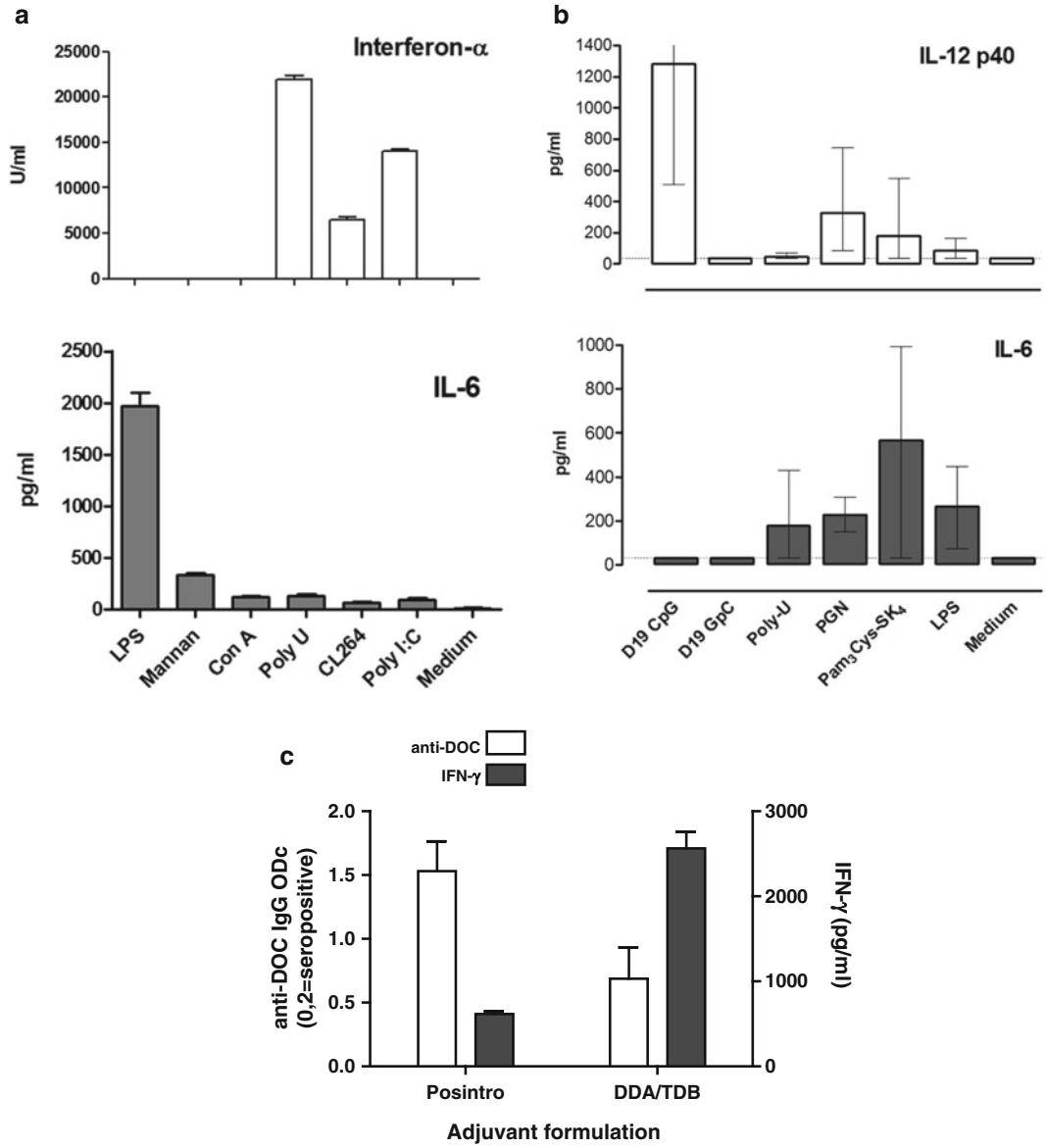

Fig. 2 (**a**) Porcine PBMCs incubated with natural stimulators (concanavalin A, yeast mannan and bacterial lipopolysaccharide (LPS)) as well as with synthetic Toll-like receptor agonists as indicated, polyU/LyoVec™ (ssRNA (with cationic phospholipid transfectant LyoVec™), TLR 7/8 agonist), CL264 (9-benzyl-8 hydroxyadenine analog, TLR 7 selective agonist), and poly I:C (LMW) (dsRNA analog, TLR 3 agonist). Cell culture supernatants were analyzed for interferon-α and IL-6 by ELISA. A typical response (cells from one animal) is shown. (**b**) Porcine PBMCs stimulated with synthetic Toll-like receptor agonists as indicated; D19 CpG (unmethylated oligonucleotide containing dinucleotide motif CpG, TLR9 agonist), D19 GpC (similar to D19 CpG except reverse sequence of dinucleotide motif), polyU (TLR7/8 agonist), PGN (peptidoglycan, gram positive PAMP, main receptor TLR 2), and Pam_3CysSK_4 (TLR1/2 agonist), as well as with bacterial lipopolysaccharide (LPS) and peptidoglycan (PGN), both obtained from natural sources. IL-12p40 and IL-6 in cell culture supernatants were analyzed by ELISA. The mean and range of six pigs are shown (**c**) Deoxycholate (DOC)-extracted membrane proteins from *Mycoplasma hyosynoviae* were included in a vaccine at 300 μg/dose and formulated with either POSINTRO (ISCOM) containing 200 μg Quilaja saponins or DDA/TDB (CAF01) and used to vaccinate two groups of three pigs each. The level of antibodies against the DOC antigen is depicted as the white bars and cell-mediated immunity against the DOC antigen, measured by whole blood IFN-γ release assay with determination of IFN-γ levels by monoclonal ELISA [49] as grey bars 14 days after second vaccination of pigs

tors as well as endogenous immune stimulating factors ("danger signals") all of which may be useful for controlling immune response types the bottle neck becomes the limited knowledge of which innate immune mechanisms are the most optimal in order to obtain good protection and memory with a given infection.

5 Concluding Discussion: Adjuvant Needs and Approval Roadblocks

Despite the continuing need for new adjuvants, especially for adjuvants inducing good cell-mediated immune responses, development of widely usable new licensable adjuvants has generally been slow. This is in part because the antigen and adjuvant combination in vaccines are registered as an entity where substitution of either antigen or adjuvant will require a new registration process and in part because adjuvants and vaccines are immune activating modalities with an inherent risk for both acute injection site and systemic adverse reactions and for long-term complications which are unacceptable for their intended use in large populations of healthy individuals [13, 46]. Furthermore, even though innate immune activation is generally quite conserved across species, as shown in the example above, differences do exist [44] putting a challenge to choosing the right preclinical animal models for safety testing, in addition to the challenge of defining the right immune activation markers for protective immunity in short term animal models.

As detailed above the immune stimulating effects of adjuvants are accomplished by a variety of mechanisms related to the "organization" of the antigen into aggregates, particles, emulsion droplets etc. by two phase-systems (emulsions or aluminum gels) or more elaborate self-organizing structures (liposomes, VLP, ISCOMs, nanoparticles etc.) incorporating antigens. In addition adjuvants may comprise substances directly stimulating innate immune responses—more or less selectively—either using endogenous control molecules such as cytokines or using pathogen associated molecules alerting and activating the innate immune system. Finally, amphiphilic molecules present in the adjuvant can activate innate immune cells and facilitate uptake of antigen by interacting with their cell membranes. Many of these mechanisms are now becoming known in sufficient detail to allow the tailored design of molecularly defined adjuvants, in principle allowing control of the induced innate immune activation, and thereby the type of obtained adaptive immune response, aiming at adaptive responses providing optimal protection and with memory towards the infection in question. Also, vaccine formulations may be optimized for mucosal administration or for delayed and sustained release of the antigen after administration.

Knowledge-based rational vaccine design and tailored adjuvant development and formulation will allow future generations of vaccines to not only induce a protective immune specificity but also to govern development of protective and recallable immune effector mechanisms in appropriate body compartments including mucosal surfaces.

6 Protocols: Adjuvant Formulation with Protein Antigen

6.1 Materials

1. Freund's complete adjuvant (Difco, Detroit, MI, USA).
2. Freund's incomplete adjuvant (Difco, Detroit, MI, USA).
3. Montanide ISA adjuvants (Seppic, Paris, France).
4. Cationic adjuvant formulation 1 (CAF01, SSI, Copenhagen, Denmark).
5. Maxisorp ELISA plates (Nunc, Thermo Scientific).
6. Concanavalin A (Sigma Aldrich, St. Louis, MO, USA).
7. Yeast mannan (Sigma Aldrich, St. Louis, MO, USA).
8. LPS (from *Salmonella enterica* Typhimurium, Sigma Aldrich, St. Louis, MO, USA).
9. polyU/LyoVec™ (InvivoGen, Toulouse, France).
10. polyU (Sigma Aldrich, St. Louis, MO, USA).
11. CL264 (InvivoGen, Toulouse, France).
12. poly I:C (InvivoGen, Toulouse, France).
13. D19 CpG (5′-ggTGCATCGATGCAGggggg-3′ (lowercase: phosphorothioate), DNA Technology, Aarhus, Denmark).
14. D19 GpC (5′-ggTGCATGCATGCAGggggg-3′ (lowercase: phosphorothioate), DNA Technology, Aarhus, Denmark).
15. Peptidoglycan (PGN) (from *Staphylococcus aureus*, Fluka, Buchs, Switzerland).
16. Pam3CysSK4 (EMC microcollections, Tübingen, Germany).
17. POSINTRO (ISCOM) (Nordic Vaccine, Copenhagen, Denmark).
18. Porcine interferon-α ELISA [43].
19. Porcine IL-6 ELISA (R&D Systems, Abingdon, UK).
20. Porcine IL-12p40 ELISA (R&D Systems, Abingdon, UK).

7 Methods

Preparation of Water-in-oil (W/O) emulsion of antigen using Freund's incomplete adjuvant, FIA (Sigma-Aldrich) or similar, such as most Montanide ISA (Incomplete Seppic Adjuvants) VG adjuvants (Seppic):

The prescribed volumes of antigen, preferably in saline, and adjuvant are mixed very thoroughly in order to form a stable emulsion. Typically equal volumes are used, but this may vary for some ISA products. Mixing can typically be prepared by one of three methods [47, 48]:

1. Mix in an Eppendorf tube and vortex at high speed for minimum 30 min (<1–1.5 ml final volumes).
2. Syringe extrusion technique where adjuvant is drawn into one syringe and antigen is drawn into another syringe. The syringes are connected with an I-Connector without air and the two liquids are emulsified by pushing the formulation back and forth between the two syringes. One cycle corresponds to the passage of the entire formulation from one container to the other through the connector, and back. The first 20 cycles are performed slowly, followed by 60 cycles performed as fast as possible (5–20 ml volumes).
3. Industrially, W/O emulsions are typically performed using high-speed homogenization, but this may also be performed at smaller scale with appropriate equipment and sterile homogenization tips (bigger volumes).

Irrespective of the method, it is important to test appropriate mixing by water-drop test: With this test a droplet of the formulation is placed on a water surface in a beaker. The drop must not disperse on the surface, but should retain its shape if appropriate emulsion has been obtained. If the drop diffuses on the surface of the water, the emulsion is not stable and the antigen will disperse from the adjuvant in the aqueous environment after injection. In this case the mixing will have to be prolonged/repeated.

Preparation of Water-in-oil-in-water (W/O/W) double emulsion of antigen in adjuvant such as Montanide ISA VG201 (Seppic):

To obtain a homogeneous and stable (W/O/W) double emulsion temperature control and mixing time are critical for the mixing process between adjuvant and aqueous antigen. For the ISA 201 VG adjuvant Seppic recommends paddle agitation in a beaker, but magnetic stirring with appropriate magnet shapes can also be used.

1. Work under sterile/aseptic conditions to prepare the equal volumes of adjuvant and aqueous phase antigen.
2. Heat both preparations to 31 °C ± 1 °C in a water bath.

3. Place adjuvant in the beaker with the propeller/magnet and set agitation to 350 rpm.
4. Add slowly (over several seconds) the aqueous phase to the adjuvant and continue stirring for another 5 min.
5. Stop the agitation and let the emulsion rest for 1 h at room temperature (21 °C).
6. The vaccine preparation is ready to use or can be stored in refrigerator to the following day.

Properly prepared emulsions of adjuvant and antigen are stable and will not separate into water and oil phases during refrigerated storage. Stability tests should be performed with storage at 4, 21, and 37 °C. While emulsified vaccines typically are stored at 4 °C, stability test at 37 °C provides an accelerated evaluation of the emulsification where no separation should be observed for at least 1 month. From a biological viewpoint, the 37 °C stability test also mirrors the stability of the vaccine after injection where antigen retention at the site of injection, as described above, is an important parameter for many oil emulsified vaccines.

Liposome cationic adjuvant formulation, CAF01 (SSI)

Cationic adjuvant formulation no. 1 (CAF01, Statens Serum Institute, SSI, Copenhagen) is composed of dimethyldioctadecylammonium (DDA) liposomes containing trehalose 6,6′-dibehenate (TDB) as a liposome stabilizing immunopotentiator (in an aqueous environment, DDA mixed with TDB self-assembles into liposomes having TDB incorporated into the DDA bilayer lipid membrane when heated above the phase-transition temperature of DDA (approx. 47 °C)) [20] . These CAF01 liposomes are stable for an extended time period and readily incorporate recombinant proteins when mixed at room temperature in aqueous solution:

1. Work under sterile/aseptic conditions to prepare equal volumes of CAF01 adjuvant and aqueous phase antigen dissolved in 10 mM Tris–HCl pH 7.4. Both preparations should have room temperature before mixing.
2. Add the aqueous phase antigen to adjuvant and mix gently at room temperature.
3. Let the emulsion rest for 30 min at room temperature (21 °C).

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Chapter 6

Polymerase Mechanism-Based Method of Viral Attenuation

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Abstract

Vaccines remain the most effective way of preventing infection and spread of infectious diseases. These prophylactics have been used for centuries but still to this day only three main design strategies exist: (1) live attenuated virus (LAV) vaccines, (2) killed or inactivated virus vaccines, (3) and subunit vaccines of the three, the most efficacious vaccines remain LAVs. LAVs replicate in relevant tissues, elicit strong cellular and humoral responses, and often confer lifelong immunity. While this vaccine strategy has produced the majority of successful vaccines in use today, there are also important safety concerns to consider with this approach. In the past, the development of LAVs has been empirical. Blind passage of viruses in various cell types results in the accumulation of multiple attenuating mutations leaving the molecular mechanisms of attenuation unknown. Also, due to the high error rate of RNA viruses and selective pressures of the host environment, these LAVs, derived from such viruses, can potentially revert back to wild-type virulence. This not only puts the vaccinee at risk, but if shed can put those that are unvaccinated at risk as well. While these vaccines have been successful there still remains a need for a rational design strategy by which to create additional LAVs.

One approach for rational vaccine design involves increasing the fidelity of the viral RdRp. Increased fidelity decreases the viral mutational frequency thereby reducing the genetic variation the virus needs in order to evade the host imposed bottlenecks to infection. While polymerase mutants exist which decrease viral mutation frequency the mutations are not in conserved regions of the polymerase, which doesn't lend itself toward using a common mutant approach toward developing a universal vaccine strategy for all RNA viruses. We have identified a conserved lysine residue in the active site of the PV RdRp that acts as a general acid during nucleotide incorporation. Mutation from a lysine to an arginine results in a high fidelity polymerase that replicates slowly thus creating an attenuated virus that is genetically stable and less likely to revert to a wild-type phenotype. This chapter provides detailed methods in which to identify the conserved lysine residue and evaluating fidelity and attenuation in cell culture (in vitro) and in the PV transgenic murine model (in vivo).

Key words RNA virus, RNA-dependent RNA polymerase, Polymerase fidelity, Live-attenuated virus, Vaccine, Attenuation, Poliovirus, Sequence homology

1 Introduction

LAVs remain the most effective strategy for vaccine design [1, 2]. However, in the past developments of these vaccines have been empirical. Blind passage of viruses in different cell types results in the accumulation of multiple attenuating mutations leaving the

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molecular mechanisms of attenuation unknown. Due to the high error rate of RNA viruses and the selective pressures of the host environment, these LAVs can potentially revert back to wild-type virulence. This not only puts the vaccinee at risk, but if shed can put those that are unvaccinated at risk as well. LAV vaccines have been created against a number of RNA viruses, such as poliomyelitis, measles, mumps, rabies, rubella, yellow fever and influenza. While these vaccines have been successful there still remains a need for a rational design strategy in which to create additional LAVs. It has been shown that by altering fidelity, the rate and speed at which the polymerase incorporates mutations, leads to viral attenuation [3–8].

RNA viruses are defined by high mutation rates, high yields, and short replication times. These viruses have an average mutation rate of 10^{-3} to 10^{-5} mutations per genome replication event [4]. As a result, RNA viruses do not replicate as a single sequence but as a "cloud" of mutant genomes, which have been dubbed *quasispecies* [9–12]. Although a high mutation rate can lead to deleterious changes in the genome, genetic diversity in RNA virus populations appears to be critical for fitness and survival and likely contributes to pathogenesis. In a heterogeneous pathogen population, some variants are able to infect primary tissues and bypass host-imposed bottlenecks. From here the remaining variants can replicate into another heterogeneous population where some are once again able to bypass another layer of bottlenecks and perform secondary infection in other tissues thus demonstrating that a heterogeneous population, or quasispecies, can be beneficial to the pathogen. This adaptability poses a unique challenge, for example, when it comes to developing antiviral drugs and vaccines.

RNA virus populations are heterogeneous due to error-prone replication by the viral RNA-dependent RNA polymerase (RdRp) which influences quasispecies evolution. This adaptability benefits the pathogen sometimes at the cost of the host. Currently, error-prone replication is known to happen in all RNA viruses that infect both plants and animals. It is also known that this error is due to rapid generation of variants and the fidelity of the viral RdRp [13–15].

In order to study the effect of polymerase mutants on RNA virus heterogeneity, we turn to a model RNA virus, poliovirus. Poliovirus (PV) belongs to the family *Picornaviridae*. This family consists of non-enveloped, positive single strand genomes many of which are important human and animal pathogens. The PV genome can be divided into three parts, the 5′-untranslated region (5′-UTR), a single open reading frame (ORF), and the polyadenylated 3′-untranslated region (3′ UTR). Upon entry into the cell, the mRNA is translated as a polyprotein of approximately 3000 amino acids and can be divided into three functionally different regions: P1, P2, and P3. The polyprotein is cleaved cotranslationally and posttranslationally by viral proteases $2A^{pro}$ and $3C^{pro}$ into 11 proteins. The PV RdRP is found in the P3 region and is termed 3Dpol.

1.1 Identification of the Conserved Lysine

The RdRp is one out of four categories of polymerases and its crystal structure shows a close evolutionary relationship not only to other RdRps but also to that of DNA-dependent DNA-polymerases (DdDps), DNA-dependent RNA-polymerases (DdRps), and RNA-dependent DNA-polymerases (RdDps) also known as reverse transcriptases (RTs). All resemble a cupped right-handed structure consisting of the thumb, fingers, and palm subdomains [16, 17]. The palm is where the active site of the polymerase lies and consists of four conserved structural motifs A–D [16]. A fifth and sixth motif, E and F, exists in the RNA-dependent polymerases but not the DNA-dependent polymerases [16]. The latter motifs are not in the active site but line this region. RdRps are error prone but they are as faithful as DNA polymerases that lack proofreading exonucleases [18]. The absence of a repair mechanism in the PV genome is what leads to an enhanced rate of mutation during viral replication.

Nucleic acid polymerases use a two-metal-ion mechanism for nucleotidyl transfer [19]. In this mechanism, two magnesium ions are used to organize the reactants. Recently, the chemical mechanism of nucleotidyl transfer has been expanded to include a general acid, which protonates the pyrophosphate leaving group of the NTP substrate and enhance the efficiency of nucleotidyl transfer [20, 21]. The general acid of PV RdRp is Lys359, located in motif D, which is conserved throughout all RdRps and RTs. Importantly, an orthologous residue at this site is known or predicted in RNA viruses for which rational design of vaccines would greatly benefit.

1.2 In Vitro and In Vivo Biological Analysis

In order to determine what effect biochemical changes have on the multiplication of the virus in cell culture we created a PV genome encoding the arginine mutation in the PV subgenomic replicon (pRLucRA) and viral cDNA (pMoVRA). Quantification of virus by plaque assay provides insight into fitness of the viral population. The subgenomic replicon permits indirect evaluation of RNA synthesis by measurement of luciferase activity. Analysis of RNA replication in the absence of virus production can provide insight on whether RNA replication is the rate-limiting step for virus production.

The characteristics of live-virus multiplication and their plaque phenotype can predict whether the virus will be attenuated in the mouse model. However, viral quantification by plaque forming units (pfu) selects variants based on phenotype and therefore can be an unreliable measure of viruses present due to phenotypic differences between viral strains. In addition to pfu, quantifying virus based on genomes accounts for total viral particles produced. This is a more accurate measurement of total viruses in the population and the number of genomes produced by the polymerase.

While these characteristics predict attenuation, actual confirmation is determined using a mouse transgenic for the PV receptor. In this system, wild-type (WT) PV is generally lethal. At the highest dose, the mutated polymerase (lysine to arginine) failed to

cause disease in the mice. To determine if the mutant virus replicated, mice surviving the initial infection were challenged with a lethal dose of WT PV. We can conclude from the mice that survive this challenge, the mutant is replication competent and elicits an immune response sufficient enough to protect against a lethal dose of WT PV [3].

Using PV as our model we have developed a rational design for polymerase-based mechanism of attenuation. By altering the nature of the general acid lysine residue to an arginine, we have shown that we maintain the ability to tune RdRp speed and fidelity creating a viral RdRp that is slower and more faithful than the WT enzyme. This results in an attenuated virus with a restricted viral quasispecies that fails to cause disease, yet elicits a protective immune response. This approach has the ability to be applied to any RNA virus given the conserved nature of the motif D lysine residue.

2 Materials

2.1 Identification of the Conserved Lysine

Table 1 is an alignment of residues found in motif D of the RdRp for positive and negative strand RNA virus families. Numbers indicate the position from the first amino acid of motif D in the RdRp domain. The conserved lysine residue is shown in boldfaced type. Other conserved residues are underlined. All sequences were obtained from the NCBI Database. Sequences were aligned using ClustalW2 and based upon alignments previously published [22].

2.2 Site-Directed Mutagenesis of pMoVRA and pRLucRA by Overlap PCR/Cloning Components

1. Poliovirus Mahoney cDNA, pMoVRA, and subgenomic replicon pRLucRA [23].
2. External primers, diluted to 5 μM concentrations.
   (a) Forward: PV-3D-BglII-for (5′-TAG AGG ATC CAG ATC TTG GAT GCC A-3′).
   (b) Reverse: PV-3D-EcoRI-ApaI-polyA-rev (5′-CGC TCA ATG AAT TCG GGC CCT TTT TTT TTT TTT TTT TTT TCT CC-3′).
3. Internal primers, diluted to 5 μM concentrations.
   (a) Forward: PV-3D-K359R-for (5′-ATG ACT CCA GCT GAC *CGT* TCA GCT ACA TTT GAA ACA-3′).
   (b) Reverse: PV-3D-K359R-rev (5′-TGT TTC AAA TGT AGC TGA *ACG* GTC AGC TGG AGT CAT-3′).
4. $T_{10}E_{0.1}$ Buffer: 10 mM Tris–HCl, pH 8.0, 0.1 mM ethylene diamine tetraacetic acid [EDTA], pH 8.0.
5. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

Table 1

Motif D sequence alignment across positive and negative strand RNA virus families

Class	Virus family	Species		Motif D
+ssRNA	Picornaviridae	Poliovirus	15	DY**G**LTMTPAD**K**SA
		Coxsackievirus B3	15	GY**G**LIMTPAD**K**GE
		Enterovirus A	15	EY**G**LTMTPAD**K**SP
		Enterovirus 71	15	EY**G**LTMTPAD**K**SP
		Human Rhinovirus A	15	KY**G**LTITPAD**K**SD
		Human Rhinovirus B	15	NY**G**LTITPPD**K**SE
		Human Rhinovirus C	15	KYGLTITPAD**K**SD
	Caliciviridae	Norovirus	10	EY**G**LKPTRPD**K**TE
	Flaviviridae	Dengue 1	10	TALNDMGKVR**K**DI
		Dengue 2	10	TALNDMGKIR**K**DI
		Dengue 3	10	LALNDMGKVR**K**DI
		Dengue 4	10	LFLNDMGKVR**K**DI
		West Nile	10	HFLNAMSKVR**K**DI
		Hepatitis C	21	RYSAPPGDPP**K**PE
–ssRNA	Togaviridae	Chikungunya	10	RCATWMNMEV**K**II
		Eastern equine encephalitis	10	RCATWLNMEV**K**II
		Venezuelan equine encephalitis	10	RCATWLNMEV**K**II
		Western equine encephalitis	10	RCATWLNMEV**K**II
		Sindbis	10	RCATWLNMEV**K**II
	Coronaviridae	SARS	22	YQNNVFMSEA**K**CW
	Paramyxoviridae	Nipah	59	YD**G**AVLSQAL**K**SM
	Filoviridae	Ebola	59	LN**G**IQLPQSL**K**TA
	Orthomyxoviridae	Influenza A	20	LVGINM.TKK**K**SY
		Influenza B	20	LL**G**INM.SKK**K**SY
		Influenza C	20	LI**G**INM.SLE**K**SY

Numbers indicate position from first amino acid in motif D of RNA-dependent RNA polymerase. Conserved residues are shown in ***boldfaced*** type. Residues conserved within a virus group are *underlined*

6. Deep Vent DNA polymerase 2000 U/mL (New England BioLabs).
7. 3 mM dNTP mix: 100 mM dATP, 100 mM dGTP, 100 mM dTTP, and 100 mM dCTP. This solution is prepared by combining 300 μL of each NTP and bringing the volume up to 10 mL with ultrapure water and can be aliquoted and stored indefinitely at 20 °C.
8. 100 mM magnesium sulfate [$MgSO_4$] solution (supplied with Deep Vent).
9. 10× ThermoPol reaction buffer (supplied with Deep Vent).
10. 3 M sodium acetate [NaOAc], pH 5.2: adjust pH with glacial acetic acid.

11. Absolute ethanol.
12. 70 % ethanol solution: 70 % EtOH, 30 % ultrapure water.
13. Omnipur Agarose (Millipore/Calbiochem).
14. 0.5× TBE electrophoresis running buffer: 33 mM Tris–HCl, 40 mM boric acid, 1 mM EDTA, pH 8.0, 0.25 μg/mL ethidium bromide [EtBr].
15. Electrophoresis chamber and power source.
16. 5× bromophenol blue [BPB]: 0.05 % bromophenol blue, 50 % glycerol in $T_{10}E_{0.1}$ buffer.
17. *Bgl*II, *Eco*RI, *Apa*I and *Pst*I restriction enzymes.
18. Shrimp alkaline phosphatase [SAP], 1000 units.
19. QIAEX II Gel extraction kit (Qiagen).
20. Spin-X Plastic Centrifuge Tube Filters (Corning/Costar).
21. T4 DNA ligase, 1 unit/μL.
22. 5× T4 DNA ligase buffer.
23. SURE competent cells (Stratagene).
24. NZCYM broth, powder (Amresco).
25. 100 mg/mL ampicillin solution: 2 g ampicillin in 20 mL ultrapure water.
26. 2 L Erlenmeyer flask.
27. 2 % agar plates prepared with NZCYM medium with 50 μg/mL ampicillin.
28. Qiagen Plasmid Midiprep Kit (Qiagen).

2.3 In Vitro Biological Analysis of Polymerase Mutation Components

2.3.1 Tissue Culture Components

1. Sterile 100 mm polystyrene tissue culture dishes.
2. HeLa S3 cells from American Type Culture Collection (American Type Culture Collection [ATCC] no. CCL-2.2).
3. Complete medium: DMEM/F12, 10 % fetal bovine serum [FBS], 100 U/mL penicillin, 100 U/mL streptomycin.
4. 1× trypsin–EDTA solution.
5. Rapid-Flow Sterile 500 mL bottle top filter with 75 mm PES membrane, 0.22 μm pore size, and 45 mm blue neck, (Thermo Scientific/Nalgene).
6. Autoclaved 1× Phosphate buffered saline [PBS]: Make a 10× PBS solution 1.37 M sodium chloride, 27 mM potassium chloride, 100 mM disodium phosphate [Na_2HPO_4], and 20 mM monopotassium phosphate [KH_2PO_4], pH 7.4. Sterilize the 10× solution using a bottle top filter. Dilute to 1× using ultrapure water and autoclave for 30 min.

2.3.2 cDNA Linearization and In Vitro T7 Transcription Reaction Components

RNA is easily degraded by RNases, which are ubiquitous in the laboratory. They are found in the air, your skin or on anything touched by bare hands. Prior to beginning in vitro transcription, take care to designate an RNase-free area of the laboratory. Wipe down surfaces and any tools (pipets, beakers, bottles, instruments, etc.) with RNase AWAY. Rinse very well with hot water and spray down with 70 % ethanol solution. Always wear gloves and spray hands with 70 % EtOH before starting any procedure. Purchase chemicals and keep them in a separate area only to be used for making RNase-free solutions. Once RNA is made it can be stored at −80 °C until ready for use. RNA made for making virus can be stored for up to 1 week. RNA made for use in luciferase assays must be used the following day.

1. RNase AWAY (Molecular BioProducts).
2. Mutant plasmid.
3. *Apa*I restriction enzyme.
4. QIAEX II Gel extraction kit (Qiagen).
5. Spin-X Plastic Centrifuge Tube Filters (Corning/Costar).
6. $T_{10}E_{0.1}$ Buffer.
7. Ultrapure water.
8. 1 M Hepes, pH 7.5.
9. 320 mM magnesium acetate [MgAcetate].
10. 400 mM dithiothreitol [DTT].
11. 20 mM spermidine.
12. 160 mM NTPs: 40 mM ATP, 40 mM, CTP, 40 mM GTP, and 40 mM UTP.
13. T7 RNA polymerase [RNAP], 0.5 mg/mL.
14. RQ1 RNase-free DNase, 1000 units (Promega).
15. Autoclaved 0.65 mL microcentrifuge tubes.
16. Agarose.
17. RNase-free 0.5× TBE with 0.25 μg/mL ethidium bromide [EtBr].
18. RNeasy Mini Kit (Qiagen).
19. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

2.3.3 Luciferase Assay Components

1. Complete medium.
2. In vitro transcribed RNA made from mutant pRLucRA.
3. Autoclaved 1.7 mL microcentrifuge tubes.
4. VWR Signature Disposable Electroporation Cuvettes, 2 mm (VWR).

5. Bio-Rad Gene Pulser Generator Model 1652076 (Bio-Rad Laboratories).
6. Bio-Rad Capacitance Extender Model 1652087 (Bio-Rad Laboratories).
7. 12 × 75 mm disposable glass borosilicate tubes (VWR).
8. Luciferase assay system: includes luciferase assay substrate, luciferase assay buffer, and 5× cell culture lysis reagent [CCLR] (Promega).
9. Junior LB 9509 Portable Tube Luminometer (Berthold Technologies).

2.3.4 Poliovirus Stock Components

1. HeLa S3 cells (*see* Tissue Culture Components).
2. Complete medium (*see* Tissue Culture Components).
3. In vitro transcribed RNA made from mutant pMoVRA.
4. VWR Signature Disposable Electroporation Cuvettes, 2 mm (VWR).
5. Bio-Rad Gene Pulser Generator Model 1652076 (Bio-Rad Laboratories).
6. Bio-Rad Capacitance Extender Model 1652087 (Bio-Rad Laboratories).

2.3.5 Quantitating Virus and Viral Genomes Components

1. HeLa S3 cells.
2. Complete media (*see* Tissue Culture Components).
3. 6-well, flat bottom cell culture plates (Corning/costar).
4. 2× DMEM/F12 complete media: two packages of 1× DMEM, powder (Gibco), 4.8 g sodium bicarbonate, 20 % FBS, 200 U/mL penicillin, and 200 U/mL streptomycin.
5. Concentrated HCl.
6. Autoclaved, Pyrex Brand 1395 media storage bottle w/screw cap, 1 L (Corning).
7. Rapid-Flow Sterile 500 mL bottle top filter with 75 mm PES membrane, 0.22 μm pore size, and 45 mm blue neck, (Thermo Scientific/Nalgene).
8. Low melt agarose (Omnipur/Calbiochem).
9. 500 mL Erlenmeyer flask.
10. Crystal violet staining solution: 0.1 % crystal violet, 3.7 % formaldehyde, 20 % ethanol made in distilled water.
11. Autoclaved 1.7 mL microcentrifuge tubes.
12. QIAamp Viral RNA mini kit (Qiagen).
13. Qiagen RNeasy Plus Kit (Qiagen).
14. Molecular biology grade β-mercaptoethanol [β-ME].

2.4 In Vivo Analysis of Viral Mutation Components

2.4.1 Lethal Dose Fifty (LD_{50})/Paralysis Dose Fifty (PD_{50}) Analysis Components

1. 4–6-week-old outbred (ICR) mice transgenic for the PV receptor (cPVR) [24].
2. Quantitated virus stock, propagated in serum-free media (*see* Quantitating virus and viral genomes components).
3. 5 mL syringes.
4. 27 gauge (G), ½ inch needles.

2.4.2 Protection

1. Immunized mice (survivors from LD_{50}/PD_{50} study).
2. Five times the LD_{50}/PD_{50} [5× PD_{50}] of wild-type poliovirus.

3 Methods

3.1 Site-Directed Mutagenesis by Overlap Extension PCR

The polymerase gene was amplified using pMoV-3D-BPKN plasmid as a template. This template has silent mutations engineered into the 3Dpol coding sequence. The "naked" viral cDNA, pMoVRA, contains 4 *Pst*I restriction sites and pRLucRA contains 3. A *Pst*I site was engineered into the 3Dpol coding sequence such that when cloned into the "naked" vectors, pMoVRA and pRLucRA, and digested with *Pst*I, positive clones containing the mutated PCR product will have 5 and 4 bands respectively when run on a agarose gel. Clones positive by restriction digest are verified for the presence of the mutation by sequencing.

3.1.1 Round One: Extension PCR

1. PCR reaction A:
   (a) External forward primer: PV-3D-BglII-for.
   (b) Internal reverse primer: PV-3D-K359R-rev.
2. Perform amplification reactions in three separate 100 µL volumes, final concentration containing: 1× Thermopol buffer, 3 mM dNTPs, 0.5 µM of each primer, 0.5 ng/µL of template plasmid pMo-3D-BPKN, and 2 U of Deep Vent Polymerase (Table 2).
3. Cycling conditions consist of a preliminary denaturing step at 95 °C for 4 min followed by a hot start cycle for 4 cycles at 95, 50, and 72 °C each for 1 min and finally, 18–20 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min and product extension at 72 °C for 2 min and a final product extension at 72 °C for 10 min.
4. Prepare 2, 1.2 % agarose gels.
5. The rest of product tubes are combined and DNA is precipitated with 100 % ethanol. Add 1/5th volume (60 µL) 3 M NaOAc, mix well with pipet then add three volumes (1080 µL) 100 % EtOH and mix well. Freeze mixture on dry ice until liquid is a slow moving "sludge" when inverted. Centrifuge at top speed for 10 min. You will observe a thick white pellet.

Table 2

Round 1–1: extension PCR—reaction A

Reagent	Volume (μL)			Reaction concentration
10× Thermopol reaction buffer	10	10	10	1×
100 mM $MgSO_4$	0	1	2	0/1/2 mM
3 mM dNTPs	10	10	10	0.3 mM
5 μM forward primer: PV-3D-K359R-for	10	10	10	0.5 μM
5 μM reverse primer: PV-3D-EcoRI-ApaI-polyA-rev	10	10	10	0.5 μM
5 ng/μL pMo-3D-BPKN	10	10	10	0.5 ng/μL
Deep Vent polymerase	1	1	1	2 U
ddH_2O	49	48	47	–
Reaction volume	*100*	*100*	*100*	

Wash the pellet with 70 % EtOH three times. Pipet off all the EtOH and allow pellet to air dry for 5–10 min. Suspend in 10 μL $T_{10}E_{0.1}$.

6. Mix 10 μL of each PCR reaction with 2 μL 5× BPB. Load onto 1.2 % agarose gel and run for 30 min at 200 V.
7. Due to the high concentration of DNA loaded, EtBr in the gel and running buffer you should be able to visualize, without a UV light, a red band where the DNA is. Extract DNA from gel and purify using QIAEX II Gel extraction kit. Suspend product in 50 μL $T_{10}E_{0.1}$. Quantitate purified PCR product using NanoDrop spectrophotometer.
8. PCR reaction B:
   (a) External reverse primer: PV-3D-EcoRI-ApaI-polyA-rev.
   (b) Internal forward primer: PV-3D-K359R-for.
9. Same procedure as for PCR reaction A (Table 3).
10. Repeat concentration and purification a for PCR A.

3.1.2 Round Two: Overlap PCR

1. Set up 3–100 μL reactions as before this time using PCR reactions A and B as templates (Table 4):
   (a) External forward primer: PV-3D-BglII-for.
   (b) External reverse primer: PV-3D-EcoRI-ApaI-polyA-rev.
2. Concentrate and purify PCR product as previously described for PCR products A and B.

3.2 Cloning PCR Fragment into pMoVRA and pRLucRA Vectors

3.2.1 Vector and PCR Fragment Digest

1. Perform 20-fold overdigestion of pMoVRA, pRLucRA vectors and overlap PCR fragment with *Bgl*II and *Apa*I restriction enzymes (*see* **Note 3**).
2. First, digest cDNA and overlap PCR fragment with *Bgl*II. Digest purified cDNA by adding 2 μg of DNA to a 1.5 mL tube containing 10 μL of the appropriate 10× restriction buffer with 4 μL (40 U) of enzyme in a total volume of 100 μL and incubate according to manufacturer's instructions. We recommend 2–4 h for an incubation time. For the purified overlap PCR product, repeat the same procedure as above, using the entire 50 μL product in the digest (Table 5).
3. Allow reaction to proceed at 37 °C for 2 h.
4. Run a sample of uncut and cut plasmids on 1 % agarose gel to check efficiency of reaction (*see* **Note 3**).
5. When you have verified that the plasmid has been linearized, purify with QIAEX II gel extraction kit. Follow the kit's protocol for purifying and concentrating DNA from an aqueous solution.
6. Clean up DNA using QIAEX II gel extraction kit. Follow the kit's protocol for purifying and concentrating DNA from an aqueous solution.
7. Suspend silica bead pellet in 50 μL $T_{10}E_{0.1}$ and incubate at 65 °C for 10 min.
8. Quick spin the tube and remove both supernatant and beads and add to Spin-X filter tube. Spin at 800 × *g* for 5 min and collect eluted DNA from tube.

Table 3

Round 1–2: extension PCR—reaction B

Reagent	Volume (μL)			Reaction concentration
10× Thermopol reaction buffer	10	10	10	1×
100 mM $MgSO_4$	0	1	2	0/1/2 mM
3 mM dNTPs	10	10	10	0.3 mM
5 μM forward primer: PV-3D-K359R-for	10	10	10	0.5 μM
5 μM reverse primer: PV-3D-EcoRI-ApaI-polyA-rev	10	10	10	0.5 μM
5 ng/μL pMo-3D-BPKN	10	10	10	0.5 ng/μL
Deep Vent polymerase	1	1	1	2 U
ddH_2O	49	48	47	–
Reaction volume	*100*	*100*	*100*	

Table 4

Round 2: overlap PCR

Reagent	Volume (μL)			Reaction concentration
10× Thermopol reaction buffer	10	10	10	1×
100 mM $MgSO_4$	0	1	2	0/1/2 mM
3 mM dNTPs	10	10	10	0.3 mM
5 μM forward primer: PV-3D-BglII-for	10	10	10	0.5 μM
5 μM reverse primer: PV-3D-EcoRI-ApaI-polyA-rev	10	10	10	0.5 μM
5 ng/μL PCR reaction A	10	10	10	0.5 ng/μL
5 ng/μL PCR reaction B	10	10	10	0.5 ng/μL
Deep Vent polymerase	1	1	1	2 U
ddH_2O	39	38	37	–
Reaction volume	*100*	*100*	*100*	

Table 5

Vector (pMoVRA and pRLucRA) and insert (overlap PCR fragment) digest

Reagent	Volume (μL)	Reagent	Volume (μL)
10× NEB 3.1 buffer	10	10× NEB 3.1 Buffer	10
*Bgl*II (10 U/μL)	5	*Bgl*II (10 U/μL)	5
2 μg cDNA	–	PCR fragment	50
ddH_2O	–	ddH_2O	35
Reaction volume	*100*	*Reaction volume*	*100*

9. Digest purified cut cDNA by adding entire 50 μL of cut DNA to a 1.5 mL tube containing 10 μL of the appropriate 10× restriction buffer with 1 μL (50 U) of enzyme in a total volume of 100 μL and incubate according to manufacturer's instructions. We recommend 2–4 h for incubation time. For the purified cut overlap PCR product, repeat the same procedure as above (Table 6).
10. Allow reaction to proceed at 25 °C (about room temperature) for 2 h.
11. After the 2 h incubation with *Apa*I to the cDNA only digest, add 4 μL (4 U) of shrimp alkaline phosphatase to reaction. Incubate at 37 °C for 4 h to overnight in order to

dephosphorylate the ends of the double cut cDNA. Afterwards heat inactivate at 65 °C for 5 min.

12. Run a sample of the double cut plasmids on 1 % agarose gel to check efficiency of reaction.
13. Clean up both the PCR and cDNA reactions using QIAEX II gel extraction kit. Follow the kit's protocol for purifying and concentrating DNA from an aqueous solution.
14. Suspend silica bead pellet in 50 μL $T_{10}E_{0.1}$ and incubate at 65 °C for 10 min.
15. Quick spin the tube and remove both supernatant and beads and add to SpinX tube. Spin at 800 × *g* for 5 min and collect eluted DNA from tube.

3.2.2 Ligation Reaction (See **Note 4**)

1. With *Bgl*II and *Apa*I digested vectors and PCR fragment set up the ligation reaction as follows: Add 50 ng of double cut cDNA (vector), 50 ng of double-cut overlap PCR product (insert), to a 0.65 mL microcentrifuge tube containing 6 μL of 5× T4 DNA ligase buffer and 1 μL (1 U) of T4 ligase in a total volume of 30 μL and incubate reaction at 15 °C for 30 min (Table 7).
2. Run 15 μL on a 1 % gel to check for successful ligation and transform 10 μL into 100 μL SURE cells. Plate on 50 μg/mL ampicillin agar plates and incubate at 30 °C overnight.
3. pMoVRA and pRLucRA are both low copy plasmids. To screen colonies, grow in 500 mL culture with 50 μg/mL ampicillin at 30 °C to an OD_{600} of 1.0. Purify 1 mL of culture and screen plasmid by *Pst*I restriction digest. Harvest cells and purify plasmid using Qiagen Midi Prep Kit.
4. Successful plasmids should be labeled as pMoV/pRLuc-PV-3D-BPKN-K359R.

Table 6

Cut vector (pMoVRA and pRLucRA) and cut insert (overlap PCR fragment) digest

Reagent	Volume (μL)	Reagent	Volume (μL)
10× NEB cut smart *buffer*	10	10× NEB Cut Smart Buffer	10
ApaI (50 U/μL)	1	*Apa*I (50 U/μL)	1
*Bgl*II digested cDNA	50	*Bgl*II digested overlap PCR fragment	50
SAP (1 U/μL)	1	–	–
ddH_2O	35	ddH_2O	35
Reaction volume	*100*	*Reaction volume*	*100*

3.3 In Vitro Biological Analysis of Polymerase Mutation

3.3.1 cDNA Linearization and In Vitro T7 Transcription Reaction

1. pMoV-3D-K359R and pRLuc-3D-K359R plasmids are first linearized with restriction enzyme *Apa*I.
2. Digest purified cDNA by adding 5 μg of mutant plasmid to a 1.5 mL tube containing 10 μL of the appropriate 10× restriction buffer with 2.5 μL (50 U) of enzyme in a total volume of 100 μL and incubate according to manufacturer's instructions. We recommend 2–4 h for incubation time (Table 8).
3. Run a sample of uncut and cut plasmids on 1 % agarose gel to check efficiency of reaction (*see* **Note 3**).
4. When you have verified that the plasmid has been linearized, purify with QIAEX II gel extraction kit. Follow the kit's protocol for purifying and concentrating DNA from an aqueous solution.
5. Suspend silica bead pellet in 50 μL $T_{10}E_{0.1}$ and incubate at 65 °C for 10 min.
6. Quick spin the tube and remove both supernatant and beads and add to Spin-X tube. Spin at 800 × *g* for 5 min and collect eluted DNA from tube.
7. Add H_2O first; subtract the DNA volume from 2.5 μL to get the volume of H_2O to be added.
8. Next add the following in this order to a total volume of 20 μL, final concentration containing: 350 mM HEPES, 32 mM MgAcetate, 40 mM DTT, 2 mM spermidine, 28 mM NTPs, 0.5 μg linearized cDNA, and 0.5 μg T7 RNAP into an autoclaved 0.6 mL microcentrifuge tube (Table 9).
9. Pre-incubate the reaction mix at 37 °C for 5 min prior to adding T7 RNAP.
10. Add T7 RNAP.
11. Incubate reaction at 37 °C. After 30 min, check reaction for cloudy, white precipitate, which is magnesium pyrophosphate forming, to ensure the reaction is progressing. Allow reaction

Table 7
Vector and insert ligation reaction

Reagent	Volume (μL)
5× T4 DNA ligase buffer	6
5 ng/μL vector	10
5 ng/μL insert	10
T4 Ligase (1 U/μL)	1
ddH_2O	3
Reaction volume	*30*

Table 8
Linearization of cDNA

Reagent	Volume (μL)
10× NEB cut smart buffer	10
*Apa*I (50 U/μL)	2.5
5 μg cDNA	–
ddH_2O	–
Reaction volume	*100*

to incubate for 4–5 h, spin the reaction for 2 min to pellet out the magnesium pyrophosphate.

12. Transfer supernatant to a new tube and then add 2 μL of RQ1 DNase (2 U) and incubate for 30 min at 37 °C.
13. Clean up RNA using Qiagen RNeasy Mini Kit following manufacturer's instructions for RNA cleanup.
14. Measure concentration of purified RNA product using a NanoDrop spectrophotometer. Store RNA at −80 °C until ready to use.

3.3.2 RNA Transfection

1. Remove media from 100 mm plate of HeLa cells. Add 4 mL 1× PBS, wash, remove 1× PBS, and add 1 mL trypsin–EDTA. Allow plate to incubate in 37 °C incubator for 3 min. Wash off cells from plate with 9 mL of complete media.
2. Count cells and prepare 1.2×10^6 cells/transfection. Pellet cells at $150 \times g$ for 4 min. Wash pellet with 1× PBS and pellet again. Suspend cells in ($n \times 400$ μL) 1× PBS. N = number of transfections needed.
3. Prepare 1.7 mL microcentrifuge tube with 5 μg of RNA transcript and place on ice. Do not add HeLa cells to RNA at this point.
4. Set Bio-Rad Gene Pulser Generator Model 1652076 (electroporator) to 0.13 kilovolts (kV) with (Bio-Rad Capacitance Extender Model 1652087) capacitance at 500 micro Farads (μFD). Remove cuvettes from individual wrapping and remove caps.
5. Add 400 μL of HeLa cell suspension to 1.7 mL tube of RNA. Quickly add the mixture to the cuvette and place cuvette into chamber and zap cells. Using media from 15 mL conical (pre-warmed from water bath) add 600 μL of media to zapped cells still in the cuvette. Gently pipet up and down multiple times to mix cells and media and to break up any cell clumps that may have formed.

Table 9

In-vitro T7 transcription reaction

Reagent	Volume (μL)	Reaction concentration
1 M HEPES 7.5	7	350 mM
320 mM Mg Acetate	2	32 mM
400 mM DTT	2	40 mM
20 mM Spermidine	2	2 mM
160 mM NTPs	3.5	28 mM
Linearized cDNA	2.5 (max volume)	0.025 μg/μL (0.5 μg)
0.5 mg/mL T7 RNAP	1	0.025 μg/μL (0.5 μg)
H_2O	2.5—DNA Volume	
Reaction volume	*20*	

3.3.3 Luciferase Assay

1. Prepare 15 mL conical tubes with 5.6 mL of complete media. Set them aside in 37 °C water bath until needed.
2. Proceed with RNA transfection as described previously (*see* RNA transfection).
3. Add 600 μL of media to zapped cells still in the cuvette. Gently pipet up and down multiple times to mix cells and media and to break up any cell clumps that may have formed.
4. Add cell and media mixture back to 15 mL conical tube. Close the tube and gently invert back and forth to mix. Aliquot 500 μL into a 1.7 mL tube and place the 15 mL conical in the 37 °C incubator until ready to take next timepoint.
5. Spin the one 1.7 mL tube at 2500 × *g* for 2 min for 0 h time point.
6. Remove media and wash pellet with 1× PBS. Spin again. Remove 1× PBS and add 100 μL of 1× CCLR (diluted to 1× in ddH_2O). Vortex tube for 10 s and place on ice.
7. Repeat this for every time point taken and leave all cells on ice until the next day. Make that each time an 500 μL aliquot is removed from the 15 mL conical tube that the tube is inverted multiple times to ensure an even distribution of cells.

8. Next day, vortex cells again for 10 s and spin down at max speed for 5 min to pellet any cellular debris.
9. Transfer 10 μL of supernatant to 12 × 75 mm glass borosilicate assay tube. Let sit for 10–15 min then add 10 μL of luciferase substrate. Immediately place into luminometer and read light output.

3.3.4 Infectious Center Assay (See Quantitating virus and viral genomes components)

The infectious center assay is used to determine the fraction of cells within a culture that are infected with virus after RNA transfection. In this case, the infected cells are suspended, counted, and plated onto monolayers of susceptible cells, which are then overlaid with agar. The number of plaques provides a measure of the number of virus infected cells in the original culture, thus how infectious the in vitro transcribed RNA is.

1. One day before seed 6-well plates with 6×10^5 HeLa S3 cells per well and cover with 3 mL complete media.
2. Next day, follow procedure for in vitro RNA transfection.
3. Prepare the 6-well plate by removing media from each well. Wash with 1× PBS, remove PBS and add another 500 μL of PBS to each well and set to the side.
4. Serially dilute, tenfold, transfected cells in complete media. Place 100 μL of virus mixture on cells in 6-well plates.
5. Allow cells to adhere to the plate for 2 h at 37 °C.
6. During incubation, prepare 2× complete media. Combine two packages of powdered media and 4.8 g of sodium bicarbonate into 1 L of ddH_2O. Adjust pH to 7.2 with concentrate HCl.
7. Inside a serological hood, filter media with 0.22 μm bottle top filter into autoclave 1 L pyrex media bottle. Next, prepare 2 % agarose solution using low melt agarose and ddH_2O. Make a 10 mL, 2 % solution for every 6-well plate prepared. Using a 500 mL Erlenmeyer flask, heat agarose mixture in microwave and be careful that solution doesn't boil over the top of the flask. Once all of the agarose is in solution, place in 37 °C water bath until cool enough to comfortably hold.
8. When cooled, add 20 % FBS and 200 U/mL of both penicillin and streptomycin then add the 2× media to final volume. Complete 2× media and 2 % agarose solution is at a 1:1 volume ratio in order to make final solution 1× complete DMEM and 1 % agarose. Completed agarose overlay can remain in the water bath until cells are ready.
9. After 2 h, cover cells with 3 mL of agarose overlay per well. Allow agarose to solidify at room temperature before incubating the plates at 37 °C for 2 days.

10. After 2 days of incubation, "pop" agarose plugs with metal spatula being careful not to scratch the cell monolayer. Once removed, wash cells with 1 mL 1× PBS. Remove PBS and add 500 µL of crystal violet stain. Let stand for 5 min, remove crystal violet and again wash with 1 mL 1× PBS. Count plaques to determine virus titer in pfu/mL.

3.3.5 Virus Isolation, Titer and One-Step Growth Curves

1. One day before, seed 100 mm plate with 3×10^6 HeLa S3 cells.
2. Next day, prepare 100 mm plate by removing media and washing once with 1× PBS. Add 9 mL of complete media to plate and put to the side.
3. Follow procedure for RNA transfection (*see* RNA transfection).
4. Add 1 mL of transfected cell mixture to HeLa cell monolayer and incubate at 37 °C until you observe cytopathic effects (CPE) under a light microscope. Complete CPE should be observed after 48 h (cells will be completely detached from the bottom of the plate and floating around in the media).
5. Upon CPE, harvest virus and cells into 15 mL conical and freeze on dry ice. Once frozen, thaw in 37 °C water bath then vortex for 30 s. Repeat this procedure two more times until you have performed "3 freeze-thaw cycles."
6. Remove cell debris by centrifugation at top speed for 10 min. Pour off supernatant into a new 15 mL conical tube. Freeze on dry ice and store at –80 °C until ready to use (*see* **Note 5**).
7. This virus culture is labeled as passage 0 (P0).
8. To titer virus, prepare a 6-well plate, the day before, with 6×10^5 HeLa S3 cells per well and cover with 3 mL complete media.
9. Next day, serially dilute virus in PBS. Place 100 µL of virus mixture on cells in 6-well plates. Allow the virus to adsorb to the cells for 30 min.
10. After 30 min, remove virus and wash cells with 1 mL 1× PBS.
11. Remove PBS and replace with complete media containing 1 % agarose (*see* Infectious center assay for *how to make overlay*).
12. Allow agarose to solidify at room temperature before incubating the plates at 37 °C for 2 days.
13. After 2 days, "pop" agarose plugs with metal spatula being careful not to scratch the cell monolayer. Once removed, wash cells with 1 mL 1× PBS. Remove PBS and add 500 µL of crystal violet stain. Let stand for 5 min, remove crystal violet and again wash with 1 mL 1× PBS. Count plaques to determine virus titer in pfu/mL.
14. Also extract RNA using QIAamp Viral RNA mini kit to quantitate genome copies/mL by RTqPCR (*see* Fecundity assay on *how to prepare the standard curve of RTqPCR*).

15. To make additional viral passages: One day before, seed 100 mm plate with 3×10^6 HeLa S3 cells.
16. Next day, prepare 100 mm plate by removing media and washing once with 1× PBS. Remove PBS and add 2 mL of fresh 1× PBS.
17. Inoculate cells with virus at MOI 0.01. Allow virus to adsorb to cells for 30 min, wash with 1× PBS and add 10 mL of complete to plate. Incubate at 37 °C until CPE is observed (with WT PV CPE should be observed after 24 h, time to CPE mutants will vary).
18. Titer by plaque assay and quantitate genome copies/mL.
19. Keep passaging virus in this manor until you have reached the desired passage number. Sequence the 3Dpol gene to check for stability of the engineered mutation (*see* **Note 6**).
20. To analyze one-step virus growth, infected cells with virus at a multiplicity of infection (MOI) 10. Allow virus to adsorb to cells for 30 min and wash with 1× PBS then add 1 mL of complete media to cells.
21. Incubated at 37 °C and harvest cells and media using a sterile disposable spatula (to scrape cells off the bottom of the well) at various time points post-infection. Virus and cell mixture is put into autoclaved 1.7 mL microcentrifuge tubes and immediately frozen on dry ice.
22. Harvest virus by three repeated freeze–thaw cycles and quantitate virus titers as described above.
23. Also extract RNA using Qiagen RNeasy Plus Mini Kit to determine genomes/mL by RT-qPCR.

3.3.6 Fecundity Assay

1. Using P0 virus, extract viral RNA using the QIAamp Viral RNA mini kit, following manufacturer's directions.
2. Determine viral genome copies by performing RT-qPCR on extracted virus sample.
3. Create a standard curve using in vitro transcribed RNA. Dilute RNA to 4 ng/μL, which is approximately 1×10^9 genome copies/μL. For a more accurate determination of genome copies/μL, use digital PCR.
4. One day before seed 6-well plates with 6×10^5 HeLa S3 cells per well and cover with 3 mL complete media.
5. Infect HeLa cells with P0 virus that corresponds to a total of 3×10^2, 3×10^3, 3×10^4, and 3×10^5 viral RNA genomes.
6. Incubate virus and cells at 37 °C for 30 min. Remove virus and wash cells with 1 mL 1× PBS. Remove PBS and add 1 mL complete media to wells and allow viral replication to proceed for 8 h.

7. After 8 h, purify total RNA from infected cells with Qiagen RNeasy Plus Mini Kit.
8. Harvested virus is at passage 1 (P1). Harvest virus by three repeated freeze–thaw cycles. Perform RT-qPCR purified RNA to calculate the amount of virus required to infect next HeLa cells with a total of 3×10^2, 3×10^3, 3×10^4, and 3×10^5 viral RNA genomes.

3.4 In Vivo Biological Analysis of Polymerase Mutants

3.4.1 Lethal Dose Fifty (LD_{50})/Paralysis Dose Fifty (PD_{50}) Analysis Components

1. House 4–6 week old outbred (ICR) mice transgenic for the PV receptor (cPVR) in standard ventilated caging for all experiments.
2. Passage 4 (P4) viral stocks are used for animal inoculations (*see* Virus isolation, titer and one-step growth curves).
3. Generate all virus stocks in serum-free media, harvested, titered, and genomes obtained.
4. Inoculate mice via intraperitoneal route (i.p.).
5. PD_{50}s are performed by infecting five mice per viral dose (1×10^7, 1×10^8, and 1×10^9 pfu) in order to calculate a PD_{50}.
6. Infect mice with virus by i.p. injection in 3 mL of serum-free media.
7. Observe mice for 14 days for signs of disease (ruffled fur and general malaise) and euthanize upon mice showing dual limb paralysis or paralysis such that their ability to obtain food and water is compromised.
8. After 14 days, determine PD_{50} values by the Reed and Muench method.

3.4.2 Protection

1. Mice previously infected with mutants, or surviving mice (from PD_{50} experiment) were challenged 1 month after initial infection using the same methods with 5× PD_{50} of WT poliovirus by i.p. injection and observed for 14 days as before.

4 Notes

1. The AAA codon encoding the lysine was changed to CGT codon encoding arginine. This genetic reversion requires two transversion mutations, which is a very inefficient event thus providing some barrier to reversion.
2. When using new primers, test the efficiency of the reaction by titrating in $MgSO_4$. Set up 3–100 μL reactions and add 0, 1, and 2 μL of the 100 mM $MgSO_4$. Run 10 μL of completed product on agarose gel to check efficiency of reaction. Combine the successful reaction tubes and precipitate out DNA for gel purification.

3. Combine water, cDNA and buffer in 1.7 mL tube and before adding enzyme to your reaction, remove 5 μL from the tube and set aside. This will be your "uncut" sample. Add the enzyme and allow reaction to proceed at proper temperature. After incubation remove another 5 μL from the tube. This will be your "cut" sample.
4. The number of colonies after successful ligation and transformation of cloned pMoVRA and pRLucRA yield different results. When plating 100 % of your transformed cells, the pMoVRA clone should yield roughly 50 colonies on a plate, whereas the pRLucRA clone will yield at most 100 colonies.
5. Multiple freeze thaws of virus stock will overtime lower the titer of the virus. To avoid this, make small aliquots of virus and store at −80 °C. Never use a stock tube that has been thawed more than three times after a titer or genome copy has been determined.
6. There are two reasons why it is important to passage virus at a low MOI. First is to check the stability of the engineered mutation by sequencing the mutated region; second is to generate a quasispecies.

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Chapter 7

BacMam Platform for Vaccine Antigen Delivery

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Abstract

Recombinant baculo viruses based on Autographa californica multiple nuclear polyhedrosis virus carrying vertebrate cell active expression cassettes, so-called BacMam viruses, are increasingly used as gene delivery vectors for vaccination of animals against pathogens. Different approaches for generation of BacMams exist and a variety of transfer vectors to improve target protein expression in vivo have been constructed. Here we describe a use of transfer vector which contains an insect cell-restricted expression cassette for the green fluorescent protein and thus enables easy monitoring of BacMam virus rescue, fast plaque purification of recombinants and their convenient titer determination and which has been proven to be efficacious for gene delivery in vaccination/challenge experiments.

Key words BacMam technology, Baculo virus transfer plasmids, Green fluorescent protein expression, In vitro and in vivo transduction

1 Introduction

The baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), belongs to the genus *Nucleopolyhedrovirus* of the family *Baculoviridae* [1]. Its large double-stranded DNA genome has size of about 134 kb and purified virus DNA is infectious. In the enveloped virions the size of the circular genome determines the length of the rod-shaped nucleocapsid which surrounds the DNA, enabling integration of large DNA sequences into the viral genome [1]. With respect to biosafety and biosecurity, Baculovirus vectors are regarded as safe because of their highly specific host range for productive replication, and lack of detectable AcMNPV-promoter driven gene expression in mammalian cells [reviewed in ref. [2]]. AcMNPV has been used successfully for the high level synthesis and purification of proteins in infected

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insect cells since the early 1980s with expression of the heterologous open reading frames (ORFs) driven by the baculoviral polyhedrin or p10 promoters [3]. In the mid-ninties, Hofmann et al. [4] and Boyce and Buchner [5] showed that recombinant AcMNPV with mammalian promoters regulating the expression of the protein of interest were suitable for delivery into and expression of genes in hepatic cells. Following these ground braking developments, a number of mammalian cell types, cells of avian [6] and even piscine [7] origin have been reported to be transduceable by the so called BacMam method which is also known as BacMam technology [for review *see* ref. 8].

Different commercially available systems for generation of recombinant baculoviruses make BacMam viruses easy to produce. These gene delivery vectors have a broad in vitro host range, are suitable for both transient and stable gene transfer, and, if mass application is envisaged, are cost-effective in comparison to chemical gene transfer procedures [2]. These benefits are mirrored by the increasing number of publications dealing with application of the BacMam technology. Surprisingly, publications reporting application of this technology in immunization challenge/experiments against pathogens are still limited so far [9–11] although direct vaccination with BacMam viruses can result in the induction of significant humoral and cell-mediated immunity against animal and zoonotic pathogens, including influenza virus, porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, West Nile virus, RABV, and hepatitis C virus [*see* ref. 12 for references].

Developments for next-generation BacMam vectors for vaccination purposes are aimed at enhancement of the transduction efficacies in vivo by increasing the antigen expression or by display of specific ligands on pseudotyped virus particles [9, 12, 13].

Here, we describe construction of BacMam viruses relying on the FastBacDual system (Invitrogen, Karlsruhe, Germany) using the new baculovirus transfer vector pMamBac-CAGGS (Fig. 1) which is based on pBacMamMCMVdual-ie [13] and contains the strong mammalian cell-active CAGGS enhancer/promoter element [14].

To facilitate isolation, plaque purification and titer determination of BacMam viruses, pMamBac-CAGGS also transfers an insect cell-restricted, polyhedrin promoter driven green fluorescent protein (GFP) expression cassette into the Baculovirus genome (Fig. 1). Thus, in insect cells, infected with respective recombinants, GFP is expressed and infection can be easily monitored using a fluorescence microscope. In vertebrate cells, however, the polyhedrin promoter is inactive.

2 Materials

Prepare all solutions using ultrapure water with a conductivity of <0.06 μS/cm and analytical grade reagents. Autoclave at 121 °C for 20 min or filtrate through a 0.2 μm filter unit. Use sterile disposable equipment for cell culture. Heat-sterilize all glass materials. Standard equipment for molecular biology laboratories is implied.

2.1 Cloning Procedures

1. Restriction enzyme *EcoR*I including 10× reaction buffers.
2. Plasmid pBacMam-CAGGS (available from the authors).
3. Calf intestine alkaline phosphatase (CIP) including 10× reaction buffer.
4. Phage T4 DNA ligase.
5. QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).
6. 60 mM ethylene glycol tetraacetic acid (EGTA), pH 7.0.
7. TE buffer: 10 mM Tris–HCl , 1 mM EDTA, pH 7.5.
8. TE-saturated phenol.
9. Chloroform–isoamyl alcohol 24:1 (v/v).
10. 3 M sodium acetate, adjusted to pH 4.8 with acetic acid.
11. Basic enzyme buffer (10× TA): 330 mM Tris–HCl, 660 mM potassium acetate, 100 mM magnesium acetate, adjusted to pH 7.9 with acetic acid, 1 mg/mL bovine serum albumin, 5 mM dithiothreitol.
12. Appropriate chemically competent *E. coli* strain.
13. LB-medium: dissolve 10 g tryptone, 5 g yeast extract, and 8 g NaCl per liter. For LB agar petri dishes add 15 g Bacto-Agar. Autoclave and cool to 56 °C in a water bath. Pour 10–15 mL into 10 cm petri dishes and invert plates after solidification. Store at 4 °C. For selection add ampicillin to a final concentration of 100 μg/mL.
14. Qiagen Plasmid Mini kit and Plasmid Midi kit (Qiagen, Hilden, Germany).

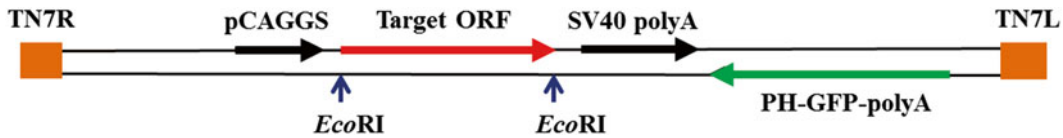

Fig. 1 Schematic of the pFastBac-Dual-based BacMam transfer plasmid pBacMamCAGGS with inserted target ORF. The hybrid human cytomegalovirus/chicken β-globin enhancer/promoter element (CAGGS) and the SV40 consensus sequence for polyadenylation (SV40 polyA) which control expression of the target ORF are indicated, as are the target ORF flanking *EcoRI* restriction enzyme cleavage sites. Also depicted is the insect cell-active, polyhedrin promoter regulated expression cassette for GFP (PH-GFP-poly A). TN7R and TN7L denote sequences required for transposition from the transfer plasmid into the bacmid. Note: Not drawn to scale

2.2 Agarose Gel Electrophoresis

1. 50-fold concentrated Tris-acetate buffer: 2 M Tris–HCl, 0.25 M sodium acetate, 0.05 M EDTA adjusted to pH 7.8 with acetic acid.
2. Agarose for gel electrophoresis.
3. Ethidium bromide solution of 10 mg/mL in water (*see* **Note 1**).
4. Electrophoresis buffer for agarose gel electrophoresis is 1× Tris-acetate. Add ethidium bromide in water to a final concentration of 100 ng/mL.
5. DNA loading buffer: 40 % sucrose, 1 mM EDTA pH 7.5, 0.05 % bromophenol blue, 0.1 % SDS.
6. Suitable DNA size marker.
7. Agarose gel electrophoresis equipment.
8. UV-transilluminator (254 and 302 nm) (*see* **Note 2**).

2.3 Transposition of the Transfer Plasmid into the Baculovirus Bacmid (Bac-to-Bac System, Invitrogen)

1. Chemically competent *E. coli* DH10Bac (Invitrogen).
2. SOC-medium (per 100 mL): 2 g tryptone/L, 0.5 g yeast extract/L, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose.
3. LB medium (*see* Subheading 2.1, **item 13**) with 10 μg/mL tetracycline, 50 μg/mL kanamycin, 7 μg/mL gentamycin.
4. LB-gar plates (*see* Subheading 2.1, **item 13**) with 10 μg/mL tetracycline, 50 μg/mL kanamycin, 7 μg/mL. gentamycin, 20 μg/mL. For blue/white selection spread 40 μL X-Gal (20 mg/mL) and 40 μL 100 mM IPTG evenly on the plate.
5. Solution 1: 10 mM EDTA, pH 8.0, 20 mM Tris–HCl, pH 8.0, 50 mM glucose. Before use add 2 mg/mL lysozyme.
6. Solution 2: 0.2 M NaOH, 1 % SDS in H_2O.
7. Solution 3: 3 M sodium acetate, pH 4.8.
8. TE-buffer with 50 μg/mL RNase A.

2.4 Insect Cell Culture

1. *Trichoplusia ni* High Five cells, grown in serum free "Insect Express Sf9-S2 with l-glutamine Medium" (PAA) in appropriate disposable tissue culture vessels.
2. *Spodoptera frugiperda* SF9 cells, grown in Grace's insect culture medium with 10 % FCS in appropriate disposable tissue culture vessels.
3. Serum free double concentrated Grace's insect culture medium.
4. Low melting point agarose (2 %) in H_2O.

2.5 Rescue of BacMam Viruses from Bacmid DNA

1. High Five cells.
2. FuGENE HD transfection reagent (Roche).
3. SF9 cells.

2.6 Transduction of Vertebrate Cells with BacMam Viruses

1. Vertebrate cells in adequate culture vessels in appropriate cell culture medium.
2. PBS^+. Contents per 1 L: 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.4 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2. Sterilize by filtration. PBS^- does not contain $CaCl_2$ and $MgCl_2$ (*see* **Note 3**).
3. 1 M Na-butyrate pH 7.0.
4. Low speed centrifuge with a plate rotor.

3 Methods

3.1 Agarose Gel Electrophoresis

We use self-constructed agarose gel apparatuses with buffer recirculation. Nevertheless, the specifications given below can be easily adapted to other formats. Thus, assemble the device as appropriate.

1. Prepare a 0.6 % gel by boiling 3 g agarose in 490 mL water in a microwave oven until fluidized and cool to 56 °C in a water bath. Replenish evaporated water (*see* **Note 4**).
2. Add 10 mL 50× Tris-acetate buffer and 5 μL of 10 mg/mL ethidium bromide solution and leave in a 56 °C water bath until use.
3. Pour the gels according to the specifics of your system (*see* **Note 5**). For examination of control of for example restriction enzyme cleavage reactions make small (6 cm × 4 cm ($l \times w$)), ~5 mm thick gels. For purification of DNA fragments larger gels (25 cm × 15 cm ($l \times w$)) and 5 mm thick) may be more appropriate. Use combs as necessary.
4. After solidifying cover the gels with electrophoresis buffer containing 0.1 μg/mL ethidium bromide and draw out the comb. Pipette the samples into the wells. Include one well with the DNA size marker.
5. The small gels run at 8 V/cm distance between the electrodes for 25 min; the large gels at 4 V/cm distance between the electrodes for 3–6 h (*see* **Note 6**).
6. Place the gel on a UV transilluminator (254 nm for documentation only, 302 nm for excision of fragments) after electrophoresis to visualize the DNA fragments. Take a picture for documentation (*see* **Note 2**).

3.2 Insertion of the Target ORF into the BacMam Transfer Vector

Among a large number of BacMam transfer plasmids, to our knowledge only those developed in our laboratory carry an expression cassette for GFP whose expression is driven by the polyhedrin promoter and thus restricted to insect cells [13]. We so far did not observe any negative effects of the presence of this cassette on

BacMam virus propagation in insect cells and infectious virus yield. Advantages of GFP expression in insect cells are monitoring of BacMam rescue, easy plaque purification and fast titer determination. For insertion of target ORFs into the transfer vector pBacMamCAGGS (*see* Fig. 1) use of the *Eco*RI cleavage site is recommended.

1. Isolate the target ORF DNA fragment, flanked by *Eco*RI cohesive ends or blunt ends, after agarose gel electrophoresis using the QIAquick Gel Extraction Kit according to the manufacturer's instructions. Elute the DNA with 50 µL 5 mM Tris–HCl/1 mM EDTA pH 7.0.
2. For the vector preparation, cleave 5 µg of plasmid pBacMamCAGGS (*see* Fig. 1) using 5 µL of 10× *Eco*RI reaction buffer, 5 U *Eco*RI enzyme, and water to a final volume of 50 µL. Incubate for 2 h at 37 °C.
3. For dephosphorylation of 5′ ends with calf intestine phosphatase (CIP) add 25 µL 10× CIP buffer, 174 µL ultra-pure water, and 1 µL CIP. Incubate for 30 min at 37 °C. Add another 1 µL CIP and incubate for 30 min at 56 °C. Add 50 µL of 60 mM EGTA and incubate for 30 min at 65 °C to inactivate the phosphatase. Add 30 mL 10 % SDS, 1 µL proteinase K (10 mg/mL) and incubate at 56 °C for 30 min.
4. Add 300 µL of TE-saturated phenol to the DNA sample. Shake vigorously 20–30 s. Centrifuge the sample for 2 min at room temperature to separate the phases (*see* **Note 7**).
5. Transfer the aqueous phase into a new 1.5 mL Eppendorf tube and add 300 µL of a 1:1 mixture TE-phenol and chloroform–isoamyl alcohol. Mix thoroughly and centrifuge the sample for 2 min at room temperature to separate the phases.
6. Transfer the aqueous phase into a 1.5 mL Eppendorf tube and add 1 mL chloroform–isoamyl alcohol. Mix thoroughly and centrifuge the sample for 2 min at room temperature to separate the phases.
7. Transfer the aqueous phase into a new 1.5 mL Eppendorf tube and determine the volume. Add TE to 360 and 40 µL 3 M sodium acetate, pH 7.0, and 1 mL 100 % ethanol. Mix thoroughly and incubate at −80 °C for about 30 min.
8. Pellet the precipitated DNA by centrifugation for 15 min at room temperature and remove the ethanol and wash the pellet with 1 mL 70 % ethanol and centrifuge for 10 min. Remove the ethanol and dry the pellet for 5–10 min by incubating the open tube at 56 °C.
9. Resuspend the dried pellet in 50 µL of TE by incubation at 56 °C for 15 min.

10. Into a new 1.5 mL Eppendorf tube, pipette 5 μL of the purified vector, up to 24 μL of the purified target ORF fragment, 5 μL BSA, 5 μL 10× TA, 5 μL 100 mM DTT, 5 μL 10 mM ATP, and 0.1 U T4 ligase (*see* **Note 8**). Adjust to 50 μL with H_2O. As a control, prepare the same ligation mixture but use water instead of the purified target ORF fragment. Incubate for 5 min at 37 °C, 1 h at 25 °C, and overnight at 4 °C (*see* **Note 9**).
11. To transform chemically competent bacteria, incubate freshly thawed aliquots on ice for 5 min and add 1–10 μL of the ligation mixtures to the recommended amount of bacteria.

    Incubate for 20 min on ice, 2 min at 42 °C, and again on ice for 5 min.
12. Add 200 μL LB medium + 2 μL 1 M KCl and 2 μL 2 M $MgSO_4$ per tube, incubate for 1 h at 37 °C and plate on LB-agar petri dishes containing ampicillin. Incubate overnight at 30 °C and then at 37 °C if colonies are too small (*see* **Note 10**).
13. Pick 6–24 (ore even more) colonies (*see* **Note 11**) with sterile pipette tips, sterile toothpicks, or an inoculation loop into 3 mL LB-medium with ampicillin and incubate overnight at 37 °C on a gyratory shaker.
14. Prepare plasmid DNA using the Qiagen Plasmid Mini kit according to the manual.
15. Determine the DNA concentration by UV spectrophotometry at 260 nm.
16. Cleave 5–20 μL plasmid DNA with appropriate enzymes for 1–2 h in the recommended buffer to determine the correct orientation of the target ORF.
17. Separate cleavage products by 0.6 % agarose gel electrophoresis for 3 h at 4 V/cm and identify correct clones.
18. Transform chemically competent *E. coli* with 1 μL plasmid DNA from a correct clone and incubate in 50 mL LB with ampicillin overnight at 37 °C on a gyratory shaker.
19. Prepare plasmid DNA with the Qiagen Plasmid Midi Kit according to the manual. Determine the DNA concentration by UV spectrophotometry at 260 nm (1 OD_{260} corresponds to a DNA concentration of 50 μg/mL) and verify identity and purity of the preparation by cleavage of 0.5 μg plasmid DNA with an appropriate restriction enzyme for 2 h followed by 0.6 % agarose gel electrophoresis.
20. Add 1.5 μg of the appropriate, purified BacMam transfer plasmid to 100 μL chemically competent *E. coli* DH10Bac in an Eppendorf tube, mix thoroughly and leave for 20 min on ice. Transfer tube to 42 °C for 2 min and chill on ice for 5 min.

21. Add 900 µL SOC medium, incubate for 4 h at 37 °C on a gyratory shaker at 300 rpm.
22. Prepare a 10^{-3} dilution in SOC medium and incubate overnight at 37 °C 300 rpm.
23. Dilute overnight culture 10^{-3}, 10^{-4} and 10^{-5} in SOC medium (500 µL each) and incubate at 37 °C, 300 rpm for 2 h.
24. Prepare IPTG und X-Gal containing agar petri dishes.
25. Plate 200 µL of the 10^{-3}, 10^{-4} and 10^{-5} dilutions on the agar plates. Incubate plates at 37 °C for 24 h and leave at room temperature for another day.
26. Pick four to six "white" colonies into 3 mL LB-medium with kanamycin, gentamycin, tetracycline and incubate overnight at 37 °C on a gyratory shaker.
27. Centrifuge 1 mL overnight culture at $4500 \times g$ for 1 min.
28. Resuspend bacteria in pellet in 100 µL "solution 1", add 100 µL "solution 2" and mix. Add 150 µL "solution 3", mix thoroughly and incubate for 20–60 min on ice.
29. Centrifuge for 5 min at $20{,}000 \times g$ and transfer the supernatant into a new tube.
30. Add 1 mL –20 °C 100 % ethanol, mix thoroughly and incubate for 15 min at –80 °C.
31. Centrifuge for 10 min at $20{,}000 \times g$ and discard supernatant.
32. Wash DNA pellet with 1 mL 70 % ethanol. Centrifuge tube at $20{,}000 \times g$ for 5 min. Dry the pellet 10 min by incubating the open tube at 56 °C for about 10 min.
33. Resolve pellet in 40 µL TE-buffer with RNase A at 56 °C for 5 min followed by shaking at 37 °C on a gyratory shaker at 1400 rpm for 30 min.
34. Determine OD_{260} and OD_{280} and use the clone with the 260/280 ratio closest to 2.0 for rescue of virus.

3.3 Rescue of BacMam Viruses from Bacmid DNA

1. Seed 1×10^6 High five cells in 1 mL "Insect Express medium" into a well of a 6 well plate and let cells attach for 1 h at 27 °C.
2. Pipette 5 µL bacmid DNA to 95 µL sterile water and add 6 µL of Fugene® HD transfection reagent. Incubate for 40 min at room temperature.
3. Wash cultures once with cell culture medium and cover cells with 1 mL cell culture medium.
4. Add 900 µL cell culture medium to the transfection mixture, mix gently and add it drop wise to the cells. Incubate plate for 5 h at 27 °C, replace supernatant with 2 mL fresh cell culture medium and incubate further at 27 °C.

5. Monitor transfected High five culture daily for appearance of autofluorescing cells and/or foci. Under optimal conditions, single autofluorescing cells (*see* Fig. 2) become visible at about 24 h after transfection (*see* **Note 12**).

3.4 Plaque Purification of BacMam Viruses

The BAC system is based on the well-studied Escherichia coli F factor. Replication of the F factor in *E. coli* is strictly controlled. However, the F plasmid is maintained in one to four copies per cell [15]. In addition, recent reports have shown that BAC vector sequences can be spontaneously excised from bacmid-derived vectors upon passage in insect cells which appears to occur primarily when foreign protein expression is interfering with virus replication. In summary, plaque purification is indispensable to as far as possible ascertain obtainment of a homologous virus isolate.

1. When a substantial amount of the transfected cells autofluoresce (usually 3 days after transfection), transfer 150 μL transfected-cell culture supernatant into an Eppendorf tube and prepare 10^{-1} and 10^{-2} dilutions. Seed each 7.5×10^5 SF9 cells in 2 mL Grace's insect culture medium with 10 % FCS into the wells of a 6-well plate and let cells attach for 20–30 min. Inoculate 100 μL of each dilution directly into one separate well and incubate for 1–2 h at 27 °C.
2. For the agarose overlay liquefy 2 % low melting agarose in a microwave oven and cool in water bath to 45 °C. Equilibrate double concentrated Grace's insect culture medium with 20 % FCS to room temperature (*see* **Note 13**) and mix directly before use 1:1 with LMP agarose.
3. Remove supernatant from the infected SF9 cells, mix equal amounts of agarose and 2× medium and overlay the cells quick but gentle with 2.5 mL per well.
4. After solidification of the overlay (approximately 10 min at room temperature) incubate plate at 27 °C and check for appearance of autofluorescing plaques.
5. Usually 3 days later, circle discrete located autofluorescing plaques on the bottom of the plate by passing a marker pen (e.g., Edding 404 black) along the objective from below (*see* **Note 14**). Aspirate cells from individual plaques using for example a Gilson P1000 (volume to 50 μL) and flush into a reaction tube with 1 mL SF9 medium. Shake for 30 min or freeze/thaw to release virions from picked infected cells.
6. Seed 1.25×10^6 SF9 cells in 4 mL medium into a 25 cm^2 tissue culture bottle and add the 1 mL plaque isolate. Incubate for 5–7 days at 27 °C until complete CPE.
7. For titer determination dilute BacMam preparations from 10^{-1} to 10^{-8} and transfer 100 μL of each dilution into the wells of a 96-well plate. Prepare four to six parallel columns and add about 1.4×10^4 SF9 cells in 25 μL Grace's insect culture

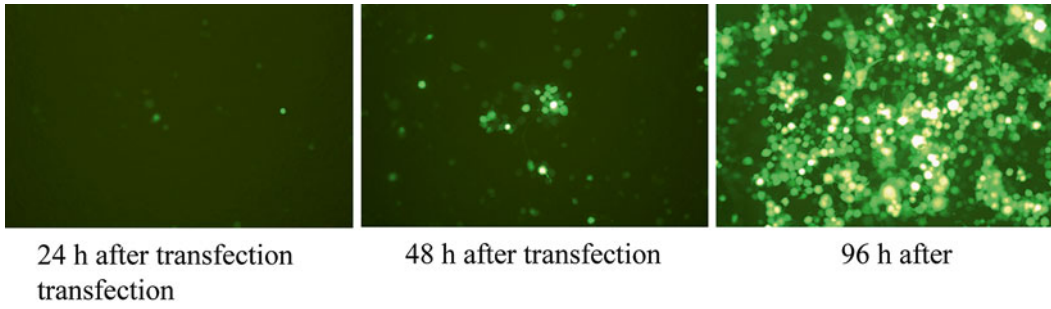

Fig. 2 Dissemination of autofluorescing cells in a High Five cell culture transfected with bacmid DNA indicates generation of BacMam viruses containing the sequences to be transferred. Cells were photographed using a Nikon fluorescence microscope and the FITC filter set

medium to the virus dilutions. Incubate for 3–5 days at 27 °C, determine wells with autofluorescent cells and calculate the $TCID_{50}$ using the Spearman and Kärber [16] algorithm.

3.5 Verification of Target Protein Expression in Vertebrate Cells

Verification of target protein expression by the individual BacMam plaque isolates should be performed prior to large scale propagation and immunization of animals. This is can be done by transduction of widely used cell lines like HEK 293T, Vero, BHK, Hela, and others reported to be efficiently transduced (reviewed by ref. [8]) followed by antigen detection using Western blotting, indirect immunofluorescence, ELISA, etc. Optimal transduction conditions need to be determined for each cell line but the following protocol should as a first approximation be applicable for most mammalian cell lines (*see* also Fig. 3)

1. Seed cells in desired plate format in a density that the cultures are confluent 24 h later (*see* **Note 15**).
2. Remove culture medium and wash cells twice with PBS^+ (*see* **Note 16**). Add 25 $TCID_{50}$ BacMam viruses per cell in PBS. Final volumes should be 2 mL per well in 6 well plates and accordingly less for other plate formats.
3. Centrifuge plates at 600 × *g* for 1 h at 27 °C. If a centrifuge with a plate rotor is not available incubate cultures for 4–6 h on a gyratory shaker with low speed agitation at 25–27 °C.
4. Replace inoculum with normal cell culture medium supplemented with 5 mM Na-butyrate [13] and incubate cultures for 24–48 h under normal conditions (*see* **Note 17**).
5. Remove medium, wash monolayers with PBS twice and process cells according to the envisaged detection method.

3.6 Large Scale Production and Concentration of BacMam Virions for In Vivo Experimentation

1. Infect 10^7 detached SF9 cells in 35 mL culture medium at a multiplicity of infection of 0.1 and seed into cell culture flasks with 150–162.5 cm growth area. Incubate at 27 °C until CPE is complete (usually 6–8 days).

2. Transfer contents of the flasks into 50 mL conical tubes and centrifuge at 4000 × *g* for 20 min. If applicable, pool supernatants from multiple tubes, remove an aliquot for titration and overlay a 7.5 mL 25 % sucrose cushion in PBS^-, filled into Beckman SW32 rotor ultracentrifuge tubes, with 25 mL of the infected culture supernatant. Centrifuge for 90 min at 25,000 rpm and 4 °C.
3. Aspirate carefully first the culture medium and then the sucrose cushion. Add 1 mL PBS+ to the pellets, seal tubes with Parafilm and leave overnight on ice.
4. Resuspend pellets and homogenize carefully using a Dounce homogenizer. Ten strokes should be sufficient. Store in aliquots at −70 °C.
5. Thaw an aliquot, sonicate for 5 s in an ultrasonic water bath at 40 W (*see* **Note 18**). Titrate together with sample taken prior ultracentrifugation and calculate recovery rate which normally is around 50 %.

3.7 In Vivo Transduction

Suitability of BacMam viruses as delivery vector for vaccines has been demonstrated, e.g., for mice [12], chicken [17] or pigs [10]. The doses for vaccination varied between 10^7 infectious units for pigs, given three times intra muscular (i.m.) in 15 days intervals and 10^9 infectious units for mice and chicken, immunized i.m. twice in a 3 week interval.

Since protocols for vaccination/challenge experiments depend markedly on specifics of the disease, of the target animal and of the laboratory, a general instruction appears not reasonable (*see* **Note 19**).

In the example for a vaccination/challenge experiment depicted below, rabbits were immunized i.m. with 5×10^8 PFU BacMam/VP60 (*see* also Fig. 4) at days 0, 7, and 12, and challenged with a lethal dose RHDV on day 42. All vaccinated animals developed VP60-specific serum antibodies and survived the challenge infection—demonstrating the potential of BacMam viruses as safe vaccine delivery vector.

4 Notes

1. Ethidium bromide powder is highly toxic when inhaled. Ethidium bromide is suspected to be a mutagen. Thus, solutions should be prepared under a fume hood and regarded as hazardous. Wear gloves while handling.
2. Short-wave UV light is hazardous for your eyes. Wear safety goggles or a face shield when examining or cutting out DNA fragments on a transilluminator to prevent damage to the eyes.

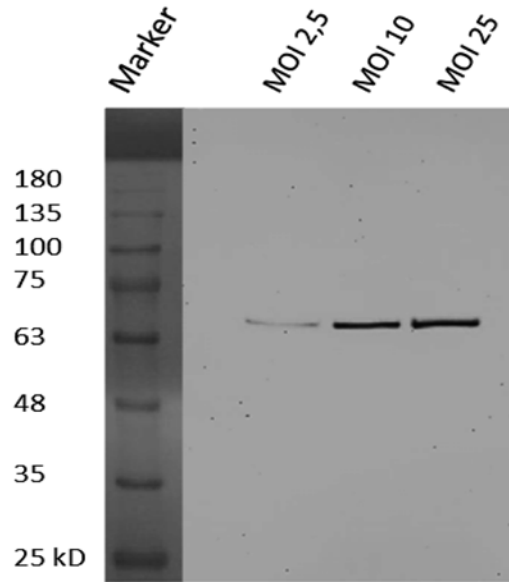

Fig. 3 Determination of BacMam transduction efficacy. Rabbit kidney cells (RK13) were transduced with BacMam/RHDV_VP60 expressing the rabbit hemorrhagic disease virus protein 60 (VP60) at the indicated MOI. Cells were harvested 24 h after transduction, lysed and proteins were transferred to nitrocellulose membranes after size separation by SDS-10 % PAGE. VP60 expression was visualized by monitoring chemiluminescence using a polyclonal rabbit serum against VP60, peroxidase labeled a-rabbit IgG serum and the Supersignal West Pico chemoluminescent kit (Pierce, Rockford, Il.) as recommended by the supplier

3. Be sure that the PBS^{+} contains calcium and magnesium. These are frequently absent in commercially available PBS solutions. Do not autoclave. Insoluble Ca^{2+} complexes may form.
4. Do not use Erlenmeyer flasks to boil agarose gels because bumping may lead to superheating may lead to sputtering of the agarose solution due to a chimney effect. Using glass beakers is safer.
5. Air bubbles on the gel surface are mainly an aesthetical problem. They can be removed with the flame of a pocket lighter or a Bunsen burner or punching with a toothpick, pipette tip, etc.
6. Electrophoresis time depends on the DNA fragment sizes (VP60) of the sample. Easy distinguishable DNA fragments require shorter, complex fragment compositions longer running times.
7. Unless otherwise noted, centrifugation of 1.5 mL Eppendorf tubes means centrifugation at about 16,000–20,000 × *g*.
8. Use 1 U T4 ligase for blunt end ligations.
9. We prefer incubation of plasmid ligations overnight at 4 °C instead of the frequently used overnight incubation at 16 °C.

10. Incubation of plates overnight at 37 °C may result in large ampicillin resistant colonies which exhaust the antibiotic and enable growth of non-transformed bacteria.
11. The number of clones to be analyzed depends on the number of colonies grown on control plates and on target plates. If for example the latter contain 30 colonies versus 5 colonies on the controls, it is theoretically sufficient to test 6 clones for presence of an insert in correct orientation.
12. In cases in which the low level expression of the target protein in insect cells [13] interferes with replication of the BacMam virus, appearance of autofluorescing cells may be delayed. However, if no autofluorescing cells appear after 3 days, at least bacmid preparation and transfection should be repeated. Although we do it not routinely, it may be worthwhile to include the empty vector as a positive process control.

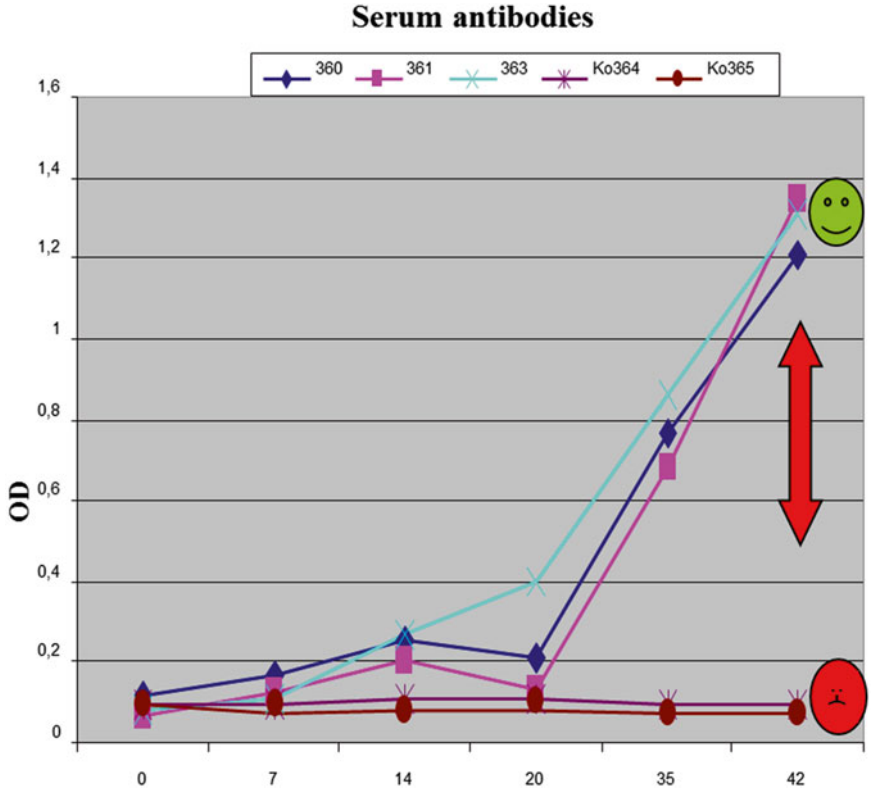

Fig. 4 Protection of rabbits against RHDV by BacMam virus vaccination. Rabbits were vaccinated i.m. with 5×10^8 PFU BacMam/VP60 at days 0, 7, and 12 (#360, #361, and #363) or left unvaccinated (#364 and #365) and challenged with a lethal dose RHDV on day 42 (*red double arrow*). Animals were bled at the indicated days and VP60-specific antibodies were quantified using an in-house indirect ELISA [18]. After challenge, all vaccinated rabbits survived (*green emoticon*), whereas the non-vaccinated animals died within 2–3 days

13. The given water bath temperature is valid if the room temperature is about 20 °C and must be reduced at significantly higher room temperatures to avoid heat damage of the SF9 culture.
14. To see the tip of the marker pen it is necessary to turn on visible light slightly.
15. We use 6-well plates if Western Blotting or antigen-ELISA is envisaged and 24-well plates for immunofluorescence.
16. It is essential to use PBS with calcium and magnesium. Otherwise most cell types will detach during the following incubation.
17. For some cells butyrate is toxic after prolonged incubation. Butyrate containing medium should then be replaced by normal cell culture medium after first signs of cytopathicity.
18. Concentrated baculovirus preparations tend to form aggregates which can be dissipated by sonication. Longer treatment times, however, reduce biologic activity.
19. If an adjuvant is foreseen to be used, its effect on transduction efficacy needs to be clarified. We observed total inactivation of BacMams by products recommended for enveloped virus-containing live vaccines.

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Chapter 8

Laboratory-Scale Production of Replication-Deficient Adenovirus Vectored Vaccines

Susan J. Morris, Alison V. Turner, Nicola Green, and George M. Warimwe

Abstract

Replication-deficient adenoviruses are potent vaccine development platforms used extensively for human and animal candidate vaccines, largely due to their very good safety and immunogenicity profile. In this chapter we describe a method that can be used in any laboratory for the scalable production of replication-deficient adenovirus vector vaccines to GLP for preclinical studies in animal models, including definitive experimental studies in large target animal species for veterinary applications. We use human adenovirus serotype 5 (HAdV5) as an example, but the method can be easily adapted for use with other adenovirus serotypes from different species of origin.

Key words Adenovirus serotype 5 viral vector, Transfection, HEK293 cells, Seed stocks

1 Introduction

Replication-deficient human adenovirus serotype 5 (HAdV5) vectors are common vehicles for the delivery of foreign genes into a diverse range of cells for gene therapy and vaccines. For instance, HAdV5-based vaccines are leading candidates for prophylactic interventions against major livestock diseases such as foot and mouth disease and Peste des petits ruminants, and a licensed HAdV5-based product, ONRAB®, is currently in use for rabies in wildlife. The large amount of information on HAdV5 biology, immunology, and safety together with the low level of preexisting antibodies to HAdV5 in animals makes this virus an ideal candidate for veterinary disease vaccines.

Here, we describe a method that can be used in any laboratory for the scalable production of replication-deficient HAdV5 vector vaccines for preclinical studies in small animals to definitive experimental studies in large target animal species such as cattle. The method uses human embryonic kidney 293 (HEK293) cells, which have been transformed with sheared HAdV5 DNA [1]. HEK293 cells express the E1 genes from adenovirus and complement the

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growth of E1-deleted, otherwise replication-deficient, adenovirus vectors [2]. Molecular cloning techniques such as recombineering allow easy manipulation of adenovirus genomes such that any foreign gene can be easily inserted into the E1 locus and DNA bulked up in bacteria. Because linear adenovirus genomic DNA is infectious [3], insertion of the adenovirus genomic DNA into HEK293 cells by transfection with cationic liposome complexes generates virus particles that can be harvested from cell lysate, propagated, and purified.

2 Materials

Prepare all materials using ultrapure molecular biology grade water, which is prepared by purifying deionized water to gain a sensitivity of 18 MΩ cm at 25 °C. All materials should be of tissue culture grade; aliquot upon receipt and store at −20 °C.

2.1 HEK293A Cell Growth Media Components

1. Dulbecco's minimum essential media (DMEM) with high glucose and pyruvate.
2. 200 mM L-glutamine.
3. 100× penicillin/streptomycin (P/S) solution (10,000 U penicillin-G and 10 mg streptomycin/ml).
4. Fetal bovine serum (FBS).
5. Preparation of D10 with and without penicillin/streptomycin (D10 ± PS): Thaw one aliquot of FCS, L-glutamine and P/S (if required) in 37 °C water bath. To a 500 ml bottle of DMEM add 50 ml FCS to give a final concentration of 10 %, 10 ml L-glutamine to give a final concentration of 4 mM and if required 5 ml P/S to give a final concentration of 100 U penicillin, ~0.1 mg strep/ml. The growth media can be stored at 4 °C for up to 1 month.

2.2 HEK 293S Cell Growth Media Components

1. CD293 media (Life Technologies).
2. 200 mM L-glutamine.
3. Preparation of CD293 media: Thaw one aliquot L-glutamine in 37 °C water bath. To a 500 ml bottle of CD293 media add 20 ml L-glutamine to give a final concentration of 8 mM. The growth media can be stored at 4 °C for up to 1 month.

2.3 Other Culture Reagents

1. Phosphate buffered saline (PBS), without Ca^{2+} and Mg^{2+}.
2. TrypLE™ Express (Life Technologies).

2.4 Transfection Reagents

1. Opti-MEM with glutamine and HEPES, without phenol red (Life Technologies).
2. Lipofectamine 2000 (Life Technologies).

2.5 Cell Lysis and Benzonase Treatment

1. Lysis buffer (10 mM Tris–HC, 1 mM $MgCl_2$, pH 7.8): Prepare 1 M solution of Tris–HCl, pH 7.8 (or use Sigma cat. No. T-2569-100 ml premade solution) and 1 M magnesium chloride solution. To a 500 ml measuring cylinder add 5 ml 1 M Tris–HCl pH 7.8 stock solution and 0.5 ml 1 M magnesium chloride solution. Make up to 500 ml with 18 MΩ water and then filter-sterilize using steri-filter cups. Aliquot in a class II cabinet and store at room temperature.
2. 25 U/μl Benzonase, purity >99 % (Merck).

2.6 Preparation of Caesium Chloride (CsCl) Purification Reagents

1. Preparation of 1.25 g/ml density CsCl in 10 mM Tris pH 7.8: Add 166.89 g CsCl to a volumetric flask. Add 5 ml 1 M Tris–HCl pH 7.8 solution and make up the volume to 500 ml with 18 MΩ water. Filter-sterilize.
2. Preparation of 1.35 g/ml density CsCl in 10 mM Tris pH 7.8: Add 233.65 g CsCl to a volumetric flask. Add 5 ml 1 M Tris–HCl pH 7.8 solution and make up the volume to 500 ml with 18 MΩ water. Filter-sterilize.
3. Preparation of Storage buffer (10 mM Tris, 7.5 % w/v sucrose. pH 7.8): Weigh 75 g sucrose and transfer to a 1 l measuring cylinder. Add 18 MΩ water up to ~800 ml. Then add 10 ml 1 M Tris–HCl pH 7.8. Seal top of measuring cylinder with Parafilm and invert cylinder repeatedly until sucrose has full dissolved. Finally, adjust volume to 1000 ml. Filter-sterilize and store at 4 °C.

3 Methods

For the generation of adenovirus vectors several replication-deficient HAdV5 vectors are available as a starting material. Life Technologies provide a destination plasmid, pAd/CMV/V5-DEST™), encoding a replication deficient (E1/E3-deleted) HAdV5 genome that can be engineered using compatible entry clones and a standard Gateway® cloning reaction to insert any antigen of choice at the E1 locus. Alternatively, bacterial artificial chromosomes (BACs) containing various modified HAdV5 genomes are provided by Adz (University of Cardiff) and can be easily engineered using standard recombineering techniques (protocols provided by Adz) to insert any antigen of choice at the E1 locus. These BAC-derived vectors contain a self-excision cassette, thus Subheading 3.1 below is not required to generate a linear genome for transfection of complementing cells.

Replication-deficient HAdV5 virus production is dependent upon the *trans* expression of the viral activating factors encoded by the E1 genes. Human embryonic kidney (HEK) 293 cells and PerC.6 cells are common E1 complementing cell lines used in the

production of replication-deficient adenoviruses. In the following protocols we use either adherent (HEK293A) or suspension (HEK293S) HEK293 cells for production of HAdV5 vectors (*see* **Notes 1** and **2** for working with this cell line). It should be noted that PerC.6 cells express E1 genes from a cassette containing less flanking homology to the HAdV5 genome thus reducing the risk of generating replication competent viruses during production.

3.1 Excision of HAdV5 Genome from Plasmid or BAC DNA

1. Adenoviruses require a linear DNA genome for the initiation of replication and genome packaging. HAdV5 genome is excised from plasmid or BAC by restriction enzyme digest. Mix the following components: 6 μg pAd/CMV/V5-DEST™ plasmid/BAC (*see* **Note 3**), 10 μl 10× restriction enzyme buffer, 20 U PacI restriction enzyme. Make up to 100 μl total in sterile molecular biology grade water (the quantities described here are sufficient for transfection of 1× T25 flask).
2. Incubate the reaction at 37 °C for 2–3 h. Then, heat the reaction to 65 °C for 25 min to inactivate the restriction enzyme. Remove 10 μl of the reaction to a separate 1.5 ml microcentrifuge tube and resolve through 1 % agarose to confirm excision of HAdV5 genome from the plasmid backbone. However, some BAC-derived vectors contain a self-excision cassette and thus restriction enzyme excision of the genome is not required.

3.2 Transfection of HEK293A Cells

This protocol describes the transfection and subsequent passaging of transfected cells to obtain replication-deficient HAdV5 vectors. The cells are passaged into larger tissue culture flasks once or twice as they reach 80–90 % confluency as this maintains the *trans* expression of the E1 genes by the cells, which is essential for virus rescue (*see* **Note 2**).

1. 24 h prior to transfection seed 2.3×10^6 HEK293A cells into T25 cm^2 flask in a total volume of 5 ml D10 + PS media.
2. On the day of transfection pre-warm an aliquot (1 ml per transfection) of Opti-MEM® for the preparation of DNA–Lipofectamine complexes to room temperature and pre-warm an aliquot (10 ml per T25 cm^2 flask) of Opti-MEM® for cell washing/media replacement to 37 °C. In addition, pre-warm D10 – PS media (~5 ml per T25 cm^2 flask) to 37 °C.
3. Prepare:
   - A sterile 2 ml screw-capped tube containing 85 μl linearized genome (*see* Subheading 3.2, **step 1**) + 215 μl Opti-MEM®.
   - A sterile 2 ml screw-capped tube containing 30 μl Lipofectamine® 2000 + 270 μl Opti-MEM®.
4. Mix gently by flicking the tubes and incubate at room temperature for 5 min.

5. Mix the contents of the two tubes, adding the DNA to the Lipofectamine, not vice versa. Flick tubes gently to mix, and incubate at room temperature for at least 20 min.
6. During the incubation remove the media from the flasks of cells and wash twice with Opti-MEM®. Add 1.4 ml of Opti-MEM® to each flask.
7. After 20 min add the 600 µl of DNA/lipid complexes to the flasks and mix by tilting the flask gently to ensure that the transfection mix covers all the cells.
8. Place the cells at 37 °C, 5 % CO_2.
9. After 4–6 h replace the transfection media with 5 ml D10 – PS and incubate at 37 °C, 5 % CO_2.

3.3 Passaging Cells to Maintain Cell Viability and Promote Virus Rescue

1. 48 h post-transfection inspect the transfected cells using a light microscope. They should be confluent and beginning to appear crowded. Some signs of cytopathic effect (cpe) may be evident (Fig. 1).
2. Pre-warm TrypLE™ Express, PBS and D10 + PS to 37 °C. The cells are fragile at this stage and must be treated very gently.
3. Transfer all the media from the T25 cm^2 flask to a fresh T75 cm^2 flask and wash the cells carefully with 5 ml PBS (aim fluids along the top of the flask to prevent dislodging the cells).
4. Add 2 ml TrypLE™ Express and place the flask at 37 °C until cells detach from flask surface (approximately 2 min).
5. Add 10 ml D10 + PS to the flask, swirl to ensure all cells are suspended in the media and transfer to the T75 cm^2 flask.
6. Incubate the T75 cm^2 flask at 37 °C, 5 % CO_2. The cells will take approximately 48 h to reach confluency. Monitor for the appearance of cpe, this should begin 48–72 h after cell passage (*see* **Notes 4** and **5**). Even if there is no obvious cpe, it is worth

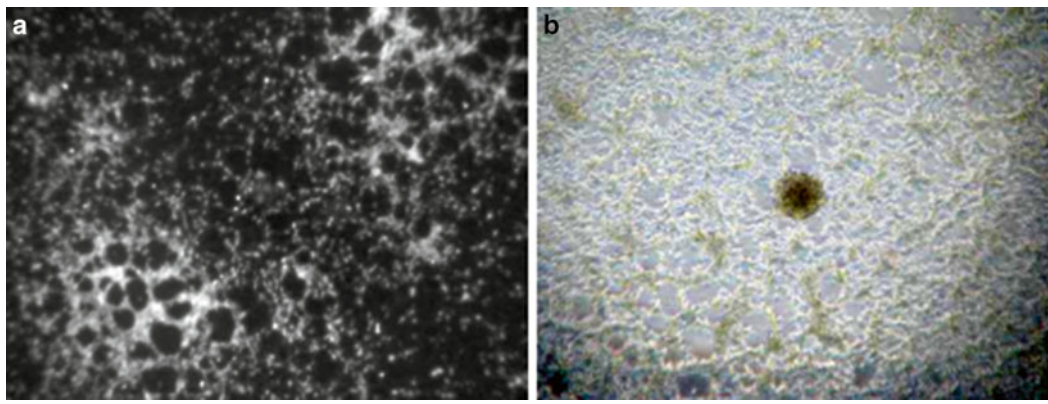

Fig. 1 Cytopathic effect of adenovirus growth in HEK293A cells. (**a**) cpe produced by a GFP-expressing adenovirus vector observed under fluorescent light. (**b**) Adenovirus vector cpe observed under light microscopy

storing some material to titrate on a 6-well plate in addition to splitting the cells. There is usually some virus present and the titration should allow this to grow out.

3.4 Harvesting of Adenovirus-Infected Cells

1. Once full cpe is observed adenovirus can be harvested. Cells showing cpe should detach from the flask with gentle tapping. Transfer cells and media to a sterile, 50 ml tube.
2. Centrifuge (1500 × *g*, 5 min) to pellet the cells and then aspirate the supernatant to waste.
3. Resuspend the cell pellet in 2 ml lysis buffer (*see* **Note 6**).
4. Freeze-thaw the suspension three times, then pellet the cell debris at 1500 × *g* for 5 min at 4 °C and store at −20 °C. This is called the transfection stock.

3.5 Preparation of a Seed Stock

To prepare a seed virus stock that can be used for bulking up, a serial dilution of the transfection stock is used to infect cells in a 6-well plate. In this way it is possible to harvest a well that is showing complete and uniform cpe at 48–72 h. It is important not to harvest cells before this as the virus will still be in early stages of replication and the yield of infectious units will be low (*see* **Note 5**).

1. 24 h prior to infection seed a 6-well plate with HEK293A cells at a density of 7.5×10^5 cells/well.
2. On the day of infection, replace media with 2 ml fresh D10 + PS media.
3. Add 200 μl transfection stock cell lysate to the first well and rock plate backward, forwards and side to side to evenly distribute the virus.
4. Take 200 μl of media from the first well, add to the second well and repeat the mixing step.
5. Repeat **step 4** for the remaining four wells.
6. Place plate at 37 °C, 5 % CO_2 for 48–72 h.
7. Harvest the cells and media from the well that shows uniform cpe using a cell lifter and 2 ml serological pipette. Transfer the material into a 2 ml tube.
8. Freeze-thaw the sample two times.
9. Pellet the cells using the microfuge (16,627 × *g* for 1 min at room temperature).
10. Transfer supernatant to a fresh 2 ml screw-capped tube.
11. Take 50 μl of the material and transfer into a new 2 ml tube to provide material for test infection.
12. Freeze all samples at −20 °C until required for bulk preparations.

For large-scale preps a larger volume of inoculum may be required. The seed stock can be generated in a similar manner to

that described above in T25 cm^2 flasks using 500 μl transfection stock in a total volume of 5 ml media and making two fold dilutions prior to infecting the cells.

3.6 Titration of Seed Stock to Determine the Inoculum for Bulk Preparations

1. 24 h prior to infection seed a 6-well plate with HEK293A cells at a density of 7.5×10^5 cells/well.
2. Number six 2 ml screw-capped tubes 1–6 and label a 15 ml tube "premaster".
3. Using a serological pipette place 15 ml of D10 + PS media into a 15 ml tube and label as "media".
4. Add 30 μl of premaster seed stock lysate to the "premaster" tube. Add 3970 μl of media from the "media" tube.
5. Mix by gently inverting ten times and avoid generating bubbles.
6. Remove 2 ml from the "premaster" tube and add to tube number 1.
7. Add 2 ml of media to the premaster tube.
8. Mix by gently inverting ten times and avoid generating bubbles. Remove 2 ml from the "premaster" tube and add to tube number 2.
9. Repeat for tubes 3–6.
10. Aspirate the media from the wells of a 6-well plate using an aspirator with a filter-less tip. Keep the plate flat whilst doing so and do not touch the bottom of the well with the tip to avoid damaging the cell layer.
11. Using a 2 ml serological pipette remove the contents of tube number 6 and place in well number 6 of the 6-well plate. Continue with the rest of the tubes, using the same pipette.
12. Place cells at 37 °C, 5 % CO_2.
13. Monitor for appearance of cpe over 48–72 h and use this information to decide on an inoculum volume for bulk virus preparation as outlined in Table 1.

3.7 Infection of Adherent HEK293A Cells or Suspension HEK293S Cells for Bulk Virus Preparations

There are many different size culture vessels that can be used for the preparation of a range of adenovirus vector stocks. Some of these, together with the culture/harvest conditions required are shown in Table 2. Here, we outline the procedure using a hyperflask and 3 l shaker flask.

3.7.1 Infection of HEK293A Cells in a Ten-Layer Hyperflask for Bulk Virus Preparations

This protocol generates a bulk virus preparation with a final yield after purification in the range of 5×10^{10} to 5×10^{11} infectious units.

1. Prepare a hyperflask (Hyperflask M; Corning) of HEK293A cells such that the cells are 70–80 % confluent at the time of infection. We recommend seeding 2×10^8 cells per flask for use 72 h post-seeding.

Table 1
Volume of inoculum required for different size preps based on the titration of the seed stock

Well number	Amount of virus in well (µl)	Amount of virus to add to a T75 flask (µl)[a]	Amount of virus to add to a T150 flask (µl)[a]	Amount of virus to add to a hyperflask (µl)[a]	Amount of virus to add to a 3 l shaker flask (ml)[a]
1	15	120	240	2400	12
2	7.5	60	120	1200	6
3	3.75	30	60	600	3
4	1.88	15	30	300	1.5
5	0.94	7.5	15	150	0.75
6	0.47	4	8	75	0.375

[a]Volumes are based on equivalent cell numbers per flask

Table 2
Cell numbers, media volumes, and adenovirus harvest details for different size preparations

Flask type	Cell type[a]	Total number of cells ($\times 10^8$)	Volume of media (ml)	Volume of cell lysis buffer (ml)	Tube for freeze–thaw (ml)	Time for freeze–thaw (min)	Total estimated yield (infectious units)[b]
T75 flask	A	0.1	20	0.4	2	10	1×10^8
T175 flask	A	0.2	40	0.8	5	10	2×10^8
Hyperflask M	A	6	500	15	50	20	5×10^{10} to 5×10^{11}
850 ml roller bottle	S	1.5	300	6	50	20	–
500 ml shaker flask	S	1	200	4	15	20	–
1 l shaker flask	S	2	400	8	50	30	1.5×10^{12} to 3×10^{12}
3 l shaker flask	S	8	1600	80	50	30	5×10^{12} to 1×10^{13}

[a]A—HEK293 adherent cells, S—HEK293 suspension cells
[b]This is an estimate of yields and will depend on various factors from cell status, nature of the antigen, and time of harvest

2. On the day of infection pre-warm a 500 ml bottle of D10 + PS media to 37 °C.
3. Add the required amount of premaster seed stock lysate (as calculated from the test infection) to a 50 ml tube.

4. Using a serological pipette, add 30 ml pre-warmed D10 + PS to the tube containing the required amount of premaster and mix by gently inverting ten times. Avoid generating bubbles.
5. Add the contents of the tube to the bottle of media using a serological pipette and mix by gently inverting ten times.
6. Carefully remove the media from the hyperflask and discard (*see* **Note 7**).
7. Refill the flasks with media containing the premaster cell lysate. This should be done by tilting the flask to about 60° and then angling the bottle of media so that the media is poured into the air dam within the neck. This minimizes the formation of bubbles as well as the washing off of cells. Flasks should be filled to within 5 mm of the top of the neck to prevent bubble formation.
8. Incubate the flasks at 37 °C, 5 % CO_2 and monitor for appearance of cpe over 24–72 h. Harvest the infected cells when they show optimal cpe (not prior to 48 h, *see* **Note 8**).
9. Dislodge the cells by tapping the side of the hyperflask and then carefully pour the media and cells into two 250 ml polypropylene centrifuge bottles (*see* **Note 7**).
10. Pellet cells by centrifugation at $1500 \times g$ for 5 min at 4 °C and discard the supernatant.
11. Refill the hyperflask with 250 ml PBS (with Ca^{2+} and Mg^{2+}) and tap the side of the flask to dislodge any remaining cells.
12. Pour the PBS into the centrifuge bottles containing the cell pellet and pellet the cells by centrifugation as in **step 10**.
13. Discard the supernatant and resuspend the cell pellet in 15 ml lysis buffer. Transfer the cell lysate to a 50 ml centrifuge tube and proceed to Subheading 3.4, **step 1** (*see* **Note 6**).

3.7.2 Infection of HEK293S Cells in a 3 l Shaker Flask for Large Bulk Virus Preparations

This protocol generates a bulk virus preparation with a final yield after purification in the range of 5×10^{12} to 1×10^{13} infectious units. We have found that five 3 l shaker flasks per batch of vaccine is a manageable amount at the lab scale. Before proceeding with a batch prep we advise that the time of harvest is determined. This can be done by inoculating a 3 l shaker flask as described below and taking samples over a 72-h time period. The virus yield at each time point should be determined by one of the titration methods outlined in Subheading 3.5.

1. Seed 8×10^8 HEK293S cells into a 3 l shaker flask in a final volume of 760 ml CD293 media. Ensure cells are growing in the log phase ($1–2 \times 10^6$ cells/ml) prior to seeding to ensure maximum virus vector growth.
2. On the day of infection pre-warm 1 l CD293 media to 37 °C in a 250 ml bottle.

3. Add the required amount of premaster seed stock lysate (as calculated from the test infection) to a 50 ml tube.
4. Using a serological pipette, make up the total volume of inoculum to 40 ml using pre-warmed CD293 media and mix by gently inverting ten times.
5. Swirl the cell cultures to ensure an even distribution of cells and then add the inoculums to the shaker flask and swirl gently.
6. Incubate the flasks at 37 °C, 8 % CO_2 with 150 rpm rotation for 3 h then add a further 800 ml pre-warmed CD293 media.
7. Incubate the flasks at 37 °C, 8 % CO_2 with 150 rpm rotation and monitor for appearance of cpe over 24–72 h. Harvest the infected cells at the predetermined time of harvest.
8. Pellet cells by centrifugation at 1500 × *g* for 5 min at 4 °C and discard the supernatant.
9. Resuspend the cell pellet in 80 ml lysis buffer and transfer the cell lysate to a 250 ml centrifuge tube before proceeding to Subheading 3.4, **step 1** (*see* **Note 6**).

3.8 Purification of Recombinant Adenovirus

Veterinary vaccines that are to be used for research purposes may not require any further purification. Many studies have used crude lysates for immunological assays. The traditional method for the purification of HAdV5, which we routinely use and describe here, is a discontinuous caesium chloride gradient followed by an isopycnic caesium chloride gradient. Other methods include the Adenopure® kit (Puresyn Inc.) for small-scale preparations or ViraBind™ kits (Cell Biolabs Inc.) and anion exchange chromatography [4, 5] for larger scale preps. These methods have all been extensively described and are compatible with the bulk preparation protocols outline in this chapter.

3.8.1 Cell Lysis and Benzonase Treatment

Most of the adenovirus vector will be cell associated. Therefore cell lysis is required to release the virus from the cells and cell debris. This is achieved by repeatedly freeze-thawing the cell-pellet in lysis buffer at −80 °C or in a dry ice bath three times (*see* Table 2 for conditions depending on initial cell volumes).

For viral vectors that require purification an additional step is added after the first freeze–thaw. Benzonase is an endonuclease that works to reduce all contaminating, free nucleic acids to oligonucleotides of between three and five bases in length. The addition of a benzonase step reduces sample viscosity due to the release of DNA during cell lysis and reduces aggregation of adenovirus due to DNA binding.

1. Freeze the cell lysate obtained in Subheading 3.3, **step 2** in an IMS/dry ice bath or −80 °C freezer.
2. Thaw the cell lysate in a 37 °C water bath.

3. Add 250 U of Benzonase per 1 ml of cell lysate and mix thoroughly by gently inverting tube ten times. Place the tube in a rotary mixer and incubate for 1 h at room temperature (*see* **Note 9**).
4. Repeat the freeze–thaw (IMS/dry ice bath and 37 °C water bath) a further two times. Whilst thawing leave at 37 °C for the shortest time possible.
5. Pellet the cell debris by centrifuging at 1500×*g* for 5 min at 4 °C.

3.8.2 Caesium Chloride Gradient Purification

1. Mix aliquots of CsCl thoroughly before use.
2. Using a 5 ml serological pipette, set up relevant number (three tubes per hyperflask) of CsCl gradients in 14 ml Beckman Ultraclear tubes (Beckman Coulter cat. No. 344060).
3. Add 3.5 ml 1.25 g/ml CsCl solution to the tubes using a 5 ml pipette.
4. Take up 4 ml of 1.35 g/ml CsCl solution into a 5 ml pipette and use this to underlay the 1.25 g/ml CsCl solution with 3.5 ml 1.35 g/ml CsCl solution. Pipette very slowly and do not add the last 0.5 ml; this way, no air bubbles are added to the tube, which may disrupt the interface. Ensure that the interface between the two solutions is visible. This is absolutely crucial to this centrifugation method as the interface remains intact during centrifugation and is the limit to which the adenovirus migrates.
5. Add the cell lysate supernatant from Subheading 3.3, **step 3** evenly to each tube containing CsCl gradients. Take care not to disturb the interface whilst adding the sample. This can be avoided by adding the sample slowly down the side of the tube. Top up the tubes if required with lysis buffer. It is important to fill the tubes to within 3 mm of the top to prevent tube failure. Place centrifuge tubes in the buckets for the SW40 Ti rotor (Beckman Coulter) and balance them using a set of scales.
6. Centrifuge for 2 h at 110,000×*g*, 4 °C in Beckman Coulter ultracentrifuge.
7. Remove the tubes using sterile forceps and place the tube in a clamp stand above a beaker of suitable disinfectant for waste disposal (*see* **Note 10**). Two virus bands should be visible close to the center of the tube; the upper band is incomplete virus and the lower band intact virus (Fig. 2).
8. Set up the second caesium gradients in 14 ml Beckman Ultraclear tubes. Using 10 ml serological pipettes, add 6 ml of 1.35 g/ml CsCl to each tube.
9. Using a 19G needle and 5 ml syringe pierce the tube from the first centrifugation step approximately 5 mm below the band by gently twisting and pushing the needle through the wall of

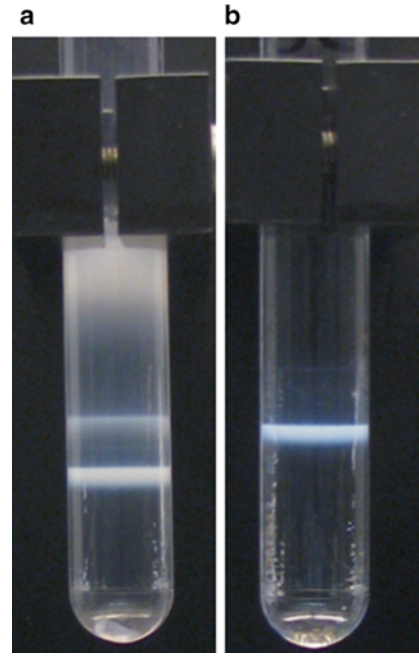

Fig. 2 Banding pattern seen after the first and second steps of caesium chloride gradient purification. (**a**) Viral banding pattern typically formed following the 2-h centrifugation step. *Lower band* is correctly formed adenovirus whereas the *upper band* contains viral capsids lacking DNA. (**b**) Viral banding pattern typically formed following the 16-h centrifugation step. The band observed contains mature adenovirus

the tube. Be careful not to hold your other hand on the other side of the tube whilst doing this for risk of injury.

10. Once the needle is inside the tube gently pull on the syringe with the bevel pointing upwards to extract the lower band. Discard the used centrifuge tube into the beaker of disinfectant. Repeat for all tubes.
11. Divide the virus supernatant between the tubes set up for the second centrifugation step (*see* **Note 11**). Top up the tubes if required with lysis buffer. It is important to fill the tubes to within 3 mm of the top to prevent tube failure. Balance the tubes with lysis buffer and place in the buckets for the SW40 Ti rotor and centrifuge at 160,000 × *g* for 16–18 h.
12. Remove the centrifuge tube using forceps and place in the clamp stand. Two bands may be visible. However, the upper band may not be present if the virus added onto the second gradient was very pure. Remove the lower band using a 19G needle and 5 ml syringe. This can be placed safely to one side to be directly loaded into the dialysis cassette (3–12 ml and 0.5–3 ml, Thermo Fisher cat. No. 66453 and 66455) or for loading onto a PD10 column for desalting (GE Healthcare).

13. For desalting by dialysis cassette, pre-wet a dialysis cassette with prechilled storage buffer. Add the purified virus band to a dialysis cassette using a needle and syringe. Place the cassette into a beaker containing 500 ml of cold storage buffer, cover with foil and place on a magnetic stir plate. Dialyze for 90 min changing the buffer every 30 min.
14. Remove virus from dialysis cassette and aliquot into suitable volumes. Store at −80 °C.

3.9 Titration and QC Analysis

The final step in HAdV5 virus vector preparation is QC analysis and titration of the bulk stock. Viral vector titration is performed in HEK293 cells and can be done by plaque forming assay [6] or immunostaining, for example using the Quicktiter™ adenovirus titre immunoassay kit (Cell Biolabs). These assays give the number of infectious viruses present in the preparation and have been extensively described previously. The number of particles is also determined, by spectrophotometric analysis [7, 8] and the particle to infectivity ratio calculated. This ratio should be below 100 for HAdV5 viral vector preparations.

Our routine QC assays include flank-to-flank PCR across the inserted antigen using primers that bind to the promoter and polyA signal, ID PCR of the inserted antigen using antigen specific primers and sterility assay by lack of growth in tryptic soya broth.

HAdV5 grown in HEK293 cells can produce replication competent adenovirus due to recombination with adenovirus genome sequences within the cell genome. This can be determined by quantitative PCR [9] or growth on normally non-permissive cell lines such as HeLa cells or A549 cells.

4 Notes

1. HEK293A cells adhere very loosely to the plasticware. Do not pipette directly onto the cells as this will cause them to be sheared from the flask surface.
2. HEK293A cells must only be allowed to reach 80 % confluency maximum (i.e., be maintained in the Log phase of growth) as the cells express proteins required for virus growth and once they start to reach the stationary phase the levels of these proteins is reduced and virus growth is compromised. HEK293A cells are immortalized lines of primary human embryonic kidney cells transformed by sheared HAdV5 DNA. The cells harbor the E1A and E1B region of the adenoviral genome, which complement, in trans, the deletion of the E1 region in the recombinant adenovirus. The cells constitutively express the adenoviral E1 proteins (E1A and E1B) while they are dividing. When they reach confluency, total protein expression, including E1, is reduced. During their natural lifecycle, adenoviruses express the

E1 proteins at all stages and the proteins are therefore extremely important for successful virus rescue. It is therefore important that cells do not reach confluency during routine passage and also that seeding densities in preparation for transfection are suitably low.

3. The adenovirus genome plasmid is large (~35 kb) and as such needs to be handled with care. Pipette very gently to prevent shearing the DNA.
4. Usually, rescued virus is apparent (as observed by a cytopathic effect (cpe)) on day 7 or 8 (i.e., approximately 5 days after subculture into a T75 flask). However, in some instances the virus has only rescued after a further passage of media and cells into a T150. The important factor is to keep the cell culture healthy and sub-culture when required.
5. An excess of the capsid proteins are produced by adenovirus. These bind to cell surface proteins and cause the cells to detach from the flask and also each other. This is evident by cells floating less than 24 h after infection, which is often mistaken for cpe. If you observe extensive cpe at 24 h discard this prep as virus yields will be low.
6. Cell lysis results in the release of proteases from the cells, which are able to degrade proteins. Importantly this includes the adenoviral capsid proteins that are required for infectivity. To minimize protease activity, once the cells are lysed ensure that the sample is kept chilled and keep incubations at 37 °C as short as possible.
7. Clean carefully around the necks of the hyperflask with 70 % ethanol to minimize risk of contamination while pouring the inoculums into the bottle or when harvesting.
8. Do not harvest hyperflasks earlier than 48 h. If extensive cpe has developed prior to this, either culture the cells until 48 h and recheck the flask or discard the hyperflask and repeat the infection using a smaller inoculum. We have tried harvesting at 24 h several times when cpe has looked well developed but the yield was too low when purified on caesium chloride gradients.
9. The optimum incubation temperature for benzonase is 37 °C. However, as a compromise between virus stability and enzyme activity, the incubation is performed at RT.
10. A black piece of card placed behind the clamp stand helps with the viewing of the bands especially if the band is faint.
11. Do not overload the second caesium chloride gradient with virus from **the first gradient** as this can lead to aggregation of the viruses.

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Chapter 9

Generation of Recombinant Modified Vaccinia Virus Ankara Encoding VP2, NS1, and VP7 Proteins of Bluetongue Virus

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Abstract

Modified Vaccinia Virus Ankara (MVA) is employed widely as an experimental vaccine vector for its lack of replication in mammalian cells and high expression level of foreign/heterologous genes. Recombinant MVAs (rMVAs) are used as platforms for protein production as well as vectors to generate vaccines against a high number of infectious diseases and other pathologies. The portrait of the virus combines desirable elements such as high-level biological safety, the ability to activate appropriate innate immune mediators upon vaccination, and the capacity to deliver substantial amounts of heterologous antigens. Recombinant MVAs encoding proteins of bluetongue virus (BTV), an Orbivirus that infects domestic and wild ruminants transmitted by biting midges of the *Culicoides* species, are excellent vaccine candidates against this virus. In this chapter we describe the methods for the generation of rMVAs encoding VP2, NS1, and VP7 proteins of bluetongue virus as a model example for orbiviruses. The protocols included cover the cloning of VP2, NS1, and VP7 BTV-4 genes in a transfer plasmid, the construction of recombinant MVAs, the titration of virus working stocks and the protein expression analysis by immunofluorescence and radiolabeling of rMVA infected cells as well as virus purification.

Key words Recombinant modified vaccinia virus Ankara, Bluetongue virus, Viral-vectored vaccine, VP2, NS1 and VP7 proteins

1 Introduction

Vaccinia viruses engineered to express foreign genes are powerful vectors for production of recombinant proteins [1]. Modified vaccinia virus Ankara (MVA) was obtained from the Chorioallantois vaccinia virus Ankara (CVA) and was isolated following more than 500 passages in chick embryo fibroblasts. After this extensive propagation the viral genome suffered several major deletions and numerous small mutations resulting in replication defects in human and most other mammalian cells, as well as severe attenuation of pathogenicity [2–4]. This is why these viral vectors have shown

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excellent safety profiles (the vector can be used at biosafety level 1), significant immunogenicity against foreign expressed antigens and ability to induce protective immune responses [5]. Poxviruses can accommodate large fragments of foreign DNA and their replication occurs within the cytoplasm of infected cells, eliminating the risk of virus persistence and genomic integration in host DNA [3]. MVA has intrinsic adjuvant capacities ant it is being widely investigated as a safe smallpox vaccine and as an expression vector to produce vaccines against other infectious diseases and cancer [4]. Recombinant MVA (rMVA) expressing immunogenic viral proteins has been shown to induce both humoral and cell mediated immunity [1, 6].

Poxviruses have the ability to induce the expression of type-I and II interferons and to express soluble receptors capable of interacting with host antiviral mechanisms. This antagonist expression is minimized owe to the deletions in the rMVA genome, which contributes to the immunogenicity of this viral-vector used as a vaccine. Type-I interferons may act as a link between the innate and adaptive immune system, including humoral and cellular responses [7, 8]. MVA has been used to construct many vectored vaccines expressing different proteins from different kind of orbiviruses [3]. The transfer plasmid pSC11 [9] was designed to place the genes of interest (in our case from *bluetongue virus*) under the control of the vaccinia virus (VV) early/late promoter p7.5. Finally, rMVAs were generated after homologous recombination in permissive cells between the TK gene sequences of pSC11 and those of wild type MVA. In our laboratory, all of these recombinant vectors have been tested as potential vaccines in IFNAR$^{(-/-)}$ mice [10–15]. We engineered rMVAs expressing VP2, NS1, and VP7 proteins from BTV-4. IFNAR$^{(-/-)}$ mice were inoculated with DNA-VP2,-NS1,-VP7/rMVA-VP2,-NS1,-VP7 in an heterologous prime boost vaccination strategy, generating significant levels of antibodies specific of VP2, NS1, and VP7, including those with neutralizing activity against BTV-4. The vaccine combination expressing VP2, NS1, and VP7 proteins of BTV-4, elicited sterile protection against a lethal dose of homologous BTV-4 infection and induced cross-protection against lethal doses of heterologous BTV-8 and BTV-1, suggesting that the DNA/rMVA-VP2,-NS1,-VP7 marker vaccine is a promising multiserotype vaccine candidate against BTV [14].

This work details the methodology applied to generate the rMVAs encoding the proteins VP2, VP7, and NS1 of BTV-4. In addition, the chapter describes the protocols to analyze the BTV protein expression in DF-1 cells infected with these rMVAs by immunofluorescence assay and radiolabeling, immunoprecipitation and SDS-PAGE.

2 Materials

1. BTV serotype 4 (SPA2004/01).
2. Modified vaccinia virus Ankara (MVA) (generously provided by Professor Francisco Rodríguez, Centro Nacional de Biotecnología, CSIC, Madrid, Spain).
3. Kidney epithelial cells extracted from an African green monkey, *Chlorocebus* sp. (Vero cells) (ATCC, Cat. No. CCL-81).
4. Chicken embryo fibroblast (DF-1 cells) (ATCC, Cat. No. CRL-12203).
5. Serum and antibiotic free Dulbecco's modified Eagle's medium.
6. DMEM with 2 mM glutamine, 10 % fetal calf serum (FCS), and 1 % Penicillin/Streptomycin (complete DMEM).
7. TRI Reagent Solution (Ambion).
8. 10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT (Life Technologies).
9. SuperScript® III Reverse Transcriptase (200 U/μL) (Life Technologies).
10. RNaseOUT™ (40 U/μL) (Life Technologies).
11. 10× PCR Buffer II, 10 mM dNTPs, specific primer (VS and RS) (Table 1) (Life Technologies).
12. AmpliTaq DNA Polymerase (1.25 U/50 μL) (Life Technologies).
13. 1 % agarose gel (Tris–acetate–EDTA buffer (TAE) and 1 % agarose).
14. Midori green DNA stain (Nippon Genetics Europe GmbH).

Table 1

Primer used for amplification of BTV genes

Gene		Sequence
VP2	VS	5′-CGCCCGGGATGAACTAGGCATCCCAG-3′
	RS	5′-CGCCCGGGCATACGTTGAGAAGTTTTGTTA-3′
NS1	VS	5′-CGCCCGGGATGGAGCGCTTTTTGAGAAAATAC-3′
	RS	5′-CGCCCGGGCTAATACTCCATCCACATCTG-3′
VP5	VS	5′-CGCCCGGGATGGGTAAAGTCATACGATC-3′
	RS	5′-CGCCCGGGTCAAGCATTTCGTAAGAAGAG-3′
VP7	VS	5′-CGCCCGGGATGGACACTATCGTCGCAAG-3′
	RS	5′-CGCCCGGGCTACACATAGCGCGCGCGTGC-3′

*Sma*I restriction site *underlined*

15. Qiaex II Gel Extraction Kit (Qiagen).
16. pSC11 plasmid (kindly provided by Professor Francisco Rodríguez, Centro Nacional de Biotecnología, CSIC, Madrid, Spain).
17. *Sma*I restriction endonuclease, shrimp alkaline phosphatase (SAP), and T4 ligase enzyme.
18. Luria-Bertani (LB) agar plates and media.
19. Ampicillin sodium salt.
20. QIAprep® Spin Miniprep Kit (Qiagen).
21. Lipofectamine® Reagent (Invitrogen).
22. Noble agar (Difco Noble Agar (DB) and distilled water).
23. X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside).
24. Complete DMEM-0.6 % Noble agar with X-Gal (0.4 μg/μL) (complete DMEM–agar–X-Gal).
25. Formaldehyde 10 %.
26. Crystal violet in 80 % methanol.
27. Acetone–methanol solution (40 %/60 %).
28. Phosphate buffered saline 1× (PBS 1×).
29. PBS 1× 20 % FCS (blocking solution).
30. Mouse polyclonal antibody against BTV-4.
31. Alexa Fluor® 594 goat anti-mouse IgG (H + L) (Invitrogen).
32. ProLong Gold antifade reagent (Life Technologies).
33. RIPA buffer: 50 mM Tris–HCl pH 7.4, 300 mM NaCl, 0.5 % sodium deoxycholate, 1 % Triton X-100, protease inhibitors.
34. Methionine-free DMEM cell culture medium.
35. [^{35}S] Methionine (800 Ci/mmol). (Amersham).
36. Dynabeads® Protein G system (Life Technologies).
37. SDS-PAGE buffer: 0.125 M Tris–HCl, 4 % SDS, 20 % v/v glycerol, 0.2 M DTT, 0.02 % bromophenol blue, pH 6.8.
38. 36 % sucrose cushion and sucrose gradient.
39. SW 28 centrifuge tube (50 mL).

3 Methods

These methods describe the generation of the recombinants MVAs encoding BTV-4 VP2, VP7 and NS1 proteins, the screening of positive recombinants, the upgrowth and quantification of virus stock, the analysis of BTV protein expression by immunofluorescence assay and radiolabeling, immunoprecipitation and SDS-PAGE in

infected DF-1 cells as well as the purification of the rMVAs generated in DF-1 cells.

3.1 Cloning of VP2, NS1, and VP7 BTV-4 Genes for Generation of Recombinant MVAs

Segments 2, 5, and 7 corresponding to VP2, NS1, and VP7 proteins will be amplified from total RNA of BTV-4 infected cells. To generate the MVA transfer plasmids pSC11-VP2, pSC11-NS1, and pSC11-VP7, the restriction site *Sma*I, must be introduced into the 5′ and 3′ ends of the PCR products, unique restriction site contained into pSC11.

1. Infect confluent Vero monolayers in M24-well plates (1.67×10^4 cells/well) with BTV serotype 4 (BTV-4) with a multiplicity of infection (MOI) of 1.
2. After virus adsorption for 1.5 h at 37 °C, 5 % CO_2, remove the medium, add 1 mL of complete DMEM and incubate for 24 h at 37 °C.
3. At 24 h post infection (h.p.i), when a clear cytopathic effect is observed, remove the supernatant and extract total RNA from infected cells with TRI Reagent Solution, according to the method recommended by the manufacturer (*see* **Note 1**).
4. The recovered RNA can be stored at −80 °C in small aliquots for later processing. RNA stored at this temperature is stable for prolonged periods of time (over 1 year).
5. Denature 5 μg of RNA in presence of 1 μL of 2 μM Reverse Sense (RS) BTV gene-specific primer (Table 1), 1 μL of 10 mM dNTP mix in a final volume of 10 μL by heating to 65 °C for 5 min and then rapidly cool on ice.
6. Add 2 μL 10× RT buffer, 4 μL 25 mM $MgCl_2$, 2 μL of 0.1 M DTT, 1 μL RNaseOUT™ (40 U/μL), and 1 μL SuperScript® III Reverse Transcriptase (200 U/μL).
7. Incubate the reaction for 1 h at 50 °C. Then, inactivate the reverse transcriptase by heating at 85 °C for 5 min. Chill on ice.
8. The cDNA produced is stable at 4 °C for short term storage, −20 °C for prolonged storage or used for Polymerase Chain Reaction (PCR) immediately.
9. Amplify the VP2, NS1, and VP7 cDNAs by PCR. Use 10 μL of 10× PCR Buffer II, 2 μL of 10 mM dNTPs, 2 μL of each specific primer (VS and RS) including *Sma*I site (Table 1), 4 μL of 25 mM $MgCl_2$ solution, 0.6 μL of AmpliTaq DNA Polymerase (1.25 U/50 μL), and 5 μL of cDNA template in a final volume of 100 μL.
10. Amplification cycle parameters are: 94 °C for 2 min (1×); 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min (30×); 94 °C for 15 min (1×).

11. Analyze the PCR products on a 1 % agarose gel stained with Midori green DNA stain (or other intercalating dye) and purify the PCR products with Qiaex II Gel Extraction Kit.
12. Digest the plasmid pSC11 and the purified PCR products VP2, NS1, and VP7 (containing the restriction site *Sma*I into the 5′ and 3′ ends) with the restriction enzyme *Sma*I as per manufacturer instructions.
13. Proceed to dephosphorylation of digested pSC11 with shrimp alkaline phosphatase (SAP) according to the method recommended by the manufacturer in order to prevent the plasmid self-ligation.
14. Purify the digested PCR products and the digested and dephosphorylated plasmid with Qiaex II Gel Extraction Kit.
15. Ligate the purified digested VP2, NS1, and VP7 with the purified digested plasmid pSC11 with T4 ligase enzyme according to the manufacturer's instructions. Perform the ligation at 16 °C overnight with a molar ratio of vector to insert of 1:3.
16. Transform the ligation products into chemically competent DH10B bacterial cells and plate out the transformants on LB agar with selection in the presence of ampicillin (100 μg/mL).
17. The next day select single colonies and grow in LB with ampicillin. Isolate the plasmid following the QIAprep Miniprep Handbook and analyze the presence and right orientation of the cloned VP2, NS1, and VP7 genes into the transfer plasmid pSC11 by sequencing (*see* **Note 2**).

3.2 Construction of Recombinant MVAs

The next step of the procedure is the generation of recombinant MVAs (*see* Fig. 1). The MVA transfer plasmids pSC11-VP2, pSC11-NS1, and pSC11-VP7 should contain the VP2, NS1, and VP7 BTV genes, flanked by thymidine kinase (TK) sequences of MVA, under the control of the vaccinia virus (VV) early/late promoter p7.5. Infection of cells with MVA and subsequent transfection with pSC-11 plasmids will generate recombinant viruses

3.2.1 Infection/Transfection of DF-1 Cells with MVA wt and pSC11 Plasmid Respectively

1. Plate DF-1 cells in p35 or six-well plates 1 day prior to infection in a 2 mL volume of complete DMEM.
2. DF-1 cells that are 60–80 % confluence are needed for infection and transfection.
3. Add 100 μL of MVA wild type (wt) in serum and antibiotic-free DMEM at 0.1 or 1 of MOI.
4. Incubate the cells at 37 °C and air–5 % CO_2 atmosphere for 1.5 h. After virus adsorption, DF-1 infected cells are transfected with pSC11-VP2, pSC11-NS1 or pSC11-VP7.
5. Mix 2 μg of plasmid in 50 μL of serum and antibiotic-free DMEM. Add to this mixture 9 μL of Lipofectamine® reagent

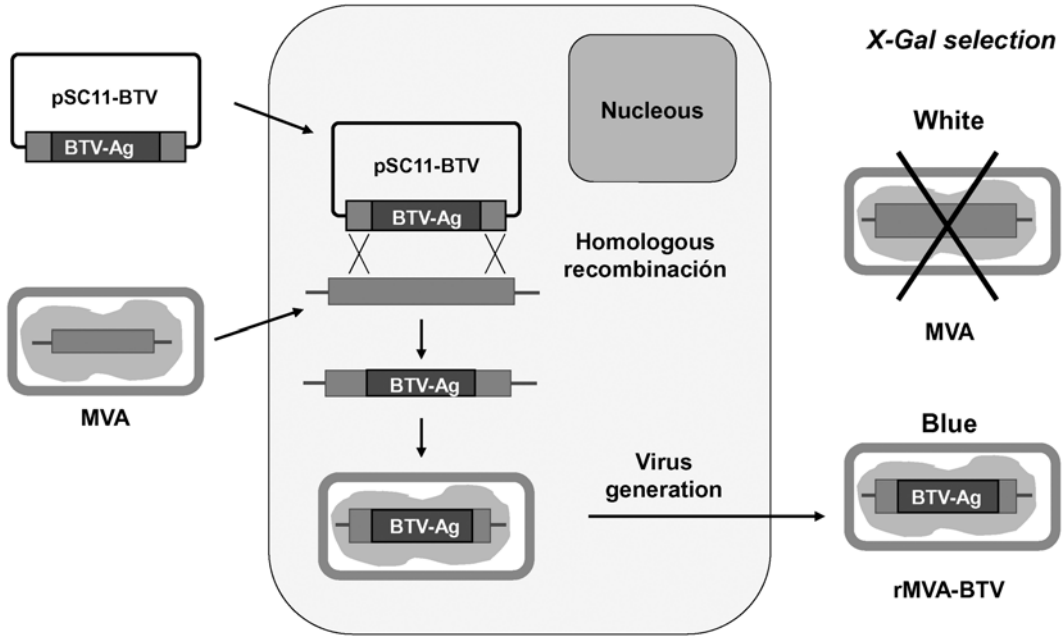

Fig. 1 General procedure for the generation of recombinant MVA. Genes VP2, NS1, and VP7 of BTV-4 were cloned into the vaccinia transfer plasmid pSC11 downstream of the p7.5 vaccinia promoter. DF-1 cells were infected with MVA virus (MOI 0.01 p.f.u./cell). After adsorption, cells were transfected with pSC11-VP2, pSC11-NS1, or pSC11-VP7 plasmids. Recombinant MVA viruses were generated by homologous recombination at the Thymidine kinase locus, allowing the analysis by using the LacZ marker

in 250 μL of serum and antibiotic-free DMEM and incubate at room temperature for 30 min.

6. Add 0.7 mL of serum and antibiotic-free DMEM to the lipid–plasmid complex. Add the total volume (≈1 mL) to the cells.
7. Incubate at 37 °C and air–5 % CO_2 atmosphere for 5 h, shaking the plate every 30 min.
8. Remove the lipid–plasmid complex and add 1 mL of complete DMEM.
9. Incubate at 37 °C and air–5 % CO_2 atmosphere for 72 h.
10. When the cytopathic effect (CPE) is apparent, harvest cells and supernatants by disruption of the monolayer (*see* **Note 3**). Carry out three cycles of thawing–freezing and sonicate twice for 10 s to disrupt the cells and release viruses.
11. Centrifuge at 2500 × *g* for 1 min. The supernatant will be used for the plaque analysis to look for recombinant MVAs as described below.

3.2.2 Plaque Purification of Recombinant MVA Viruses

In this step, we try to find clear, well-separated plaques for isolating and screening cloned viruses (*see* Fig. 2).

1. Plate DF-1 cells in six-well plates and incubate until they reach 80 % confluence.

2. Use the supernatants from the infected-transfected cells to do tenfold dilutions on the DF-1 cells from undiluted to 10^{-7} (*see* **Note 4**).
3. Allow the viruses to adsorb at 37 °C for 1 h.
4. Aspirate the supernatants and add 1.5 mL of complete DMEM.
5. Incubate at 37 °C and air–5 % CO_2 atmosphere for 72 h.

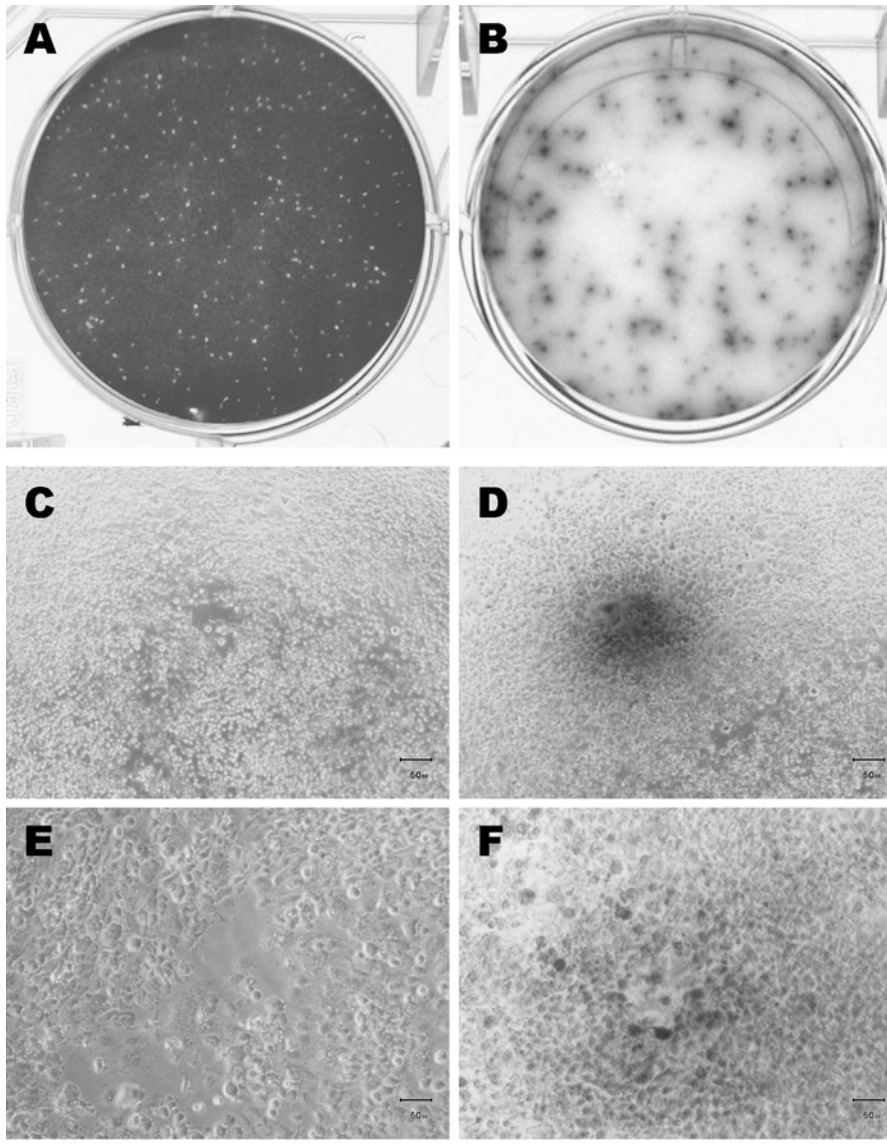

Fig. 2 Plaque formation of rMVA-VP2 and wild-type MVA in avian DF-1 cells. DF-1 cells were infected with 100 pfu/well of rMVA-VP2 (**b**, **d**, and **f**) or MVA-wt (**c** and **e**). 72 h post infection, titration was performed (**a**) or DMEM-0.6 % and Noble agar with X-Gal was added over the monolayer (**b**–**f**). *White plaques*: MVA-wt; *Dark plaques*: rMVA-VP2

6. Remove the medium and add complete DMEM–agar–X-Gal (*see* **Note 5**) over the monolayer.
7. Allow the overlay to solidify.
8. Incubate at 37 °C for 8 h.
9. Pick only well-separated blue plaques, about six per clone (*see* **Note 6**). To pick the plaques, insert the tip of the micropipette into the agar overlay just over the plaque, and draw the agar plug into the pipet. Transfer it into a small tube with 0.5 mL of complete DMEM and pipette up and down a few times to ensure that the plug does not remain in the pipette tip.
10. Carry out three cycles of thawing–freezing and sonication.
11. Repeat this cloning procedure (**steps 9** and **10**) about six times with each clone.
12. Amplify the cloned plaques using DF-1 cells.

3.3 Preparation and Titration of Virus Working Stocks

1. Passage DF-1 cells in 175 cm^2 flasks such they are confluent in 1 or 2 days.
2. Remove the old medium, add fresh medium and inoculate 0.1 MOI (*see* **Note 7**) of seed virus stock and Incubate DF-1 cells at 37 °C for 2–3 days until all cells show clear CPE; often, most cells will be floating.
3. Remove part of the medium (*see* **Note 8**), detach the cell monolayer and disrupt the cells with three cycles of thawing–freezing at –80 °C, transferring the medium and the disrupted cells to a new tube.
4. Sonicate in water bath the tube twice for 10 s, aliquot the rMVAs working stocks in volumes suitable for your purposes. We usually prepare aliquots of 1 mL each. Store at –80 °C.
5. For plaque assays of stocks prepare DF-1 cells in six-well plates 1 or 2 days prior to virus titration.
6. Thaw virus in 37 °C water bath, and make tenfold dilutions of the stock in complete DMEM. Each dilution must be mixed carefully and pipet tips changed between tubes (*see* **Note 4**). Transfer 100 μL of each dilution in each well.
7. Adsorb virus at 37 °C for 1.5 h, gently tilting back and forth every 15–20 min.
8. Aspirate the inoculum from higher to lower dilution wells, add 1.5 mL of complete DMEM from higher to lower dilution wells, and incubate for 3 days at 37 °C and air–5 % CO_2 atmosphere.
9. Fix the plates with 1 mL of 10 % formaldehyde for 30 min. Remove the medium and add 1 % crystal violet to stain the monolayers and count plaque numbers to calculate the virus titers (*see* **Note 9**).

3.4 Protein Expression Analysis

There are various methods to analyze the expression of the BTV proteins in DF-1 cells infected with the generated rMVAs. The two most common methods used in our laboratory are the immunofluorescence assay (*see* Fig. 3) and the immunoprecipitation of proteins in radiolabeled infected cells (*see* Fig. 4).

3.4.1 Immunofluorescence Assay

1. Plate DF-1 cells in 24-well plates with coverslips and incubate until they reach 80 % confluence.
2. Infect these cells with the rMVAs at an MOI of 1.
3. After 24 h of infection, fix the infected cells with acetone–methanol and store the plate at −20 °C for 20 min.
4. Remove acetone–methanol and wash once with 1 mL of PBS 1× (*see* **Note 10**).
5. Incubate the fixed cells with 1 mL blocking solution for 1 h.

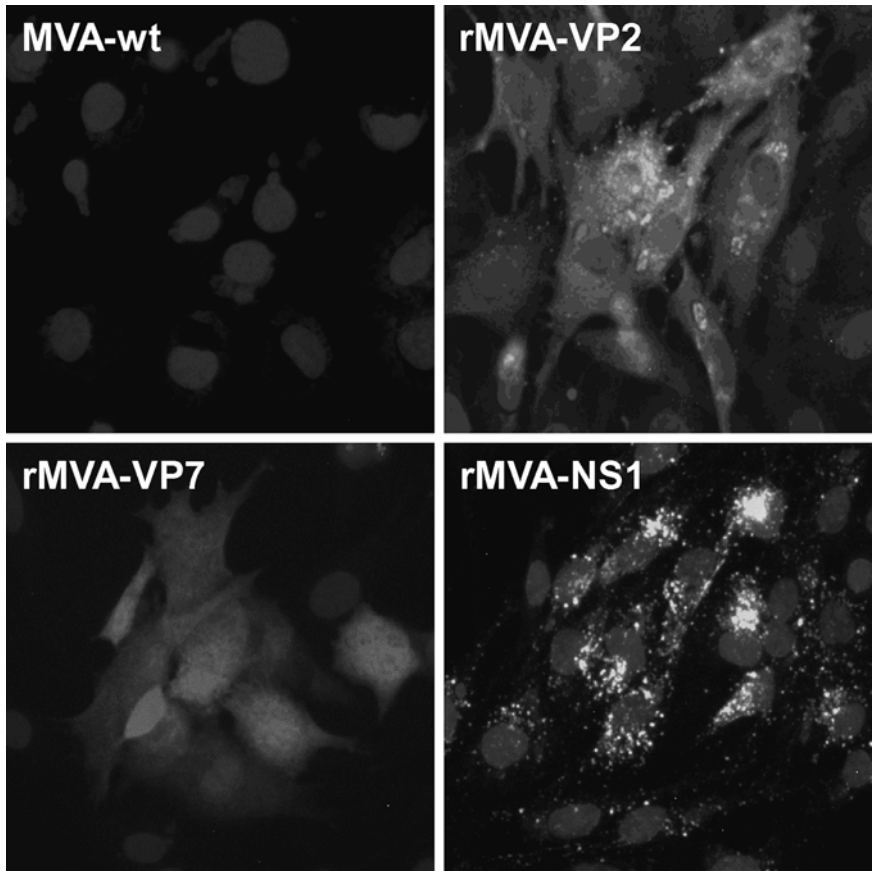

Fig. 3 Analysis of BTV-4 VP2, NS1, and VP7 expression by immunofluorescent staining. DF-1 cells were infected with recombinant MVA containing BTV-4 VP2, NS1, or VP7 genes. At 24 h.p.i, cells were fixed and analyzed by immunofluorescence by using a mouse polyclonal antisera specific of BTV-4

6. Remove the blocking solution and add the primary antibody. We use a mouse polyclonal antibody against BTV-4 diluted 1:500 or 1:1000 in blocking solution (it depends on each stock of sera) (*see* **Note 11**). We usually use 250 μL/well. Incubate at 4 °C o/n or room temperature for 3 h.
7. Remove the polyclonal antibody and wash with PBS 1× three times for 10 min preferably with shaking.
8. Add the conjugated secondary antibody (Alexa Fluor® 594 goat anti-mouse IgG (H + L)) specific of mouse primary antibody diluted 1:1000 in blocking solution over the cells. Incubate for 30 min at room temperature in dark conditions.
9. Remove the secondary antibody and wash with PBS 1× three times for 10 min with shaking preferably.
10. Mount the coverslips on slides using ProLong Gold antifade reagent and visualize using an immunofluorescence microscope.

3.4.2 Analysis of BTV Proteins Expression by Radiolabeling, Immunoprecipitation, and SDS-PAGE

Radiolabeling followed by immunoprecipitation is useful to analyze the expression of BTV proteins. VP2 protein contains conformational epitopes and polyclonal antibodies specific of BTV are not able to recognize the denatured protein by immunoblot. VP2, NS1, and VP7 can be immunoprecipitated with BTV-specific polyclonal antibodies from either BTV or MVA-VP2, MVA-NS1, and MVA-VP7 infected cells (*see* Fig. 4).

1. Infect DF-1 cells in 35-mm dishes with MVA-VP2, MVA-NS1, or MVA-VP7 at an MOI of 1.
2. After 90 min of virus adsorption, remove the culture medium and rise the cell monolayers with PBS 1× and once with methionine-deficient medium.
3. Add 1.5 mL of fresh methionine-deficient medium and incubate for 60 min (to starve of methionine).
4. At the end of starvation period, replace the medium and add medium containing [^{35}S] methionine (100 μCi/mL).
5. Incubate the cells for 16 h at 37 °C.
6. At the end of the incubation, remove the labeling medium and rinse the cells twice with PBS 1×.
7. Add 300 μL of RIPA buffer to each dish.
8. Leave the dishes on ice for 10 min.
9. Harvest the cell lysate to microfuge tubes. Vortex for 5 s and incubate on ice for another 10 min.
10. Centrifuge the tubes for 10 min at 8,050 × *g* to remove the cell debris and nuclei.
11. Transfer the supernatant to new microfuge tube and stand on ice or store at −20 °C.

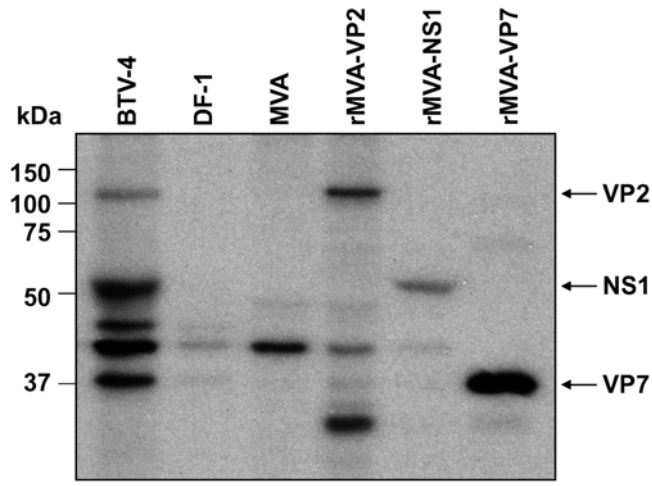

Fig. 4 Analysis of BTV protein expression by radiolabeling, immunoprecipitation, and SDS-PAGE. [^{35}S] methionine-labeled BTV proteins were isolated by immunoprecipitation using polyclonal antibody specific of BTV-4. The expression of BTV proteins in DF-1 cells infected with rMVA-VP2, rMVA-NS1, or rMVA-VP7 was then analyzed by SDS-PAGE

12. Immunoprecipitate BTV proteins with 10 μL of mouse polyclonal antibody specific of BTV-4 by using the Dynabeads® Protein G system and according to the protocol recommended by the manufacturer.
13. After the immunoprecipitation process, boil the beads in SDS-PAGE buffer for direct characterization of proteins on SDS-PAGE.

3.5 Purification of rMVAs by Using a Sucrose Gradient

There are various forms to purify and to separate viruses. Sucrose gradient is frequently used for separating virus, and the use of a sucrose cushion allows the possibility to concentrate the virus.

1. Layer 19 mL of the sonicated lysate onto 19 mL of a 36 % sucrose cushion (in PBS) in a sterile SW 28 (or SW 27) centrifuge tube (50 mL). Centrifuge for 90 min at 30,000 × *g* (SW 28 rotor) at 4 °C. Aspirate and discard the supernatant.
2. Resuspend the viral pellet in 0.5 mL of PBS 1× for a T150 flask (*see* **Note 12**).
3. Sonicate once for 1 min, and prepare a sterile 24–40 % continuous sucrose gradient in a sterile SW 27 centrifuge tube the day before it is needed by carefully layering 6.8 mL each of 40, 36, 32, 28, and 24 % sucrose. Let it sit overnight in the refrigerator.
4. Overlay the sucrose gradient with 1 mL of sonicated viral pellet and centrifuge for 50 min at 26,000 × *g* (11,500 × *g* an SW 27 rotor), 4 °C.

5. Observe the virus as a milky band near the middle of the tube. Aspirate the sucrose above the band and discard. Carefully collect the virus band (~10 mL) with a sterile pipet, place in a sterile tube, and save.
6. Collect aggregated virus from the pellet at the bottom of the sucrose gradient after aspirating the remaining sucrose from the tube. Resuspend the viral pellet by pipetting up and down in 1 mL of 1 mM Tris·Cl, pH 9.0.
7. Sonicate the resuspended pellet once for 1 min, reband the virus from the pellet as in **steps 5** and **6** and pool band with band from **step 6**. Add 2 volumes of 1 mM Tris·Cl, pH 9.0, and mix. Transfer to sterile SW 27 centrifuge tubes (*see* **Note 13**).
8. Centrifuge for 60 min at 32,900 × *g*, 4 °C, then aspirate and discard supernatant, resuspending the virus pellets in 1 mL of 1 mM Tris·Cl, pH 9.0. Sonicate as in last steps and divide into 200–250-μL aliquots. Store at −80 °C.

4 Notes

1. We found that 1 mL of TRI reagent is suitable for lysis of $5–10 \times 10^6$ cells.
2. Sequencing was performed by using a plasmid specific primer located 214 nucleotides downstream of the *Sma*I restriction site: *pSC11-A(VS): GTGGTGATTGTGACTAGCGTAG*.
3. The CPE caused after MVA infection consist of vacuolae formation spreading the cell cytoplasm. By using a Pipetman is easy to disrupt the monolayer, transferring the supernatants to a new tube to facilitate virus processing. It is important to use negative controls (infecting cells with MVA wt and MVA wt + Lipofectamine) and include a positive control (recombinant plasmid pSC11 + Lipofectamine).
4. We usually add 20 μL of the virus stock diluted in 180 μL of complete medium, and successively transfer 20 μL of the prior dilution to 180 μL of complete medium until dilution 10^{-7}).
5. In order to allow cell spreading, we use a proportion 1:1 between agar and medium. Do not try to do too many assays at the same time because the agar–DMEM mixture could solidify.
6. It is advisable to confirm the presence of the plaques by light microscopy.
7. In order to obtain a high viral titer is convenient to use low MOI to avoid a prompt damage in the cells.
8. We usually maintain 4 mL of medium/flask.

9. It is convenient to count the plaques of the intermediate dilutions, because it is easier to count (the size of MVA plaques are small).
10. In this step is possible to stop the procedure and the coverslips can be stored in PBS 1× at 4 °C for at least 1 month.
11. It is convenient to do tenfold dilutions of the sera in order to find the best dilution.
12. At this stage, the virus may be sufficiently pure for some purposes—e.g., isolation of DNA.
13. The total volume should be ~60 mL, which is enough to fill two SW 27 centrifuge tubes. If less volume is obtained, fill the tubes with 1 mM Tris–HCl, pH 9.0.

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Chapter 10

Generation of Recombinant Capripoxvirus Vectors for Vaccines and Gene Knockout Function Studies

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Abstract

The ability to manipulate capripoxvirus through gene knockouts and gene insertions has become an increasingly valuable research tool in elucidating the function of individual genes of capripoxvirus, as well as in the development of capripoxvirus-based recombinant vaccines. The homologous recombination technique is used to generate capripoxvirus knockout viruses (KO), and is based on the targeting a particular viral gene of interest. This technique can also be used to insert a gene of interest. A protocol for the generation of a viral gene knockout is described. This technique involves the use of a plasmid which encodes the flanking sequences of the regions where the homologous recombination will occur, and will result in the insertion of an EGFP reporter gene for visualization of recombinant virus, as well as the *E. coli* gpt gene as a positive selection marker. If an additional gene is to be incorporated, this can be achieved by inserting a gene of interest for expression under a poxvirus promoter into the plasmid between the flanking regions for insertion. This chapter describes a protocol for generating such recombinant capripoxviruses.

Key words Capripoxvirus, Recombinant virus, *E. coli* gpt selection, EGFP, Virus titration

1 Introduction

The genus *Capripoxvirus* consists of sheeppox virus (SPPV), goatpox virus (GTPV), and lumpy skin disease virus (LSDV). Sheep and goat pox are endemic in Africa (excluding Southern Africa) as well as the Middle East and Asia, whereas lumpy skin disease virus is endemic throughout Africa [1]. All capripoxviruses share a high degree of sequence homology [2, 3]. Sheep and goat pox affect sheep and goats and generally have a host preference, although some isolates can affect both sheep and goats; whereas lumpy skin disease (LSD) affects cattle. Upon infection into their respective hosts, these viruses cause clinical signs of disease such as fever, increased heart rates, increased nasal and mucosal discharge, as well has the formation of skin macules affecting the majority of the skin surface in severe cases [4–6]. Mortality rates vary but can reach over 90 % in outbreaks with sheep and goat pox, whereas

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mortality caused by lumpy skin disease (LSD) is lower but can approach 50 %. The high morbidity and mortality associated with these diseases are of particular concern to the livestock industries where these diseases are endemic [7]. Attenuated vaccines have been developed for sheep and goat pox and LSD [4, 8, 9]; while effective in preventing disease outbreaks, the mechanism of attenuation is not known. Recombinant viruses that specifically target and inactivate genes suspected of being virulence factors could be used as vaccines once they have been developed and demonstrated to be effective. To date, the complete genomes of several LSDV, SPPV, and GTPV isolates have been sequenced and annotated [2]; and several putative virulence factors have been identified (*see* Table 1). Furthermore, the use of viral gene deletion has also been shown to be a valuable tool in the elucidation of the role of

Table 1

Selected Open Reading Frames (ORF) deduced from the lumpy skin disease virus (LSDV) genome[a]

LSDV ORF number	Length (amino acids)	Putative function/homologue
003	240	ER-localized apoptosis regulator
005	170	IL-10
006	231	IL-1 receptor
009	230	α-Amanitin sensitive protein
011	381	G-protein coupled chemokine receptor
013	341	IL-1 receptor
014	89	eIF2α-like PKR inhibitor
015	161	IL-18 binding protein
026	302	Ser/Thr protein kinase; virus assembly
034	177	dsRNA-binding PKR inhibitor
057	373	Virion core protein
066	177	Thymidine kinase
067	198	Host range protein
117	148	Fusion protein, virus assembly
128	300	CD47-like protein
135	360	Interferon α/β binding protein
139	305	Ser/Thr protein kinase, DNA replication
142	135	Secreted virulence factor
154	240	ER-localized apoptosis regulator

[a] The following ORFs were deduced based on previous genomic work by Tulman et al. [2]

individual genes of poxvirus genomes [10]. The ability to manipulate poxvirus genomes has been previously demonstrated for capripoxvirus through the creation of a gene knockout to sheeppox virus kelch-like gene SPPV-019, which was then subsequently shown to affect virulence [11]. The principle method to generate gene knockouts using homologous recombination is the same for all poxviruses [10] (*see* **Note 1**). The main differences in the methods with different poxviruses are the host cells used to grow the viruses and the DNA target sequences used for the homologous recombination.

Aside from the need to generate more effective capripox vaccines, the past decade has seen the use of recombinant capripoxviruses used as efficient vaccine vectors, expressing foreign antigens of other non-related ruminant diseases. In experimental settings, capripoxvirus-based vaccines have been shown to protect against bluetongue virus (BTV) [12, 13], Rift Valley fever virus (RVFV) [14, 15], peste des petits ruminants virus (PPRV) [16], and rinderpest virus (RV) [17–19]. These vaccines have been designed based on the insertion of an antigen of interest into the viral genome through gene recombination, targeting the viral thymidine kinase (tk) gene of capripox. In this chapter, the protocol to generate these recombinants will be described, and will incorporate the use of a reporter gene such as enhanced green florescent protein (EGFP) as well as a positive selection marker [10]; in this case, the *E. coli* guanine phosphoribosyl transferase (gpt). The selective media to be used in this protocol includes mycophenolic acid and aminopterin, inhibitors of purine metabolism; and when grown in the presence of xanthine, which can be metabolized via gpt into guanine [20]. In this protocol, the gpt gene will be flanked by two vaccinia 7.5K early/late promoters, which upon removal of selective pressure, will enable for its removal through recombinant deletion.

2 Materials

Unless indicated, all reagents and solutions were prepared according to the manufacturer's instructions. All reagents used pertaining to cell culture were either pre-sterilized, or filtered using a 0.22 μm syringe/bottle top filters. Furthermore, all cell culturing was performed using sterile technique, and inside biosafety cabinets designed specifically for cell culture.

2.1 Cell Lines

OA3.T cells were obtained from ATCC, and cultured in Dulbecco's Modified Eagle Medium (DMEM), and supplemented with 10 % fetal bovine serum (FBS), 1× penicillin/streptomycin (Pen-Strep) and, 1× nonessential amino acids (NEAA). Unless otherwise indicated, cells were cultured in Costar/Corning 6-well plates, and

incubated at 37 °C at 5 % CO_2. The culture media and reagents used in this protocol are from Wisent (Saint-Bruno, Canada); however, it should be noted that these reagents are available from a wide variety of manufacturers, which are also acceptable for this protocol.

2.2 Capripoxvirus Strains

Depending on the purpose of the individual experiment, the capripoxvirus strains to be used can either be virulent or attenuated (*see* **Note 2**). In both cases, it is suggested that the gene target and the required flanking genome sequences are sequenced prior to cloning, in order to ensure that the target sequences are identical to the flanking sequences of the transfer vector.

2.3 Plasmid Construction of the Transfer Vector

Plasmid construction was performed using the sequences described in the previous section, with open reading frames (ORF) encoding for a gene of interest (*see* **Notes 3 and 4**), EGFP and gpt synthesized in tandem (Fig. 1), and flanked genes encoding for a viral target of interest. A list of potential LSDV targets for functional and/or attenuation is described in Table 1.

2.4 Transfection Reagents

While previous studies of recombinant capripox have described the use of various types of transfection reagents for the uptake of the transfer vector in various cell lines, the transfection described in the protocol will involve the use of X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics, Mannheim, Germany; also *see* **Note 5**).

2.5 Buffers and Media

1. For all steps involving washing cells, calcium and magnesium free phosphate buffered saline (PBS) was used (Wisent, Saint-Bruno, Canada); however, it should be noted that PBS is available from a wide variety of manufacturers.
2. The transfection procedure will involve the use of optimized, low-serum medium. To this end, Opti-MEM (Invitrogen/Life Technologies, Carlsbad, USA) is used for incubations with the transfection reagent (*see* **Note 6**).
3. For gpt selection, 500× mycophenolic acid solution and 100× aminopterin (containing xanthine and hypoxanthine) were purchased from EMD Millipore. Following addition to culture media, the selective medium was then syringe filtered prior to use.
4. Recombinant capripoxviruses will be selected and purified using semisolid medium containing selective chemicals (i.e., mycophenolic acid). The semisolid medium used in this protocol is ClonaCell (StemCell Technologies). However, it should be noted the DMEM/carboxymethyl cellulose (CMC) medium may also be used.

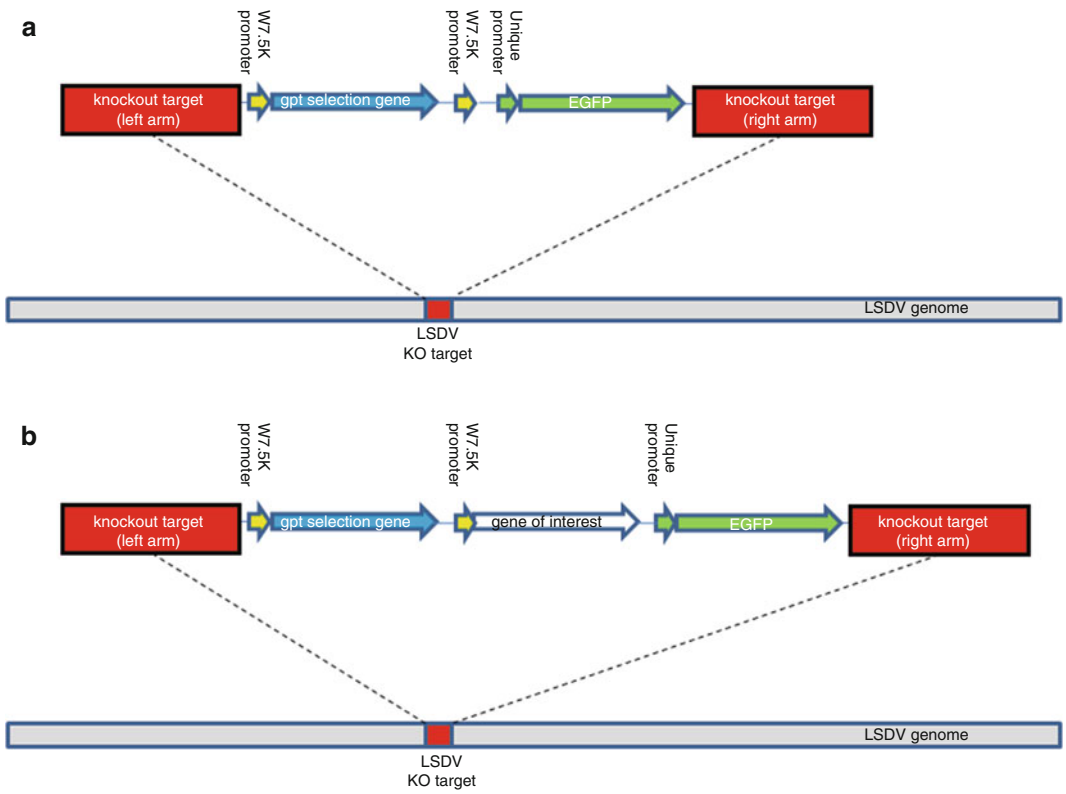

Fig. 1 The insert of a shuttle vector for generating capripox recombinant virus. A viral gene is targeted for gene knockout (Panel **a**) by cloning an insert containing the following: (1) selection and reporter genes (in this case, gpt and EGFP, selectively); (2) vaccinia early/late promoter 7.5K, enabling for both the expression of gpt and the gene of interest, as well as enabling for the subsequent deletion of the gpt gene; (3) flanking sequences, encoding for the 5′ and 3′ ends (knockout target left/right arm) designed for the knockout of a specific viral gene (in *red*). The shuttle vector may also include a gene of interest to be expressed (Panel **b**)

3 Methods

3.1 Infection of Transfected OA3.T Cells with LSDV (See Schematic of the Whole Process in Fig. 2)

Seed OA3.T cells in a 6-well plate, at 60–70 % confluency 24 h prior to transfection. Cells should be cultured in DMEM/10 % fetal bovine serum, supplemented with 1× penicillin/streptomycin (Pen-Strep) and 1× nonessential amino acids (NEAA).

1. Prior to addition of LSDV, verify the condition of the cells, and ensure that the confluence is between 70 and 80 %.
2. Remove medium, and add 1 mL of fresh medium (DMEM/1× Pen-Strep) to each well.
3. Add 1000 $TCID_{50}$ (100 μL of a 10^4 $TCID_{50}$/mL stock) of capripox virus (or use at an MOI of 0.1). Incubate at 37 °C for 4 h.
4. Remove medium, then add 2 mL of fresh medium (DMEM/1× Pen-Strep) to each well.

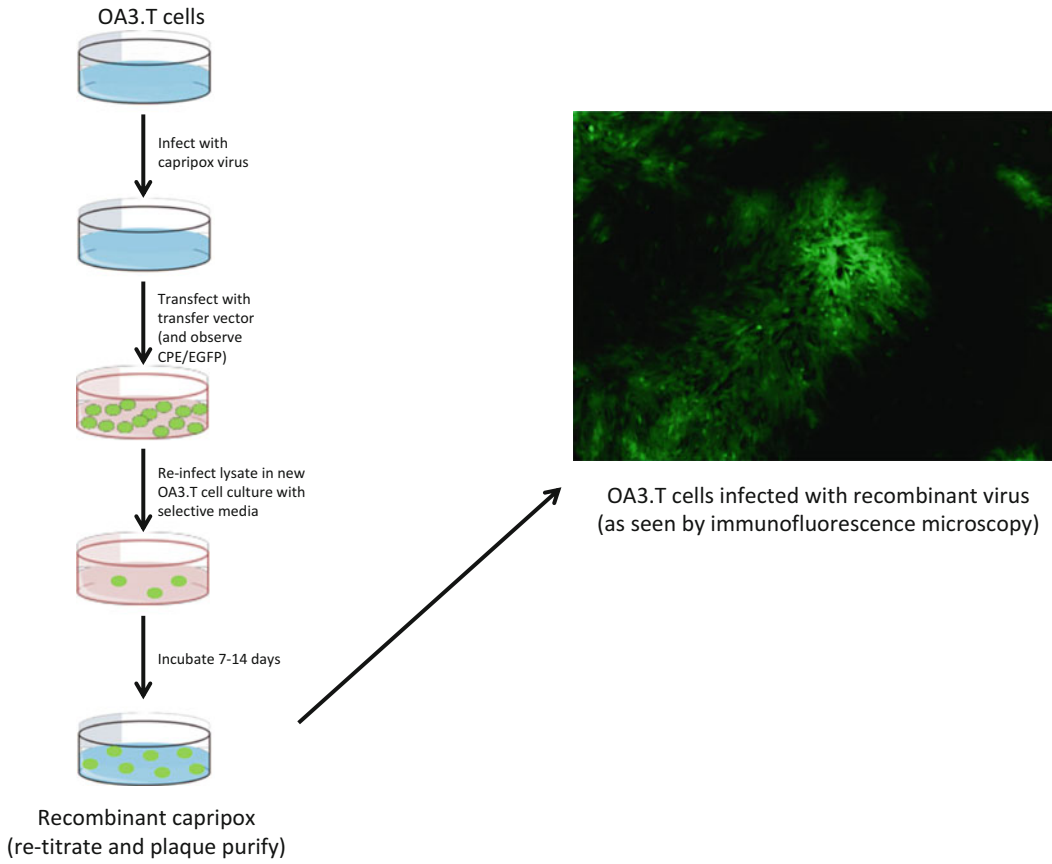

Fig. 2 Schematic of the process of generating recombinant capripox virus. OA3.T cells are transfected with a transfer vector containing the gene of interest, as well as the elements needed for recombination (*see* Fig. 1). Upon transfection of the transfer vector, cells are then cultured in selective media, following by infection with a capripox strain of interest (i.e., LSDV, SPPV, or GTPV). Recombinant viral plaques are visible using fluorescence microscopy, and are re-titrated and plaque purified until an individual recombinant viral plaque is isolated

3.2 Transfection of OA3.T Cells

1. The day of transfection, warm up a tube of X-tremeGENE HP transfection reagent to room temperature 1 h before use, as well as 10 mL of Opti-MEM.
2. Wash cells with 1× PBS. Remove PBS and add 800 μL of Opti-MEM in each of the six wells. Incubate at 37 °C.
3. In a sterile Eppendorf tube, add 1 μg of plasmid DNA with 100 μL of Opti-MEM.
4. In another sterile Eppendorf tube, add 4 μL of X-tremeGENE HP transfection reagent to 100 μL of Opti-MEM (note, thoroughly vortex the transfection reagent prior to use).
5. Leave both tubes at room temperature for 10 min.
6. Add the tube containing the X-tremeGENE reagent to the tube containing the plasmid DNA.

7. Incubate at room temperature for 30 min.
8. Add the transfection the reagent/plasmid DNA mixture dropwise into one well of the 6-well plate.
9. Incubate the plate at 37 °C for 4 h.
10. Remove the media. Add 2 mL of fresh medium into each well. Incubate for 4–6 days at 37 °C, until the cytopathic effect (CPE) and EGFP is observed through the appearance of fluorescent green plaques.

3.3 Selection of Recombinant Viruses

1. Replace medium with selective media: DMEM/10 % fetal bovine serum, supplemented with 1× penicillin/streptomycin (Pen-Strep) and 1× nonessential amino acids (NEAA)/1× gpt selective agents (note: verify the pH of the medium prior to use) and incubate for 5–6 days. Note, that within 3–4 days, fluorescent plaques (i.e., expressing EGFP) should be visible by microscopy.

   Collect supernatant from infected wells when extensive EGFP expression and plaque formation is observed. Depending on the experimental conditions, this can take anywhere between 7 and 14 days. Green-fluorescent plaques should indicate the generation of recombinant LSDV (*see* **Note 7**).
2. Remove the plate, and perform two freeze–thaw cycles (i.e., freezing the culture plate at −80 °C and thawing at 37 °C). This step will liberate viral particles into the resulting cell lysate.
3. Remove the lysate and centrifuge at 3000 × *g* for 15 min.
4. Aliquot the supernatant into sterile cryotubes and store at −80 °C.
5. Increase the titers of the recombinant virus by infecting a new culture of OA3.T cells in 6-well plates using selective media. Repeat **steps 1–5**, this time using 100 μL of the supernatant.
6. After 6–7 days, collect the supernatant. This will be used for the subsequent plaque purification and gpt removal, as described in the following section (*see* **Note 8**).

3.4 Isolation of Individual Recombinant Plaques

1. On a new culture plate, seed OA3.T cells, as previously described (i.e., with 1 mL of selective media in each well).
2. Incubate 100 μL of the viral supernatant obtained in the previous section (i.e., infection/transfection step) in the first well. In the second well, incubate 100 μL of a tenfold dilution (note: the dilutions should be done in selective medium).
3. Using 100 μL of the diluted supernatant, perform another tenfold dilution, and add to the third well.
4. Incubate at 37 °C for 4 h. During this time, warm a bottle of semisolid medium at 37 °C.

5. After incubating for 4 h, remove the supernatant from all the wells of the culture plate. Add 3 mL of semisolid medium to each well.
6. Incubate at 37 °C for 7–14 days. Verify the appearance of plaques after 4 days, monitoring for plaque size thereafter. Note: While a standard fluorescent microscope can be used to identify smaller plaques, larger plaques can be seen using a blue light source (*see* **Note 9**).
7. When plaques are at least 1–2 mm in diameter (i.e., enough to see with the naked eye), mark the plaques to be picked by using a fine black marker, marking the bottom of each plate. If possible, try to isolate at least five well-separated plaques.
8. To pick each individual plaque, cut off a sterile 100 or 200 μL micropipette tip with a sterile blade/scalpel, approximately 5 mm away from the tip (*see* **Note 10**).
9. Place the cut tip over the plaque and pipet the semisolid medium plug. Dispense of the plug into a sterile Eppendorf tube containing 500 μL of selective media.
10. Vortex the Eppendorf tube several times, and keep at 4 °C.
11. Prepare a new 6-well plate of OA3.T cells, as described in Subheading 3.2.
12. Remove 100 μL of the contents of the medium in the tube and use to reinfect the cells, as previously described.
13. Incubate for 7–14 days, until significant plaque formation can be observed via fluorescence microscopy.
14. Collect the supernatant containing the amplified recombinant virus, as described in **steps 5–8** (*see* **Note 11**).
15. The recombinant virus can then be analyzed by PCR, using primers specific to the 5′ and 3′ of the targeting viral (i.e., knockout) gene and the flanking sequences.

3.5 Removal of gpt Gene Through Selective Pressure

1. Seed a 6-well plate with OA3.T cells, with a confluency between 80 and 90 %, using media free of selection agent (10 % FBS/DMEM/1× Pen-Strep).
2. The following day, replace media with fresh medium (again, without selective agent) at a volume of 1 mL/well.
3. Using a stock of recombinant virus from the previous section, add 100 μL of virus to the first well of plate, performing 100-fold serial dilutions to the next four wells (leave the final well without virus).
4. Incubate for 4 h at 37 °C.
5. Replace medium with fresh medium free of selection agent.

6. Incubate for 7–14 days, until significant plaque formation can be observed via fluorescence microscopy.
7. Determine the well where the highest dilution where plaque formation is observed.
8. Remove the plate, and perform two freeze–thaw cycles (i.e., freezing the culture plate at −80 °C and thawing at 37 °C). This step will liberate viral particles into the resulting cell lysate.
9. Remove the lysate and centrifuge at 3,000 × *g* for 15 min.
10. Collect the supernatant. Keep 10 μL for PCR. See PCR conditions at the end of this section.
11. Repeat **steps 1–10** in this section. Recombinant virus stocks free of gpt will be determined when gpt screening by PCR is found to be negative.
12. PCR conditions for gpt screening: 37 cycles at, 95 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min; 1 cycle at 72 °C for 5 min.
13. Primers for detection of gpt selection marker: -Primer 1 (5′-ATGAGCGAAAAATACATCGTCACC-3′) -Primer 2 (5′-TTAGCGACCGGAGATTGGCGGGA-3′).
14. At this point, confirmation of the recombinant gene can be determined by PCR, using primers and conditions specific to the gene of interest. Expression of the recombinant protein of interest can then be determined by western blotting or enzymatic assays (if possible).

4 Notes

1. This method can be modified for other poxviruses that affect animals by using cells that support replication of the pox virus of interest, and designing a plasmid that uses the DNA sequences for insertion for the specific pox virus of interest. For instance, camelpox knockout viruses could be generated by substituting Vero cells instead of OA3.Ts cells.
2. Due to the extremely infectious nature of capripoxvirus, all manipulations involving LSDV, SPPV, GTPC should be performed in a Biosafety Level-3 Agriculture laboratory in non-endemic countries, and a Biosafety Level-2 laboratory in endemic countries.
3. Any cloned gene of interest should encode for the complete open reading frame (ORF), containing both start and stop codons: poxviruses are not capable of RNA splicing. ORFs should also be driven by a poxvirus promoter (doesn't matter which poxvirus).

4. It should be noted that in the case of gene knockout studies, a similar strategy can be used with the additional gene of interest. While earlier techniques described restriction fragment cloning techniques, gene synthesis companies (Genscript, Piscataway, USA) can generate the entire construct de novo.
5. Other transfection agents, such as JetPEI (Polyplus Transfection Co.) or Lipofectin (Invitrogen) or Lipofectamine (Invitrogen) may also be used, provided that conditions for plasmid uptake are optimized for OA3.T cells prior to use.
6. Serum-free DMEM may also be used during the transfection step.
7. It is important to stress that, at this step, not all green fluorescent cells contain recombinant virus; in fact most of the EGFP observed is a result of EGFP being expressed *in trans* by capripox polymerases. Recombinant viruses represent only a small fraction of observed EGFP, and are only amplified following additional gpt selection steps.
8. If performing gene knockout studies, it is possible that the inactivated gene may be essential for viral replication. If that is the case, no EGFP plaques will be seen in subsequent steps. In order to overcome this problem, OA3.T cells should be transfected with an expression vector (i.e., a plasmid expressing the gene of interest, under a constitutively expressed promoter—e.g., CMV or CAG) prior to infection with the capripox knockout.
9. This step is primarily intended to determine which dilution of recombinant virus is needed to generate well-spaced plaques (to enable for easier plaque purification). If a high population of EGFP plaques is still present following these tenfold dilutions, it would be advised to repeat this step, with several additional tenfold dilutions.
10. It should be stressed that all steps should be performed in a biosafety cabinet, using sterile technique.
11. All generated viruses (including those generated during the intermediate generation/amplification steps) can be stored at −80 °C; and it would be well advised that the intermediate products be kept until the final recombinant virus has been obtained (and confirmed by PCR).

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Chapter 11

Recombinant Swinepox Virus for Veterinary Vaccine Development

Hong-Jie Fan and Hui-Xing Lin

Abstract

Poxvirus-vectors have been widely used in vaccine development for several important human and animal diseases; some of these vaccines have been licensed and used extensively. *Swinepox virus* (SPV) is well suited to develop recombinant vaccines because of its large packaging capacity for recombinant DNA, its host range specificity, and its ability to induce appropriate immune responses.

Key words *Swinepox virus*, Homologous recombination, Vaccine

1 Introduction

Swinepox virus (SPV) is the only member of the *Suipoxvirus* genus, one of eight genera in the *Chordopoxvirinae* subfamily of *Poxviridae*. SPV infects only swine [1], and replicates in the epidermal keratinocytes of the stratum spinosum. Tissues other than the skin are rarely affected. It causes swinepox, a disease that occurs worldwide and is associated with poor sanitation [2–5]. Swinepox is characterized by slight fever and inflammation of local lymph nodes [6]. Generalized infection and viremia are not observed. Adult pigs generally develop a mild, self-limiting form of swinepox, with lesions in hairless skin areas that remain localized at the sites of entry [5, 7]. Macroscopic cutaneous lesions pass through the characteristic stages of poxviral lesions, with a very short vesicular phase that usually does not exhibit fluid exudates [8].

SPV possesses a 146 kb double-stranded DNA genome [9] that can accommodate large amounts of extra DNA; thus, several transgenes can be expressed simultaneously, providing a multivalent vaccine approach [10]. SPV replicates within the cytoplasm of infected cells and does not integrate into the host genome, which

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eliminates the potential for insertional mutagenesis. The host range specificity of SPV, and its ability to induce solid protective immunity, have stimulated interest in using it as a host range-restricted vaccine vector [1, 11, 12].

2 Materials

Prepare the SDS-PAGE gel, electrophoresis running buffer, and immunoblotting components etc. as described in Molecular Cloning: A Laboratory Manual [13]. Prepare all solutions with ultrapure water and analytical grade reagents. Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 Bacteria and Plasmid

The pUC19 plasmid backbone and host *Escherichia coli* (*E. coli*) DH5α were purchased from Takara Biotechnology Co., Ltd. (Dalian, China).

1. Luria–Bertani (LB) medium (tryptone 10.0 g/L, yeast extract 5.0 g/L, NaCl 10.0 g/L) is used as the growth medium for *E. coli*. Dissolve 25 g of the mixture in 1 L of distilled water and adjust the pH to 7.2. Sterilize by autoclaving at 121 °C for 15 min. Store prepared media at 4–8 °C, protected from direct light.
2. *E. coli* were routinely cultured in LB medium or on LB agar plates (containing 1.5 % agar, m/v) at 37 °C. Ampicillin (100 mg/L) was added to bacterial cultures as required.
3. Restriction endonucleases *Eco*RI, *Kpn*I, *Xho*I, *Hin*dIII, *Not*I, *Sal*I, *Bam*HI.

2.2 Viruses, Cells, and Media

Wild-type *swinepox virus* (wtSPV, Kasza strain, VR-363™) and PCV-free porcine kidney PK-15 cells (CCL-33™) were purchased from the American Type Culture Collection (ATCC).

1. The PK-15 cells are routinely cultured at 37 °C in 5 % CO_2 in Eagle's Minimum Essential Medium (EMEM), supplemented with 10 % fetal bovine serum (FBS).
2. The crude stock of SPV is prepared by infecting the cultured PK-15 cells for 3–4 days at 37 °C, followed by freezing and thawing the cells three times.
3. The viral titer is determined by determining the number of the plaque-forming units (PFU) on PK-15 cells. The plaque assay was conducted in EMEM-2 % FBS, containing 1.5 % methylcellulose, until a visible plaque was observed.
4. Hank's Balanced Salt Solution (HBSS).

3 Methods

Carry out the procedures of gene amplification, plasmid extraction, plasmid construction, and identification as described in Molecular Cloning: A Laboratory Manual [13]. The primers used in this study are listed in Table 1. All PCR-amplified DNA fragments are initially cloned in the Simple T-vector (Takara) before restriction enzyme (RE) digestion and ligation procedures.

3.1 Construction of the Transfer Vector pUSG11/ P28Cap

Construct the transfer vector pUSG11/P28Cap with the pUC19 plasmid (Takara) backbone (Fig. 1).

1. Extract the SPV Genomic DNA using a virus DNA extraction kit (Geneaid).
2. Amplify the left flanking sequences (LF), a 1.1 kb flanking region upstream of *SPV016* (GenBank: AF410153), containing *SPV020* (*see* **Note 1**), *SPV019*, *SPV018*, and *SPV017* from the SPV genomic DNA, using primers LF1/LF2 and the following conditions: preheat to 95 °C for 5 min; denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and elongation at 72 °C for 1 min for 30 cycles; and a final elongation at 72 °C for 5 min.
3. Amplify the right flanking sequences (RF), a 1.4 kb flanking region downstream of *SPV022* (GenBank: AF410153), containing *SPV021* (*see* **Note 1**) and *SPV020* from the SPV genomic DNA, using primers RF1/RF2 and the following conditions: preheat to 95 °C for 5 min; denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 90 s) for 30 cycles; and a final elongation at 72 °C for 5 min.
4. Analyze the PCR products of LF and RF using 1.2 % agarose gel electrophoresis with Goldenview (Vazyme Biotech Co., Ltd.) at 8 V/cm. Identify the target bands under ultraviolet (UV) light, excise them from the gel, and cut them into small pieces. Extract the DNA from the gel using a DNA purification kit (Geneaid).
5. Insert the LF and RF sequences into the *Eco*RI-*Kpn*I sites and the *Xho*I-*Hin*dIII sites of pUC19 plasmid, respectively, to construct the plasmid pUS01.
6. Identify the plasmid pUS01 by digestion with *Eco*RI-*Kpn*I and *Xho*I-*Hin*dIII restriction, enzymes respectively (Fig. 2a).
7. Detect all the inserted genes LF and RF by subjecting plasmid pUS01 to PCR using primers rSPV1/rSPV2 (*see* **Note 2**). Sequence the PCR fragment to make sure there are no mutations in the inserted sequences.

Table 1

Primers used in this study

Primer	Sequence (5′–3′)	Restriction enzyme	Target gene
LF1	*GAATTC*TAAATCTACTTCTTCAACGG	*Eco*RI	LF
LF2	*GGTACC*TATAACTACTAGGTCCACAC	*Kpn*I	
RF1	*CTCGAG*AGGCGATTATTTATGTTATTA	*Xho*I	RF
RF2	*AAGCTT*ATTTTTATCCTATTGTTGTTC	*Hin*dIII	
11G1	*GCGGCCGC*TTTACTTGTACAGCTCGTCCAT	*Not*I	P11; GFP
11G2	*CTCGAG*ATATAGTAGAATTTCATTTTGTTTTTTTCTATGCTAT AAATGAACATGGTGAGCAAGGGCGAGGAG	*Xho*I	
28M1	CAGATCTTTTTTTTTTTTTTTTTTTTTTGGCATATAAATGGTCGA CTCGAGAGCTCCCGGGGATCCATCGATGC		P28 promoter; MCS
28M2	*GCGGCCGC*ATCGATGGATCCCCGGGAGCTCTCGAGTCGACCATTTATA TGCCAAAAAAAAAAAAAAAAAAAAGATCT*GGTACC*	*Not*I; *Kpn*I	
CAP1	*GTCGAC*ATGACGTATCCAAGGAGGC	*Sal*I	*cap*
CAP2	CGC*GGATCC*TTAAGGGTTAAGTGGG	*Bam*HI	
rSPV1	GTGTGGACCTAGTAGTTATAGGTACCAG		All inserted genes
rSPV2	GCAAAGACCCCAACGAGAA		

Italic = restriction enzyme site

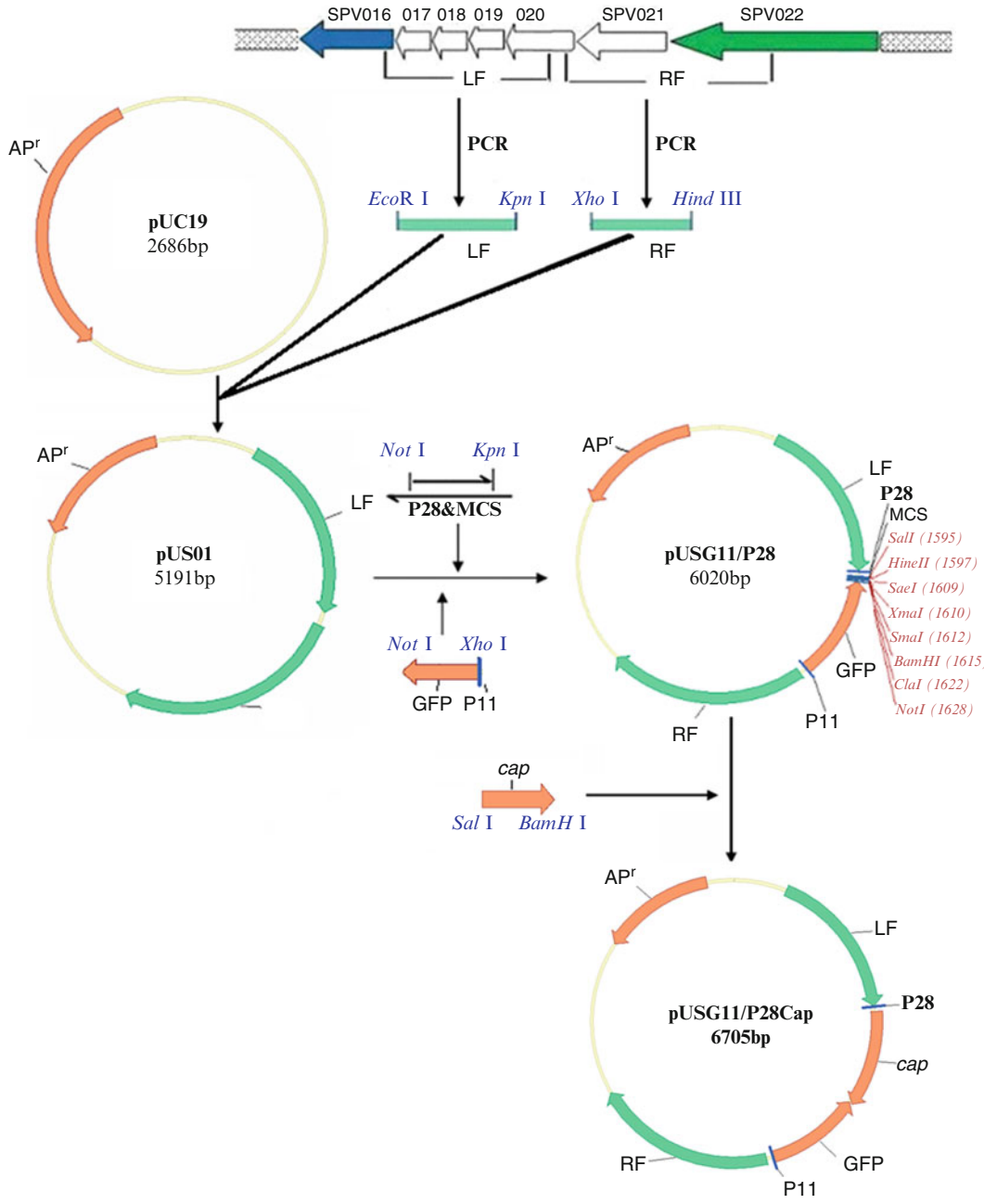

Fig. 1 Construction of the transfer vector pUSG11/P28Cap. LF and RF respectively indicate left flanking sequences and right flanking sequences of SPV. P11 and P28 are *Vaccinia virus* (VV) promoters. The GFP reporter gene is also included in the plasmid. The *cap* gene is the target gene for recombination into the SPV genome

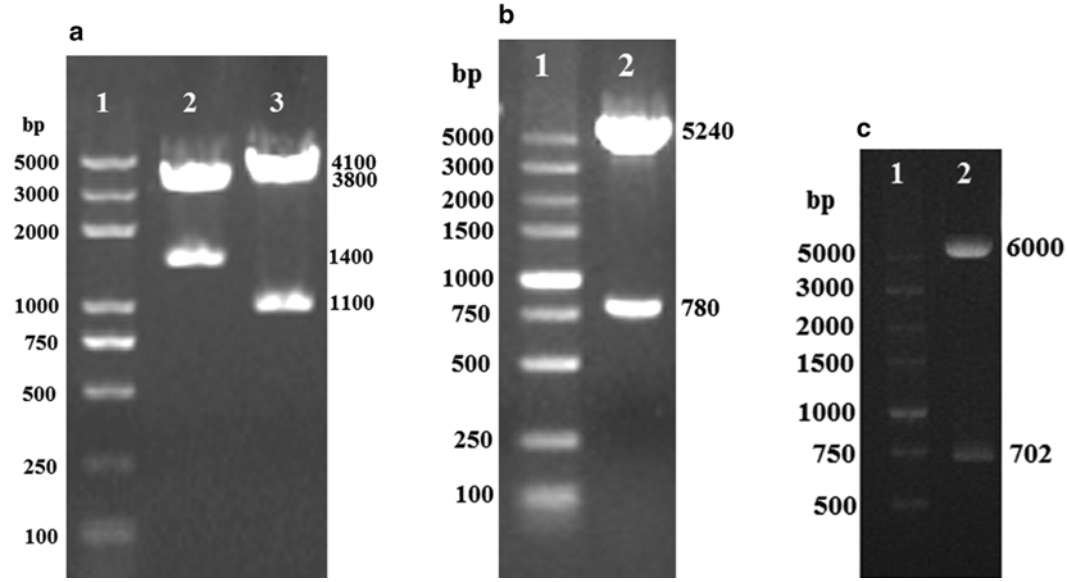

Fig. 2 Restriction endonuclease (RE) of intermediate plasmids generated for transfer vector construction. (**a**) RE digestion identification of pUS01; *1*: DNA Marker; *2*: *Xho*I-*Hin*dIII digestion; *3*: *Eco*RI-*Kpn*I digestion. (**b**) RE digestion identification of pUSG11/P28; *1*: DNA Marker; *2*: *Not*I-*Xho*I digestion. (**c**) RE digestion identification of pUSG11/P28Cap. *1*: DNA Marker; *2*: *Sal*I-*Bam*HI digestion

8. Amplify P11GFP, a 774 bp GFP gene with the promoter P11 sequence from the pEGFP-N1 plasmid (Clontech), using primers 11G1/11G2 and the following conditions: preheat to 95 °C for 5 min; denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 72 °C for 45 s for 30 cycles; and a final elongation at 72 °C for 5 min.
9. Form P28MCS, a 78 bp DNA fragment containing the promoter P28 sequence and a multiple cloning site (MCS) is formed by annealing of oligonucleotides 28M1/28M2.
10. Insert P11GFP and P28MCS into the *Not*I-*Xho*I sites and the *Not*I-*Kpn*I sites of plasmid pUS01, respectively, to construct the plasmid pUSG11/P28.
11. Identify the plasmid pUSG11/P28 by digestion with *NotI-XhoI* restriction enzymes (Fig. 2b).
12. Detect all the inserted genes LF, RF, P11GFP, and P28MCS by subjecting plasmid pUSG11/P28 to PCR using primers rSPV1/rSPV2 (*see* **Note 2**). Sequence the PCR fragment to make sure there are no mutations in any of the inserted sequences.
13. Amplify the targeted gene (gene of interest) (*see* **Note 3**), such as the 702 bp *cap* gene (GenBank: JN382185.2) of *Porcine circovirus* type 2 (PCV2), from the PCV2 genomic DNA using primers CAP1/CAP2.

14. Insert the *cap* gene into the *Sal*I-*Bam*HI sites of plasmid pUSG11/P28 to create the recombinant plasmid pUSG11/P28Cap.
15. Identify plasmid pUSG11/P28Cap by digestion with *SalI-BamHI* restriction enzymes (Fig. 2c).
16. Detect all the inserted genes LF, RF, P11GFP, P28MCS, and *cap* by subjecting plasmid pUSG11/P28Cap to PCR using primers rSPV1/rSPV2 (*see* **Note 2**). Sequence the PCR fragment to make sure there are no mutations in any of the inserted sequences.

3.2 Construction and Purification of the Recombinant *Swinepox Virus*

Construct the recombinant *swinepox virus* rSPV-Cap by homologous recombination of wtSPV with pUSG11/P28Cap.

1. Inoculate 3 mL (0.3×10^6/mL) of PK-15 cells per well in a 6-well plate; 12–18 h later the cells will be 90–95 % confluent.
2. Infect the monolayer of PK-15 cells with the wtSPV (multiplicity of infection (m.o.i) of 0.05); 1–2 h later, discard the infection liquid and replace the cell culture medium with serum-free media.
3. Transfect the cells with 4.0 μg of the pUSG11/P28Cap plasmid using Exfect™ Transfection Reagent (Vazyme Biotech Co., Ltd), according to the manufacturer's instructions (*see* **Note 4**).
4. Collect the cells after 3–4 days of incubation in 2 mL EMEM medium with 2.0 % FBS.
5. Release the virus by freezing and thawing of the cell suspension three times.
6. Prepare the lysate of serial tenfold dilutions from 1:100 to 1:1,000,000 (*see* **Note 5**).
7. Infect every dilution into PK-15 cells grown in a 6-well plate, for further purification of the recombinant virus.
8. After adsorption of the virus for 2 h, discard the infection liquid and wash the cells three times with Hank's Balanced Salt Solution (HBSS).
9. Add 3 mL of EMEM medium containing 1 % methylcellulose (Sigma-Aldrich, St. Louis, MO, USA) to each well and incubate the cells for 3 days until the green foci became visible under a fluorescence microscopy (Fig. 3).
10. Resuspend the plugs of methylcellulose surrounding the stained foci in 0.5 mL EMEM medium (without FBS), and release the recombinant virus by three rounds of freezing and thawing.
11. Repeat foci isolations for 8–10 rounds until all foci in a given well are stained green (*see* **Note 6**).

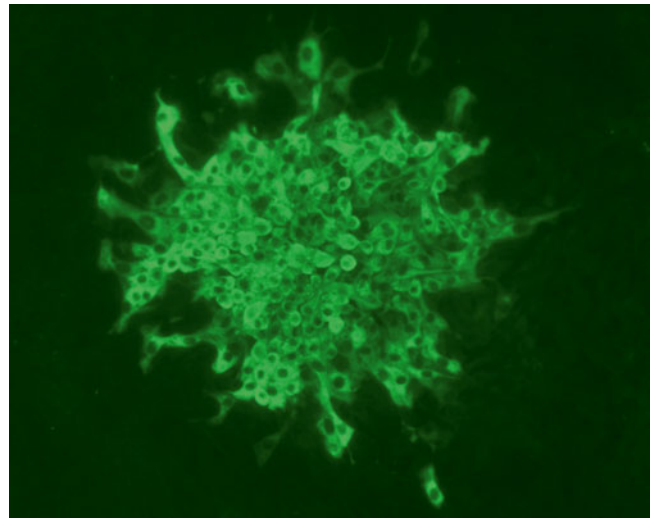

Fig. 3 The green foci of the recombinant *swinepox virus* under fluorescence microscopy

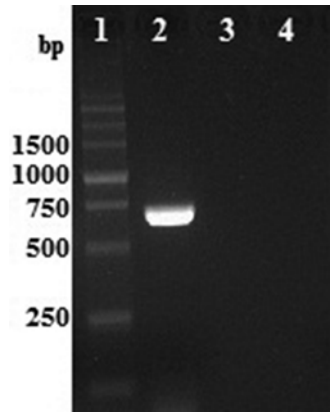

Fig. 4 PCR analysis of rSPV-Cap using primers CAP1/CAP2. *1*: DL5000 DNA marker; *2*: rSPV-Cap; *3*: wtSPV; *4*: uninfected PK-15 cells

3.3 PCR Analysis of the Recombinant Swinepox Virus

1. Extract the rSPV-Cap genomic DNA using a commercial DNA extraction kit (Geneaid).
2. Detect the *cap* ORF by amplifying the rSPV-Cap DNA using primers CAP1/CAP2 (Fig. 4).
3. Detect all the inserted sequences by amplifying the rSPV-Cap genomic DNA with primers rSPV1/rSPV2 (*see* **Note 2**).
4. Sequence all the PCR fragments from **steps 2** and **3** above to make sure there are no mutations in rSPV-Cap.

3.4 Western Blotting Analysis of the Recombinant Swinepox Virus

1. Inoculate PK-15 cells with rSPV-Cap and the wtSPV (m.o.i. of 5), separately, for approximately 60 h at 37 °C.

2. Discard the infection liquid and wash the cells three times with D'Hanks.
3. Resuspend the cell extract in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (*see* **Note 7**), and then incubate the extracts at 100 °C for 5 min to denature them.
4. Electrophorese them on a 12 % SDS-PAGE gel. Initially at a voltage of 80 V for approximately 30 min, and then at 120 V for approximately 45 min (*see* **Note 8**).
5. 5. After electrophoresis, soak the gels in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10 % methanol, pH 11.0) for 5 min to reduce the amount of Tris and Glycine.
6. At the same time, rinse a PVDF membrane with 100 % methanol for about 10 s or until the membrane appearance changes uniformly from opaque to semitransparent (*see* **Note 9**).
7. Wash the PVDF membrane in distilled water and store it in transfer buffer (*see* **Note 10**).
8. Sandwich the gel between a sheet of PVDF membrane and several sheets of blotting paper (*see* **Note 11**), assemble them into the blotting apparatus (GE Healthcare) and perform electroelution for 2 h at 0.8 A/cm^2 in transfer buffer.
9. Block the membrane with 5 % (w/v) skimmed milk dissolved in TBST (137 mM sodium chloride, 20 mM Tris, 0.1 % Tween 20, pH 7.6), for 2 h at room temperature.
10. Incubate the membrane with a monoclonal antibody against Cap (1:1000 dilution with TBS) for 90 min at room temperature and subsequently wash three times with TBST for 10 min.
11. Perform an immunoassay using Staphylococcal Protein A-HRP (1:5000 dilution with TBS) at room temperature.
12. Wash the membrane three times with TBST for 10 min before and after the addition of the secondary antibody.
13. Develop the membrane with the 3,3′-diaminobenzidine substrate until the optimum color development is observed (Fig. 5).

3.5 Immunofluorescence Analysis of the Recombinant Swinepox Virus

1. Inoculate PK-15 cells grown on a 24-well plate with rSPV-Cap and the wtSPV, separately (about 15 PFU per well).
2. At 72 h post-infection, wash the cells three times in phosphate-buffered saline (PBS) and fix them with cold methanol for 10 min at −20 °C.
3. Wash the cells three times with PBS–Tween 20 (PBST) and block them by the addition of 10 % bovine serum albumin (BSA) in PBST.

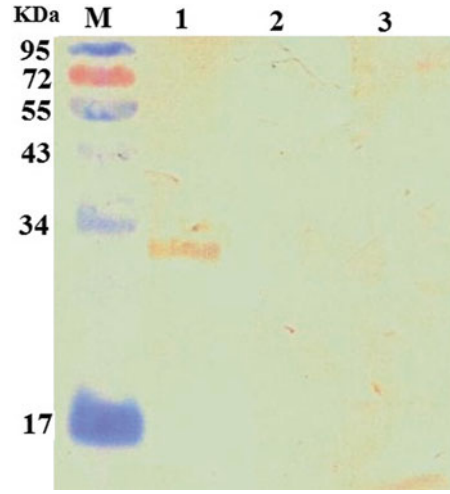

Fig. 5 Western blot analysis of rSPV-Cap. *M*: prestained protein ladder; *1*: PK-15 cells infected by rSPV-Cap; *2*: PK-15 cells infected by wtSPV; *3*: uninfected PK-15 cells

4. Incubate the preparation for 1 h at 37 °C with the monoclonal antibody against Cap in dilution buffer (1 % BSA in PBST).
5. Wash the cells with PBST three times, and then treat the cells with a rhodamine-conjugated secondary antibody (goat anti-mouse IgG-R) at a 1:5000 dilution with PBS for 30 min at 37 °C.
6. Wash the cells with PBST three times, and examine all wells under a fluorescence microscope (Fig. 6).

3.6 Evaluating the Genetic Stability of rSPV-Cap

1. Infect PK-15 cells grown in 25 cm^2 cell culture bottles with rSPV-Cap (100 μl) for 2 h.
2. Wash the cells three times with D'Hanks, add 10 mL EMEM with 2 % FBS to the bottles.
3. Culture the cells at 37 °C in 5 % CO_2 for 5 days, and then release the virus by freezing and thawing the cell suspension three times.
4. Carry out PCR, western blotting, and immunofluorescence analysis of the lysate (as described in Subheading 3.3–3.5) to evaluate the genetic stability of rSPV-Cap.
5. Repeat the evaluation for more than 30 generations.

3.7 Evaluating the Replication Capacity of rSPV-Cap

1. Dilute the lysate of tenfold with EMEM without FBS.
2. Use every dilution to infect PK-15 cells grown in a 6-well plate.

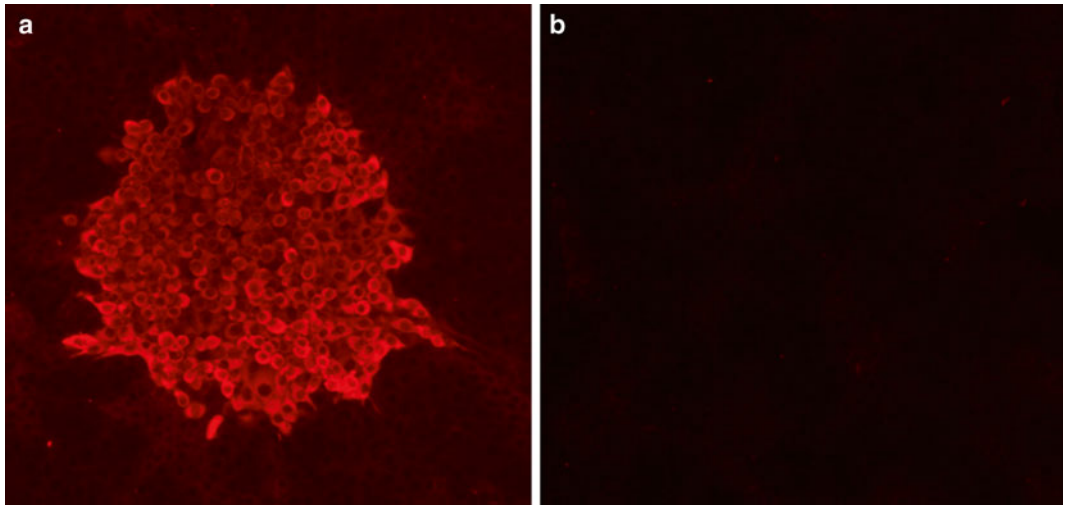

Fig. 6 Indirect immunofluorescence assay of rSPV-Cap. (**a**) Red fluorescence is observed in rSPV-Cap infected cells, with the fluorescence localized to the cytoplasm. (**b**) No fluorescence was observed in cells infected with wtSPV

3. 2 h later, add 3 mL of EMEM containing 2 % FBS and 1 % methylcellulose (Sigma-Aldrich) to each well.
4. Incubate the cells at 37 °C in a 5 % CO_2 for 3 days, count the green foci under a fluorescence microscopy, and calculate the virus titer, by PFU of the lysate, to evaluate the replication capacity of each generation of rSPV-Cap.
5. Repeat the evaluation for more than 30 generations.

4 Notes

1. There is a 366 bp noncoding region between the SPV20 and SPV21 regions of the SPV genome [9], which is chosen as the insertion site. The insertion sites of foreign genes should be noncoding regions or regions not essential for replication, such as the viral thymidine kinase (TK) gene [14].
2. Primers rSPV1/rSPV2 contain flanking sequences of the insertion site; therefore, the PCR fragment amplified with primers rSPV1/rSPV2 could be sequenced to make sure there are no mutations in any of the inserted sequences.
3. *Swinepox virus* vectors can accommodate large amounts of extra DNA; thus, several transgenes can be expressed simultaneously, providing a multivalent vaccine approach.

4. There are many kinds of commercial applied transfection reagents; however, some of them are toxic to cells. Therefore, choose a reagent that minimizes cellular toxicity, observe the cells every 12 h and collect the cells before half of them have shed.
5. The titer of the first generation of the recombinant *swinepox virus* usually is not high; therefore, during the first two rounds of purification, prepare the lysate of serial tenfold dilutions from 1:10 to 1:1000.
6. After six rounds of purification, the recombinant *swinepox virus* will be more than 95 % pure in the cell suspension; after ten rounds it will be more than 99 % pure.
7. Prepare the cell extract on ice, or add protease inhibitors to prevent the protein degradation.
8. The duration protein electrophoresis is depended on the molecular weight of the target proteins.
9. Alternatively, if working with a nitrocellulose membrane, proceed directly to the following step, because nitrocellulose membranes do not require prewetting.
10. The membrane must always be kept wet. Should it dry out, rewet the membrane in methanol and water as previously described.
11. Avoid touching and folding the membrane. To avoid contamination, always handle the filter papers and the membrane wearing gloves and using blunt end forceps.

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Chapter 12

Generation and Selection of Orf Virus (ORFV) Recombinants

Hanns-Joachim Rziha, Jörg Rohde, and Ralf Amann

Abstract

Orf virus (ORFV) is an epitheliotropic poxvirus, which belongs to the genus *Parapoxvirus*. Among them the highly attenuated, apathogenic strain D1701-V is regarded as a promising candidate for novel virus vector vaccines. Our recent work demonstrated that those ORFV-based recombinants were able to induce protective, long-lasting immunity in various hosts that are non-permissive for ORFV. In this chapter we describe procedures for the generation, selection, propagation, and titration of ORFV recombinants as well as transgene detection by PCR or immunohistochemical staining.

Key words Orf virus (ORFV), Parapoxvirus, Recombinant vector vaccine

1 Introduction

Poxviruses are used manifold as viral vectors. Vaccinia virus (VACV) was among the earliest eukaryotic viruses to be engineered for expression of foreign genes already more than 30 years ago [1, 2], and also paved the way for the general development of recombinant viral vectors (reviewed in ref. [3]). Increasing knowledge of poxviral gene regulation and developments in molecular biological techniques commonly facilitated the generation of poxvirus recombinants including VACV, fowlpox virus, and canarypox virus (reviewed in ref. [4]). Subsequently, recombinant poxviruses also became attractive live vaccine vectors against various infectious diseases, for human gene therapy, and for anticancer immunotherapy [5–7].

The use of recombinant poxviruses as excellent candidate vaccine vectors is reasoned mainly by (1) their stability, (2) their large genomic size allowing flexible integration of multiple foreign genes, (3) their exclusive cytoplasmic gene expression, which is independent from the host cell machinery and therefore, (4) essentially no risk of integration into the host genome and subsequent insertional cellular gene inactivation, (5) the very low mutation

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rates of the recombinants' genome, and (6) most importantly their ability to stimulate long-lasting transgene-specific B- and T-cell immunity. However, replication-competent attenuated VACV vectors caused some inadvertent, serious complications after immunization [6, 8]. Due to their inability of replication in mammalian cells, the highly attenuated VACV strains modified vaccinia virus Ankara (MVA) and NYVAC turned out to promising poxvirus vectors for human use [4, 8, 9]. Concern exists that the profound attenuation of MVA or NYVAC might be responsible for reduced immunogenicity observed in some clinical trials, and several strategies have used to enhance their immunogenic capacity [10–14].

Recently the *Parapoxvirus* Orf virus (ORFV) has been recognized as a valuable new virus vector system combining several important demands for a safe recombinant vector virus: The very restricted host range, the absence of systemic virus spread, the short-term vector-specific immunity and the lack of serum antibodies efficiently neutralizing ORFV enabling repeated immunizations, still unraveled immune-modulating properties, and the induction of strong and long-lasting immune responses against vector-encoded foreign antigens [15, 16]. During recent years, we demonstrated the successful use of this novel virus vector system based on the apathogenic, Vero cell culture-adapted highly attenuated ORFV strain D1701-V, which is used to generate recombinants by substituting the viral vegf-e gene with a foreign gene, and thereby removing an ORFV virulence gene [15, 17]. We have chosen the original vegf-e early ORFV promoter for the transgene control, which allows its expression before ORFV DNA replication and consequently does not need production of infectious progeny of ORFV recombinant. Therefore, transgene expression is also achieved in cells non-permissive for ORFV [18]. Adaption for growth in the non-ruminant cell line Vero led to additional genomic deletions, which are most probably responsible for the strongly reduced pathogenicity of D1701-V [17]. Various D1701-V recombinants were reported to mediate excellent protective immunity against a number of different viral infections [18–25].

Here we describe up-dated procedures for the generation and selection of recombinant ORFV derived from strain D1701-V or D1701-VrV. The protocols include two different selection techniques and optimized procedures for production, titration, and identification of recombinant ORFV.

1.1 General Considerations

Since poxvirus DNA is not infectious and the large genome is not efficiently taken up by cells, poxvirus recombinants must be generated by homologous recombination taking place between a transfer plasmid, which contains the transgene(s) of interest under the control of defined poxviral promoter, and flanking poxviral genomic DNA. To this end, cells infected with the parental ORFV

are transfected with the transfer plasmid at the approximate time point of ORFV DNA replication. Because infection of too many cells ultimately leads to high background of parental ORFV impeding efficient selection of new recombinants, low multiplicities of infection (moi) like for example 0.01–0.2 must be pretested. Consequently, the low moi demands for highest transfection rates allowing to target a sufficient number of infected cells with transfer plasmid DNA.

After assaying various transfection reagents and techniques including Lipofectamine, Fugene, or magnetofection, the best recombination rates were reproducibly obtained using nucleofection [26]. The electroporation-based principle of nucleofection allows most efficient transfection of different cell types either as cell monolayer or single cell suspension with high cell viability. To our knowledge two versions of nucleofector devices are available, either the original Amaxa nucleofector or the CLB-Transfection system, which both are used with excellent results.

To achieve highest possible titers of ORFV progeny, the so-called simultaneous ORFV infection is preferred to standard infection of monolayer cells. To this end, the necessary amount of ORFV is mixed together with the needed amount of trypsinized cells in growth medium and seeded directly into the culture dishes or flasks. Cells in best condition and regularly tested to be free of mycoplasma must be used not only for transfection.

2 Materials

2.1 Virus and Cell Culture

1. *Virus*: The attenuated ORFV strain D1701-V and the β-galactosidase expressing derivative D1701-VrV has been described earlier [18, 27].
2. *Vero cells*: The African green monkey kidney cell line was originally obtained from the ATCC (CCL-81).
3. *Growth medium*: Minimum Essential Medium (MEM) Eagle, supplemented with Earle's salt, nonessential amino acids, and l-glutamine, including 5 % fetal calf serum (FCS), 10^5 units penicillin per liter, and 100 mg streptomycin per liter.
4. *Versene-trypsin (VT)*: 0.125 % (w/v) trypsin, 0.025 % (w/v) EDTA, 0.4 % (w/v) NaCl, 0.01 % (w/v) KCl, 0.01 % (w/v) KH_2PO_4, 0.057 % (w/v) Na_2HPO_4 (*see* **Notes 1** and **2**).
5. *Fetal calf serum (FCS)*: Endotoxin-free, sterile filtrated, before use heat-inactivated (30 min, 56 °C) and stored at –20 °C.
6. *TB, Trypan-blue*: 0.25 % w/v TB in PBS.
7. *PBS*: Dulbecco's PBS, phosphate-buffered saline without Mg^{2+} and Ca^{2+}.
8. *384-well plate*: Perkin-Elmer, viewplate (OptiPlate), suited for fluorescence.

9. *Multi(12-)channel reagent reservoir*: Best with V-shaped troughs, which facilitates the serial dilution and mixing of virus lysate with cells etc.
10. *Nucleofection*: The nucleofection solutions and cuvettes are delivered with the kit.

2.2 Agarose Overlay

1. 2× MEM: or 2× medium T199, without phenol red.
2. *Low melting temperature (LMT) agarose*: Agarose is solved in aqua bidest. by boiling in a microwave to obtain 2 % (w/v), and after cooling down to approximately 37 °C distributed into 6 mL portions into tubes and subsequently sterilized by autoclaving. These portions can be stored tightly closed at RT (*see* **Note 3**).
3. *BluoGal*: Stock solution contains 30 mg/mL DMSO or DMF (Dimethyl-formamide), store at –20 °C in the dark, i.e., wrapped in aluminum foil. For use dilute 1:100 to obtain 0.3 mg/mL final concentration (*see* **Note 4**).

2.3 DNA

1. *The transfer plasmid* used for nucleofection should be of high quality, endotoxin-free.
2. *Phenol*: Tris-equilibrated phenol for molecular biological use.
3. *CIA*: Mixture of 24 parts of chloroform and 1 part isoamyl alcohol, nucleic acid grade.
4. *Glycogen-blue*: Life Technologies.
5. *7.5 M Ammonium acetate*: Autoclaved.
6. *Eppendorf tabletop centrifuge*, refrigerated.
7. *DNA isolation kit*: For instance Master Pure DNA isolation kit, Epicentre, including lysis buffer, RNase and proteinase K enzyme solution.
8. *Isopropanol*.
9. *Ethanol*: absolute and 70 % (v/v) in sterile H_2O; can be stored at –20 °C.
10. *Filter-tips* (aerosol-tight) recommended for PCR.

2.4 Immune Staining

1. *FALDH*: 16 % methanol-free formaldehyde, tightly sealed in glass ampoules.
2. *TBST (Tris-buffer saline plus Tween 20)*: Can be prepared from a 10× concentrated TBS stock solution (0.5 M Tris, 1.5 M NaCl solved in H_2O dest., pH adjusted with HCl to 7.4–7.6) by 1:10 dilution and adding Tween 20 to a final concentration of 0.05 % (v/v).
3. *Block solution*: TBST plus 10 % FCS or 10 % BSA (bovine serum albumin).

4. *Peroxidase staining substrate*: 1 mg DAB or DAB-black per mL 0.1 M Tris–HCl, pH 7.4, shortly before use add 0.01 % (v/v) H_2O_2.
5. Vector VIP stain kit is commercially available from Vector Laboratories.
6. *Beta Blue staining kit*: Available from Novagen-Merck.

3 Methods

3.1 Nucleofection of ORFV-Infected Cells

To obtain best possible transfection efficiency, Vero cells are seeded the day before and grown to an approximately 80 % confluent monolayer. After trypsinization (VT) the number of viable cells is determined by Trypan Blue (TB) exclusion, and cells are diluted in MEM plus 5 % (v/v) FCS to obtain 1.5×10^6 cells per mL. This is achieved as follows:

1. Pre-incubate the VT solution in a 37 °C water bath.
2. Pour off the medium of the fresh overnight cell culture (T75 flask).
3. Wash the cell layer once with 5 mL PBS or medium without FCS (tempered at 37 °C).
4. Rinse the monolayer with 2 mL VT, remove the VT completely with a pipette.
5. Add 1 mL fresh VT, slightly move the flask to distribute the VT solution evenly over the cell layer.
6. Place in a 37 °C incubator until cells start to detach (*see* also **Note 2**).
7. When monolayer cells are beginning to detach, some taps against the side of the flask help to dislodge the cells.
8. Add 0.5 mL FCS to stop trypsinization, vigorously suspend the cells by strongly flushing the solution on that side of the flask where the cells were grown.
9. Add 3.5 mL medium plus FCS, again vigorously suspend the cells.
10. Remove 50 µL of the cell suspension and mix with 50 µL of TB solution.
11. Pipette the mixture at the edge of a counting chamber covered with a coverslip to allow suspension to spread evenly by capillary action.
12. Place the chamber under a light microscope and count the viable, not stained cells in four large quadrants (*see* **Note 5**).
13. For each transfection, 1 mL containing 1.5×10^6 suspended Vero cells are infected with 3.0×10^5 plaque-forming units (PFU) of the ORFV strain D1701-VrV (moi = 0.2) and incu-

bated at 37 °C in a 2 mL Eppendorf cup under continuous slow rotation for 2 h (*see* **Note 6**).

14. Prepare a 6-well culture plate by filling 2.5 mL of MEM plus 5 % FCS per well for each nucleofection.
15. Let the nucleofection solution equilibrate to room temperature.
16. Two hours after infection (**step 13**) centrifuge the cell-virus suspension at 90×*g* for 10 min at room temperature and remove the supernatant completely (*see* **Note 7**).
17. Resuspend the cell pellet carefully with 0.1 mL nucleofection solution.
18. Add immediately 2 μg plasmid DNA, gently mix and transfer into a cuvette included in the nucleofection kit avoiding air bubbles (*see* **Note 8**).
19. Close the cuvette and insert it into the cuvette holder of the apparatus.
20. Select the correct pulse program for Vero cells and start.
21. After the pulse is finished, remove the cuvette from the holder, and add 0.2–0.5 mL of the pre-warmed medium-FCS under a laminar flow hood by using sterile Pasteur pipettes.
22. Gently transfer the solution back to the well of the 6-well plate and incubate at 37 °C, 5 % CO_2.
23. After 2–5 days, up to 80 % cpe (cytopathogenic effect) should be recognized.
24. Medium and detached cells from each well are transferred to sterile tubes and placed on ice.
25. Cells remaining on the bottom of the well are harvested by treatment with 0.5 mL VT and combined with the medium from **step 24**.
26. The obtained nucleofection-cell lysates (NL) are broken by three times freezing at −70 °C and alternating briefly thawing at 37 °C.
27. Preferably the NL is sonicated (100 W) 5–7 times 20 s on ice (with a 10 s break between) to release infectious virus, and stored at −70 °C until use.

3.2 Selection of lacZ-Negative Recombinant Virus

The following procedure describes our original protocol for selecting new recombinants starting with the parental β-galactosidase expressing, blue ORFV D1701-VrV [18, 23, 24].

1. The nucleofection lysate (NL; as described in Subheading 3.1) is thawed on ice.
2. Five dilutions of NL (1:4–1:2500) are prepared on ice in PBS or medium (*see* **Note 9**).

3. For each NL dilution 3×10^5 Vero cells in 2 mL MEM plus 5 % FCS are freshly prepared as described in Subheading 3.1.
4. Added per well of a 6-well plate and mixed with 0.1 mL of each NL dilution. One well receiving non-infected cells is used as negative control.
5. After approx. 3 days incubation at 5 % CO_2, 37 °C plaques should become visible.
6. Pre-warm 2× MEM in 37 °C water bath.
7. For each well of a 6-well plate 1.5–2.0 mL agarose overlay is needed, i.e., 12 mL agarose-medium-BluoGal overlay must be prepared per plate.
8. To this end boil one 6 mL portion of LMT agarose followed by equilibrating to approximately 37 °C in a water bath (*see* also **Note 2**).
9. Thoroughly mix with 6 mL of 2× MEM equilibrated at 37 °C, but be careful to avoid creating air bubbles.
10. Finally mix 0.12 mL of the BluoGal stock to obtain 0.3 mg/mL final concentration.
11. Carefully remove the medium, take care not to let dry out the cells, and slowly pour 2 mL of the overlay from the edge of each well followed by carefully moving the plate to prepare an evenly distributed overlay covering the complete cell monolayer.
12. Let the overlay harden for a short time at room temperature (*see* also **Note 4**), before incubating at 5 % CO_2, 37 °C.
13. Blue parental D1701-VrV plaques should become visible after 4–48 h.
14. Isolation of potentially new recombinant plaques: Identify single, colorless plaques under the light microscope, and label them with a lab pen outside on the bottom of the well.
15. For each virus plaque fill 0.2 mL PBS per well of a 48-well plate.
16. Under the sterile work bench pick the plaques for example with a sterile Pasteur pipette.
17. Transfer each plaque agarose block into the PBS of the individual wells.
18. Incubate the 48-well plate overnight at 4 °C to elute virus from the agarose blocks.
19. Next day, freshly prepare Vero cells (*see* Subheading 3.1), and add 0.5 mL MEM plus 5 % FCS containing 1×10^5 cells to each well containing the picked plaques.
20. Incubate the plate at 37 °C, 5 % CO_2 for 3–5 days until cpe or plaque formation becomes visible (*see* **Note 10**).

21. The plate is frozen and thawed three times (−70 °C, 37 °C), in order to break cells and to release virus.
22. Medium and cells are harvested from each well and stored at −70 °C until used for: PCR screening (Subheading 3.6), virus plaque titration (Subheading 3.8), X-gal staining, and additional plaque-purification by re-isolation of white plaques (*see* **Note 11**).

    For DNA preparation 0.1 mL of the lysates can be used, too (Subheadings 3.5 and 3.6).

3.3 Fluorescence-Based Positive Selection

As compared to the blue-white screening described in Subheading 3.2, the positive selection based for instance on fluorescence has several advantages:

- Less expensive and laborious, no agarose overlay containing special substrate (like X-Gal) is necessary.
- The fluorescent signal can be detected earlier and faster.
- The initial screening for new fluorescence-positive recombinants can be performed by limiting dilution procedure, which is clearly easier and faster. The strategy of the "endpoint-dilution" method is to detect in the best case a dilution of the NL, which contains per well one single pfu, here derived from one fluorescent recombinant infectious particle.
- Several fluorescent colors can be used, which might facilitate the generation of polyvalent vectors. Various fluorescent marker genes are commercially available, which can be used not only as marker genes expressed alone or in addition to another gene of interest, but of course also for tagging or fusing with the foreign gene.

The nucleofection of Vero cells infected with D1701-VrV (moi = 0.2) is performed exactly as described in Subheading 3.1. In the following procedure the selection of an ORFV recombinant is described, where the LacZ gene of D1701-VrV is replaced for the AcGFP gene. The used transfer plasmid pdV-AcGFP contains the AcGFP gene under the control of the early vegf-e promoter of ORFV (*see* **Note 12**).

After freeze-thaw and sonication the obtained NL is diluted and screened for AcGFP-positive recombinants as follows:

1. Freshly prepared Vero cells (*see* Subheading 3.1) are diluted to (a) 1×10^5 cells per mL and (b) 1.5×10^5 cells per mL.
2. In trough one of a multichannel reservoir prepare a 1:3 dilution of NL achieved by mixing 1.0 mL of NL thoroughly with 2.0 mL MEM containing 5 % FCS and 3×10^5 Vero cells.
3. Troughs 2–12 of a multichannel reservoir are filled each with 2.0 mL medium plus 5 % FCS and 2×10^5 Vero cells.

4. Next, transfer 1.0 mL from trough 1 to trough 2 containing the 2.0 mL cells-medium, mix thoroughly to obtain the next 1:3 dilution.
5. This 1:3 dilution steps are identically repeated ending up in trough 12 with a dilution of 1:531,441.
6. Using a multichannel pipette (12 channels) 50 µL of the different dilutions from troughs 1–12 can be easily transferred to the 384-well plate as follows:

   Wells A to P of rows 1 and 2, i.e., 32 wells of the plate receive the first 1:3 dilution from trough 1.
7. Wells A to P of rows 3 and 4 receive the next dilution from trough 2 (1:9 dilution), and so on.
8. Ending with wells A to P, rows 23 and 24 with the highest dilution from trough 12 (*see* **Note 13**).
9. After 24 h of incubation at 37 °C And 5 % CO_2 the plate can be monitored under a fluorescence microscope (*see* **Note 14**).
10. Wells exhibiting green fluorescent cells in the highest NL dilutions are recorded.
11. Continue incubation until virus plaque formation has proceeded (usually 72 h after seeding).
12. Determine the ratio of the number of green plaques to white plaques (fluorescence versus bright field).
13. Harvest those wells showing the highest ratio of green plaques by transferring medium and cells (detached with 30 µL VT per well) into single wells of a 48-well plate (*see* **Note 15**).
14. Freeze-thaw the harvested lysates three times (as described before).
15. Add 0.5 mL of MEM containing 5 % FCS and 1×10^5 Vero cells to each well.
16. Incubate at 37 °C, 5 % CO_2 until clear cpe and plaque formation can be visualized (usually after 72 h).
17. Harvest the medium and cells (treating with 0.1 mL VT per well), freeze-thaw three times.
18. Use 0.1 mL for DNA preparation (Subheading 3.6), store the lysates at –70 °C.

Negative selection:

It is self-evident that the above described procedure of selection can be identically used for a negative selection. Here a parental D1701-V is applied, which for instance is expressing a fluorescent marker gene and new nonfluorescent recombinant ORFV are screened. The latter might require some expertise, because starting ORFV-specific cpe or ORFV plaques are not always unambiguously identifiable.

3.4 DNA Isolation from Single ORFV Plaques for PCR

The following method modified from Pasamontes et al. [28] we found reliably working with good success. However, other reported methods or commercially available DNA purification kits might be suitable, too.

Following important points should be strictly considered:

- Work sterile at a place reserved for PCR and use filter-tips recommended for PCR.
- Use clean pipettes reserved for PCR, which are never used for pipetting of DNA templates.
- Extreme care must be taken to prevent contamination or carry-over with virus or DNA (plasmid or viral DNA).
- All solutions used must be reserved for PCR and bottled in single use portions.
- Working with phenol must be performed under a fume hood.

1. Mix 0.1 mL of the plaque virus lysates, as obtained in Subheading 3.2 or 3.3, with 0.1 mL PCR grade H_2O.
2. Add successively 0.1 mL phenol and 0.1 mL CIA, vortex, and centrifuge 3–5 min at 12,000 × *g* in an Eppendorf centrifuge.
3. Save the DNA containing supernatant, add 0.2 mL CIA, vortex and centrifuge as above (*see* **Note 16**).
4. Repeat **step 3**, take the supernatant and add 1–3 μL Glycogen-Blue (*see* **Note 16**).
5. Ethanol precipitate the DNA (0.2 mL) by mixing successively with 0.1 mL 7.5 M ammonium-acetate, and adding 0.6 mL absolute ethanol, mix and chill 10–30 min on ice.
6. Centrifuge at 10,000 rpm in an Eppendorf centrifuge, 4 °C for 20–30 min, pour off ethanol and wash pellet twice with 0.2 mL 70 % (v/v) Ethanol (*see* **Note 17**).
7. Remove the ethanol completely and dry DNA in the opened cup at room temperature or 37 °C (*see* **Note 17**).
8. Thoroughly dissolve DNA in 12 μL PCR-H_2O.
9. Use 3 μL containing approximately 100–500 ng DNA for PCR as described in Subheading 3.7.

3.5 Quick Preparation of Viral DNA

The protocol is adapted from the Master Pure DNA isolation kit from Epicentre Biotechnol (Biozym Scientific). In our hands, this method results in reproducibly good quantity and quality of ORFV DNA, and can be also used for other DNA analyses like Southern blotting.

The given volumes refer to infected cells taken from one well of a 6-well plate (1.0–1.5 mL medium per well for infection is sufficient); however, for the use of smaller cell numbers as derived from 48- or 96-well plates the volumes can be adapted proportionally.

1. Harvest cells when cpe has proceeded to approximately 80 % (*see* **Note 18**).
2. Transfer cells and medium in a 2 mL Eppendorf cup, keep on ice.
3. Trypsinize remaining cell monolayer with 0.3 mL VT at 37 °C, thoroughly suspend cells and combine with the corresponding medium from **step 2**.
4. Spin down cells by brief centrifugation and discard supernatant.
5. Add 1.0 mL PBS, vortex to resuspend cells completely, and centrifuge again.
6. Remove supernatant but leave one drop (approximately 50 μL) to suspend cell pellet thoroughly by vortexing.
7. Add 0.3 mL lysis buffer premixed with Proteinase K (kit) and completely suspend the cell pellet (*see* **Note 19**).
8. Heat for 15 min at 65 °C and mix by inversion every 5 min.
9. Equilibrate the lysate to 37 °C before adding 1 μL RNase A (kit) and incubate additional 30 min at 37 °C.
10. Place on ice, add 0.15 mL MPC reagent (kit), and mix thoroughly by inversion.
11. Centrifuge, for 10 min at 4 °C and 12,000 × *g* in an Eppendorf centrifuge.
12. Transfer supernatant without any precipitate into a fresh Eppendorf cup (1.5 mL) and add 0.5 mL isopropanol.
13. Precipitate DNA by inverting the cup 30-times (can be stored overnight at 4 °C).
14. Centrifuge for 10 min at 4 °C and 12,000 x g in an Eppendorf centrifuge, and pour off ethanol (*see* also **Note 17**).
15. Wash DNA pellet twice with 0.2 mL 70 % ethanol, centrifuge for 5 min, and drain off ethanol as above (**step 14**).
16. Dry DNA pellet until ethanol is completely evaporated (*see* also **Note 17**).
17. Solve the DNA pellet with 10–50 μL TE (kit) and leave overnight at 4 °C for completely resolving DNA. Thoroughly suspend with a cut yellow tip (*see* **Note 20**), and determine DNA concentration.

3.6 Screening by PCR

The successful isolation and purification of new recombinants is monitored by PCR assays, which are (1) specific for the new inserted foreign gene of interest, (2) specific for the marker gene of the parental virus (the lacZ gene in the case of D1701-VrV), and (3) ORFV-specific. The result of such a PCR analysis is representatively shown in Fig. 1.

Specific PCR primers are selected, which amplify internal gene fragments of 300–700 bp in size, and which allow most sensitive and specific detection of the corresponding gene. We recommend establishing PCRs that are able to detect less than 50 fg of the inserted gene. Using such PCR sensitivity, we never detected growth of parental blue or fluorescent virus from plaque-purified ORFV recombinants after several cell culture passages, which have been PCR-negative for the marker gene of the parental virus. In that respect, we recommend to test recombinant virus passages also routinely by PCR for the absence of the parental virus and to verify stable insertion of transgene.

In the very most cases, we found that ready-to-use double-concentrated Taq polymerase-based PCR mixes, which already contain gel loading dye, are most suitable for screening of larger numbers of DNA isolated from potential recombinant virus plaques or virus lysates. The only exception represents detection of the lacZ gene of D1701-VrV, which needs for highest sensitivity a Pfx or Pfu polymerase (*see* protocol in Subheading 3.6.1).

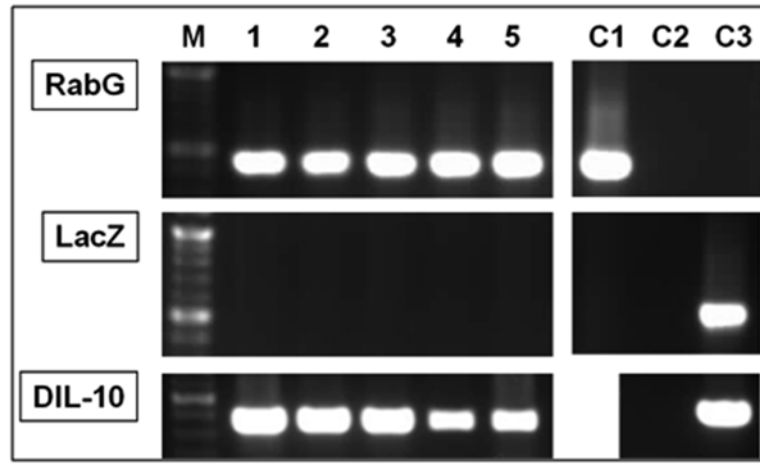

Fig. 1 PCR screening of recombinant ORFV plaques. *Lanes 1–5* represent PCR products of DNA directly isolated from single recombinant ORFV plaques, which are positive for the inserted Rabies virus G gene (RabG, 433 bp), negative for the parental LacZ gene (508 bp), and positive for the ORFV-specific IL-10 gene (DIL-10, 363 bp). As controls PCR was performed with RabG plasmid DNA (*C1*), with DNA from non-infected cells (*C2*), and with lacZ plasmid DNA (*C3*-LacZ) or with IL-10 plasmid DNA (*C3*-DIL-10). *M* represents 1 kb size marker (BioLabs)

The chosen times for denaturation, annealing, and extension of the amplification protocol is depending on the apparatus used. Due to the relatively high G+C content of the ORFV DNA (on the average 64 %), we recommend to start with a first denaturation step at *98 °C for 2 min*, which helps to obtain complete single strand denaturation of ORFV DNA. The following 35 amplification cycles consist of denaturation at 96 °C, the appropriate annealing temperature and extension at 72 °C using Taq polymerase.

Hot-start PCR is used to improve the performance of PCR and to increase specificity as well as target yield. Several hot-start PCR systems are commercially available. These approaches avoid DNA polymerase extension at lower temperatures and minimize nonspecific amplification and formation of primer dimers. Similar hot-start effect can be achieved by starting the thermocycler with the chosen PCR program, but stop the program after reaching 80 °C during the first step. At this temperature all samples just lacking the template but already including primers and polymerase mix are preheated in the thermoblock at 80 °C for a couple of minutes. Next, the template is added to each vial and placed back to the thermoblock. After adding DNA to the last vial the program is continued for cycling.

3.6.1 LacZ Gene-Specific PCR

The following PCR has been established for the sensitive detection of the parental D1701-VrV (as used in Subheadings 3.1–3.3).

1. The primer mix LacZ-FR is prepared by mixing together 3.95 pmol of the forward primer lacZ-F (5′-CGA TAC TGT CGT CGT CCC CTC AA-3′) and 4.13 pmol of the reverse primer lacZ-R (5′-CAA CTC GCC GCA CAT CTG AAC T-3′).
2. Per PCR reaction use 1 µL LacZ-FR, 1 µL PCR-H_2O, 3 µL (ca. 100 ng) template DNA isolated from the NL or virus lysates, and 5 µL twofold concentrated AccuPrime II (Life Technologies).
3. The PCR program consists of 98 °C and 2 min, 35 cycles consisting of 96 °C and 60 s, 62 °C and 30 s, 68 °C and 90 s, ending with a final step at 68 °C for 2 min.
4. The expected amplicon size amounts to 508 bp, as seen in Fig. 1, LacZ.

3.6.2 ORFV-Specific IL-10 PCR

Negative PCR results cannot be excluded to result from poor quality of the DNA isolated from the different virus isolates (Subheadings 3.1–3.3). This can be tested by the use of an ORFV-specific PCR to verify the presence of ORFV DNA. ORFV encodes a functional IL-10 homologue (PP42) not present in other poxviruses. We found that ovIL10-specific PCR is very sensitive for D1701-V, which of course does not exclude the suitability of other ORFV genes.

1. The primer mix DIL10-FR is prepared by mixing 4 pmol forward primer DIL10-F (5′-CAC ATG CTC AGA GAA CTC AGG G-3′) and 4 pmol reverse primer DIL10-R (5′-CGC TCA TGG CCT TGT AAA CAC C-3′).
2. Per PCR reaction 3 µL DIL10-FR are mixed with 100 ng template DNA (2 µL) and 5 µL 2× DreamTaq Green PCR Master Mix available from Thermo Scientific-Fermentas (*see* **Note 21**).
3. The PCR program consists of 98 °C and 2 min, 35 cycles consisting of 96 °C and 30 s, 65 °C and 30 s, 72 °C and 30 s, ending with the final step at 72 °C for 2 min.
4. The expected amplicon size amounts to 363 bp, as seen in Fig. 1, DIL-10.

3.7 Production of Concentrated ORFV Preparation

ORFV titers obtained by in vitro cell culture propagation, not only with ORFV D1701-V, are generally hardly exceeding 10^6–10^7 pfu/mL. In order to achieve virus stocks with higher titers the following procedure can be recommended (*see* **Note 22**).

1. Vero cells are seeded in a T175 culture flask to obtain an almost confluent cell monolayer of approximately 2×10^7 cells after overnight incubation at 37 °C and 5 % CO_2.
2. After pouring off the medium, briefly wash the cell monolayer with VT, add 2.0 mL VT and incubate 3–5 min at 37 °C until cells are detached completely.
3. Add 0.9 mL FCS and resuspend cells by vigorous pipetting.
4. Add 1×10^7 pfu virus, finally corresponding to 0.5 moi, fill up with medium to 9.0 mL, and swirl the flask for mixing.
5. Divide the virus–cell suspension into three T150 flasks (3 mL for each).
6. Fill up to 40–50 mL with medium plus 5 % FCS.
7. Incubate 3–4 days at 37 °C, 5 % CO_2 until cpe has developed to approximately 80 % (*see* **Note 23**).
8. Tap the flasks vigorously to dislodge infected cells, pour medium plus cells into a rotor cup (Beckman, JA-14, 250 mL),
9. In case cells are still retained in the flask, add 2.0 mL VT and incubate briefly at 37 °C.
10. Vigorously suspend the trypsinized cells with the medium transferred in **step 8** to the rotor cup.
11. Centrifuge 2 h at 26–30,000 × *g* and 4 °C, remove supernatant carefully without disturbing pellet.
12. Add 1 mL PBS and leave the rotor cup at 4 °C overnight in a sloped position to cover and to resolve the pellet completely.
13. Transfer the crude virus preparation into appropriate vials on ice.

14. Freeze-thaw three times (−70 °C, 37 °C).
15. Immerse the sterile bar of an ultrasonic device into the upper part of the virus suspension on ice, sonicate with 8–10 pulses (100 W, 20 s each) with a 5–10 s break between each pulse.
16. Now immerse the bar into the lower part of the virus suspension and again pulse four times.
17. Centrifuge 5 min at 4 °C and 500–700 × *g* to remove cell debris.
18. Save supernatant on ice.
19. Suspend the pellet in 1.0 mL PBS and transfer it to an Eppendorf cup.
20. Sonicate (100 W) again in an ultrasonic bowl, on ice 20 s twice (10 s break) and once for 30 s.
21. Centrifuge at 2000 × *g* for 10 min at 4 °C and combine this supernatant with supernatant from **step 17**.
22. Determine the virus titer, which should be higher than 10^8 pfu/mL. Store in aliquots at −70 °C.

3.8 ORFV Plaque Titration

The classical virus plaque titration assay is applied to determine the titer of D1701-V derived recombinant virus preparations. Vero cells are seeded the day before use and prepared as described in Subheading 3.1 (*see* **Note 24**).

1. Prepare on ice 1.0 mL tenfold virus dilutions ranging from 1:10^2 to 1: 10^8 in medium,
2. Pipette 0.1 mL of each dilution into wells A–F of rows 1–7 (sextuple) of a 48-well plate.
3. Wells 8A–8F represent the non-infected controls and are filled with cells only.
4. Pipette 0.5 mL cell suspension containing 5×10^4 cells in MEM plus 5 % FCS into each well.
5. Mix with the virus dilution by careful manual agitation, and incubate at 37 °C, 5 % CO_2.
6. After 3–4 days plaques can be counted and the mean plaque-forming unit (pfu) titers are calculated.
7. For additional virus plaque staining remove the medium, carefully wash once with PBS without destroying the cell monolayer, and proceed as described in Subheading 3.9.

3.9 Immunostaining of Infected Cells and Virus Plaques

The availability of appropriate specific antibodies allows the proof of successful transgene expression by performing immunohistochemical staining of infected cells (shown in Fig. 2). In the following, immune peroxidase monolayer assays (IPMA) are described for antigen detection in fixed or non-fixed, live cells.

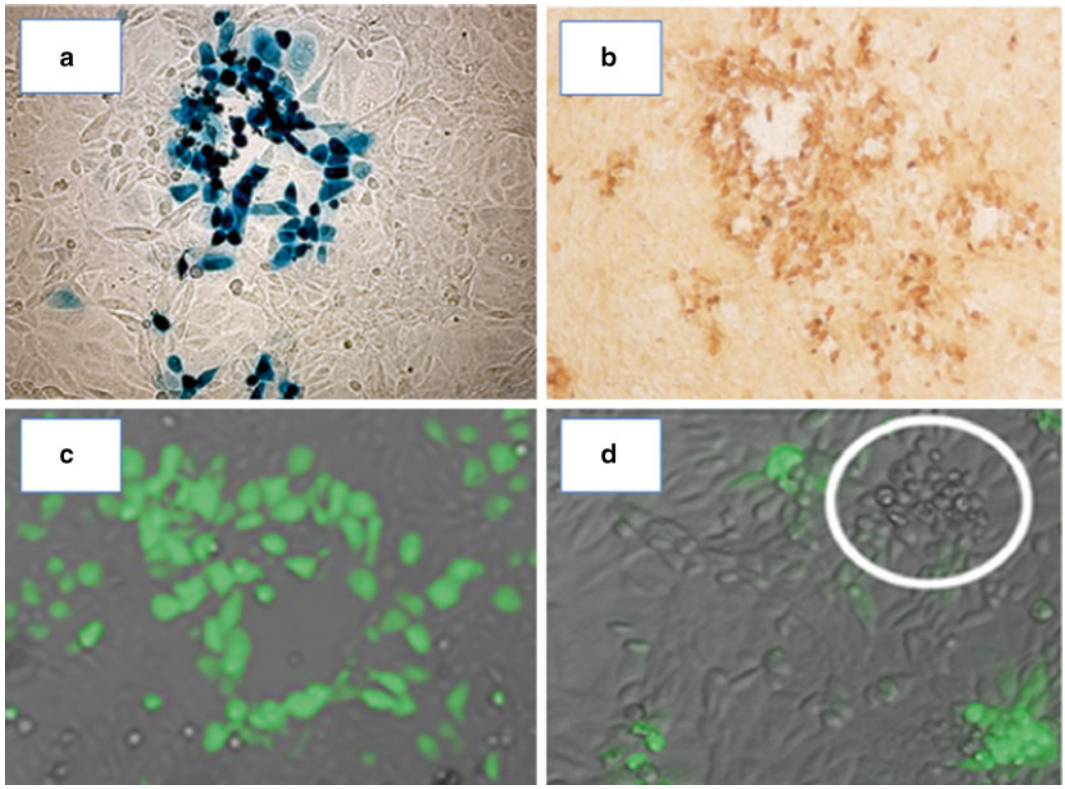

Fig. 2 Identification of recombinant ORFV plaques. (**a**) LacZ gene expression from D1701-VrV is detected by X-Gal staining using agarose-overlay. (**b**) RabG gene expressing recombinant virus plaque detected by specific IPMA, as described in Amann 2013 [24]. (**c**) Exchange of the lacZ gene for the AcGFP gene leading to green fluorescent recombinant virus plaques of D1701-V-AcGFP as described in Subheading 3.3. (**d**) Nonfluorescent, white recombinant virus (*circled*) was obtained by substitution of the AcGFP gene (Microscopic magnification: 40-fold)

3.9.1 IPMA Plaque Assay

This assay allows the discrimination of new foreign gene expressing recombinant ORFV plaques or foci from plaques of parental, transgene-negative ORFV (Fig. 2b).

1. Vero cells are prepared as described in Subheadings 3.1–3.3.
2. Virus lysate dilutions are prepared on ice.
3. For each well of a 24-well plate 0.1 mL virus dilution is mixed with 0.5 mL MEM plus 5 % FCS containing 1×10^5 cells (*see* **Note 25**).
4. One well contains non-infected and parental virus-infected cells as negative controls.
5. The plate is incubated for 2–4 days at 37 °C and 5 % CO_2 until distinct cpe or virus plaques have been formed.
6. Aspirate medium carefully not to damage the intact cell monolayer.

7. Let dry cells by placing the uncovered plate in the laminar flow hood for ca. 10 min, which helps to maintain intact monolayer.
8. Add slowly 0.5 mL ice-cold 100 % methanol (prechilled at –20 °C) to each well.
9. Fix the cells for 15 min at –20 °C.
10. Aspirate methanol, wash twice with ice-cold PBS containing either 1 % (w/v) BSA or 1 % (v/v) FCS.
11. Blocking unspecific antibody binding sites is achieved with 0.5 mL PBS plus 10 % FCS per well for 60–90 min at room temperature or overnight in the refrigerator.
12. Remove block solution and without washing add 0.2 mL per well of first, antigen-specific antibody diluted in TBST-BSA/FCS (*see* **Note 26**).
13. Incubate for 1 h at room temperature under constant slow shaking.
14. Wash three times for 5 min with 0.5 mL TBST-BSA/FCS at room temperature for instance on a rocking platform.
15. Incubate for another 30–60 min at room temperature with 0.2 mL appropriate peroxidase-labeled second antibody, diluted for example 1:2000 in TBST (*see* also **Note 26**).
16. Wash the wells three times 5 min with 0.5 mL TBST and once with PBS at room temperature.
17. Add substrate (e.g., DAB or DAB-black) prepared as recommended by the manufacturer until distinct brown or black color had developed.
18. Stop reaction by rinsing with tap water (which helps to intensify brownish staining) before counting stained plaques or foci.
19. The tightly closed plate can be stored at 4 °C and also used for further histological staining.

3.9.2 X-Gal Staining

Instead of using a X-gal-agarose-overlay (Subheading 3.2), β-galactosidase can be also monitored in fixed, infected cells as follows. Instead of the BetaBlue Staining kit (Novagen-Merck) other comparable kits should be also suitable for similar quick, direct visualization of β-galactosidase expression.

1. Vero cells are infected in 24-well plates and incubated until cpe and/or plaque formation is achieved as described above (Subheading 3.9.1).
2. Aspirate culture medium from cells.
3. Wash the cells twice with PBS plus NP-40 (*see* **Note 27**).
4. The cells are fixed by the addition of 0.5 mL methanol-free FALD for 15 min at room temperature.

5. Remove the fixative and wash cells four times with PBS.
6. Gently add 0.5 mL of the BetaBlue staining solution and incubate at 37 °C (*see* **Note 28**).
7. Staining is finished after 1–3 h and stopped by washing with PBS.
8. For storage, the stained cell layer can be covered with 15 % (v/v) glycerol in PBS.

3.9.3 Combined Detection of β-Galactosidase and Antigen

ORFV D1701-VrV expressing β-galactosidase can be discriminated from new transgene-expressing recombinants by the simultaneous staining for β-galactosidase and the foreign antigen.

1. Remove supernatant from cells infected in a 24-well plate as described above (Subheading 3.9.1).
2. Wash at room temperature three times 5 min with PBS (0.5 mL).
3. Dry the cell monolayer by leaving the open plate for ca. 10 min in laminar flow hood.
4. Cells are fixed at 4 °C (in the refrigerator) after adding 0.5 mL cold methanol-free FALD.
5. Gently add 0.5 mL of the BetaBlue staining solution and incubate for 1 h at 37 °C (*see* **Note 28**).
6. Wash three times 5 min at room temperature with 0.5 mL TBST containing 1 % FCS.
7. Incubation with first and peroxidase-labeled secondary antibody as well as substrate reaction is performed exactly as described from **steps 12–19** in Subheading 3.9.1.

3.9.4 Plaque Staining of Unfixed Infected Cells

The following procedure can be used to detect foreign gene expression in non-fixed, viable cells forming virus plaques, which can be subsequently isolated. This method works very well for recombinant proteins expressed on the infected cell surface; staining of internally expressed proteins has not yet been tested.

1. Vero cells, $3–5 \times 10^5$ cells in 3 mL MEM plus 5 % FCS are mixed with 0.1 mL virus lysates (dilution series from 10^{-2} to 10^{-6}).
2. Seeded simultaneously in wells of a 6-well plate and incubated until plaque formation is clearly visible.
3. After removal of medium, cells are washed carefully twice with sterile PBS.
4. Incubated with 1 mL first antibody (specific for the foreign gene product), diluted in MEM plus 2 % FCS for 2 h by gentle rocking.

5. After removal of antibody solution, the cells are washed once with PBS (2 mL).
6. Incubated for 1 h with 1 mL secondary peroxidase-labeled antibody diluted in MEM.
7. Cells are washed once with PBS (2 mL).
8. Incubation can be performed with for example the peroxidase substrate "VECTOR VIP" substrate kit (Vector) until purple plaque staining becomes visible during the next 1–3 h.
9. Wash twice with 2 mL PBS.
10. Cover the wells with LMT-Agarose overlay, which is prepared as described in Subheading 3.2, **steps 6–9**.
11. After cooling down the overlay to 37 °C, slowly pour 1.5 mL per well.
12. Allow the agarose-overlay to harden in the refrigerator.
13. Now the antigen-positive, purple virus plaques can be picked with a sterile Pasteur pipette and used for virus isolation exactly as described (Subheading 3.2, **steps 15–22**).

4 Notes

1. Vero cells should not be overgrown or completely confluent for splitting, because that might cause sticking together and clumping of cells.
2. Incubate cells not too long in VT (versene-trypsin solution) at 37 °C. Check after approximately 3 min; too long trypsin treatment or too high trypsin concentration can lead to damaged, clumped or stuck cells. VT pre-warmed at 37 °C leads to reproducible times for cell displacing. Forcing cells to detach by harsh, too extensive tapping or pipetting can cause permanent cell damage. Before counting or seeding, carefully check that the cells are evenly suspended.
3. LMT, low melting point agarose has the advantage to become completely molten at 65 °C and to remain fluid even between 37 and 30 °C. Weigh the needed amount of agarose (e.g., 2 g) into water (100 mL), and after boiling take care to refill the evaporated amount of water.
4. Agarose-BluoGal-overlay: We found BluoGal to result in fast and strong blue staining; however, other X-Gal substrates work equally.

   The pH of the agarose overlay should be between 7.0 and 7.4.

   Cool the agarose overlay solution down to ca. 30 °C, pour it very slowly from the edge of the wells, in order to prevent cell damage that can be erroneously noted as plaques or

cpe; be aware that the cell layer does not dry out after removal of the culture medium.

After pouring the overlay, allow the agarose to harden briefly at room temperature before incubating at 37 °C.

5. For cell counting preparation of more dilutions might improve calculation of cell density. The calculation depends on the type of counting chamber used.

   Neubauer improved chamber:

   Number of cells/4 quadrants × 2× dilution × 10^4 = cells/mL.

   Fuchs-Rosenthal chamber:

   Number of cells/4 quadrants × 2× dilution × 5 × 10^3 = cells/mL.

6. The optimal multiplicity of infection (moi) might be pretested, in our hands moi of 0.2–0.01 works well. If it is not possible to place a rotator in the incubator, the virus–cell suspension can be slowly shaken by hands several times during incubation. Two hours of incubation are chosen to become ORFV DNA replication started and increasing the possibility of recombination with transfer plasmid DNA in the infected cells.
7. Caution, the cell pellet is very unstable.
8. We found 2 μg plasmid DNA optimal. Pilot tests can be performed by monitoring the number of fluorescent or X-gal stained cells 24 and 48 h after nucleofection.

   We recommend performing not more than two nucleofections simultaneously, because incubation of cells plus DNA in transfection buffer should not exceed 15 min to avoid cell death. Prepare in advance the needed amount of cuvette, Eppendorf cups, pipettes, and cut filter tips.
9. Nucleofection lysates dilutions: That dilution must be found resulting in a reasonable number of separated virus plaques to allow single virus plaque picking. Because the virus titers in the different NL can vary, additional dilutions might be needed.
10. In case that no cpe or clear plaque formation becomes visible, nevertheless harvest the cells by trypsinization, freeze-thaw and sonicate, and use a 1:5 or 1:10 dilution for simultaneous infection of Vero cells in a 24- or 48-well plate. Due to very low amount of virus in the plaque eluate, such a "blind passage" can increase the virus titer.
11. After this point, at least additional three up to five more rounds of virus plaque purification are necessary to obtain genetically homogeneous recombinant viruses.
12. Genetic variants of the green fluorescent protein (GFP) are available with improved photostability, fluorescence strength, or spectral characteristics. The AcGFP gene encodes a GFP

consisting of human codons to enhance translation and expression in mammalian cells (Clontech, BD).

13. The described dilution series can be changed or adapted. The reason for preparing 32 wells of identical dilutions is to enhance the chance of identifying a sufficient number of wells with single green plaques. Similar endpoint dilution series can be of course also performed for example in 96-well plates.
14. Twenty-fold microscopic magnification is recommended to allow clear visualization of beginning plaque formation in the bright field and identification of green fluorescent cells and/or plaques by UV fluorescence.
15. For reasonable handling, maximally 24 wells are recommended to harvest.
16. Instead of transferring each supernatant into fresh cups after each extraction, the removal of the lower phenol-CIA and CIA phase by withdrawing it with a pipette from the bottom of the cup and using it for the next extraction step, represents a good alternative. Finally, the supernatant of last CIA extraction is transferred to a fresh cup.

    Glycogen-blue can be added to facilitate recovery of low amounts of DNA. Alternatively, yeast tRNA (10 μg) could be also used.
17. To prevent loss of the tiny DNA precipitate, for instance by swapping out during ethanol removal, we recommend the following procedure:

    Immediately after centrifugation slowly turn the closed cup upside down, which separates the DNA pellet from the ethanol solution. After placing it in that position onto a clean filter (e.g., Kleenex) paper, slowly open the lid, let the ethanol drain off by putting the cup with the opened lid upside down on the filter paper.

    Depending on the amount of remaining traces of ethanol, drying can take from several to 10 min. Take care that the ethanol is completely evaporated, which can be recognized by the DNA pellet becoming translucent.
18. Harvesting the infected cells at the appearance of approximately 80 % cpe should result in the best yield of ORFV DNA.
19. Complete suspension of the cell pellets is important, because unsolved cell clumps will drastically reduce the DNA recovery.
20. To cut the filter tips for DNA solving avoids mechanical shearing of high mol. wt. poxviral DNA.
21. Other available ready-to-use double-concentrated Taq polymerase-based PCR mixes could work equally.

22. Only a relatively crude, partially purified virus preparation is obtained. However, we found no detectable differences in the strength and quality of the protective immune response as compared to sucrose-gradient purified preparation (unpublished data). Nevertheless, for more routine use as vaccine more purified virus preparations are advised.
23. We found that that simultaneous infection with moi 0.5 and harvesting at the appearance of ca. 80 % cpe resulted in highest final virus titers.
24. One T75 flask containing 80–90 % confluent Vero cell should be sufficient for three 48-well plates.
25. Virus dilutions should be used that lead to maximally 10–20 plaques per each well. Of course this procedure can be adapted to 12- or 6-well plate.
26. Optimal dilution of first and second antibodies has to be determined; the peroxidase-labeled secondary antibody must be directed against that species used for production of the first antibody.
27. For higher sensitivity, we use PBS containing 0.02 % (v/v) NP-40; however, PBS without detergent can work properly, too.
28. Be careful by using a tissue culture incubator as the CO_2 may alter the pH and can lead to unacceptable background staining.

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Chapter 13

Polycistronic Herpesvirus Amplicon Vectors for Veterinary Vaccine Development

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Abstract

Heterologous virus-vectored vaccines, particularly those based on canarypox virus vectors, have established a firm place in preventive veterinary medicine. However, herpesvirus-based vaccines have paved the way for DIVA vaccines (discrimination of infected against vaccinated animals), which are particularly desirable for highly contagious livestock diseases that are otherwise combatted by culling of infected animals.

In this chapter, we describe the design, the preparation, and the testing of a polycistronic herpesvirus amplicon vaccine against rotaviruses with a particular emphasis on generating heterologous virus-like particles for immunization. After the design, the procedure consists of three steps, first, transient expression of the construct in cell cultures, second, expression and antibody response in a mouse model, and third, application of the system to the desired host species. As a whole, the present information will facilitate the design of novel vaccines of veterinary interest from the designing process until pre-licensing.

Key words Polycistronic herpesvirus amplicons, Virus-like particles, Vaccine

1 Introduction

Herpesvirus vaccines for animals have played pioneering roles in the past, particularly in terms of developing DIVA vaccines (discrimination of infected against vaccinated animals), which have revolutionized the possibilities in campaigns for eradicating certain viruses from life stock animals [1–4]. Accordingly, herpesviruses may be used for developing different types of vaccines, i.e., inactivated [5], modified live [6, 7], and amplicon-vector-based monocistronic [8] or polycistronic [9]. The selection largely depends on what type of immunity is to be achieved in the target animal species. The development of new vaccines for veterinary medicine has become technically much easier in recent years but much more demanding in terms of quality management, including safety, potency, efficacy, and batch-to-batch reproducibility [10]. Therefore, it pays off to include the principles of future quality management issues at early times in vaccine development.

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Helpervirus-free herpesvirus-vectored polycistronic amplicon vaccines offer the following three main advantages:

- Safety, due to exclusion of replication competent virus, combined with the potential to generate a definable balance between cellular and humoral immunity.
- Almost unlimited space in the vector (up to 150 kbp), allowing for a selection of desirable antigen combinations as well as for dealing with structural issues of vaccine delivery, i.e., particle formation [11, 12].
- Application of synthetic DNA, encoding the antigens of interest in a codon-optimized manner to suit best the purpose in the targeted animal species [13, 14]. This approach also diminishes the need to adapt the targeted viruses to growing at high titers in cell cultures.

The present chapter describes how these issues have been addressed in the context of a rotavirus vaccine, a group of viruses whose wild types may grow poorly in cell cultures and for which in situ particle formation is important, considering the desired type of immunity.

2 Materials

2.1 Transient Expression of Amplicon Vectors in Cell Cultures

2.1.1 Cell Culture

1. Vero 2-2 cells, a derivative of Vero cells that stably express HSV-1 ICP27 [15].
2. Phosphate buffered saline (PBS).
3. Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % of penicillin/streptomycin (P/S).
4. DMEM supplemented with 10 % FBS, 1 % P/S, and 500 μg/ml G418 (Geneticin).
5. 0.05 % trypsin–EDTA (1×).
6. Hemocytometer.

2.1.2 Western Analysis

1. DMEM supplemented with 2 and 10 % FBS and 1 % P/S.
2. 24-well tissue culture plate.
3. PBS.
4. Protein loading buffer (1x PLB): Dissolve 0.6 g tris(hydroxymethyl)aminomethane and 1.6 g sodium dodecyl sulfate (SDS) in 20 ml dH_2O, add 4 ml β-mercaptoethanol, 8 ml glycerin, and 0.4 ml bromophenol blue.

2.1.3 Immune Fluorescence

1. DMEM supplemented with 2 and 10 % FBS and 1 % P/S.
2. 24-well tissue culture plate.
3. 12 mm (diameter) glass coverslips.

2.1.4 Kinetics of Antigen Production

1. DMEM supplemented with 2 and 10 % FBS and 1 % P/S.
2. White or black 96-well tissue microplates (*see* **Note 1**).

2.1.5 Luciferase Assay

1. NanoGlo luciferase assay components (NanoGlo luciferase assay buffer and NanoGlo luciferase assay substrate).
2. Microplate luminometer.

2.1.6 Harvesting of Virus Like Particles

1. DMEM supplemented with 2 and 10 % FBS and 1 % P/S.
2. 6 cm diameter tissue culture dishes.
3. Cell scraper.
4. PBS.
5. Liquid nitrogen.
6. 37 °C water bath.
7. 0.22 μm syringe membrane tip filter.
8. 20 ml disposable syringe.
9. 10 % sucrose in PBS.
10. PBS-PI: PBS with protease inhibitor (protease inhibitor cocktail tablets complete, EDTA free), sterile filtered.
11. Beckman Ultra-Clear 14×95 mm centrifuge tubes.
12. Ultraspeed centrifuge with an SW40 rotor (or equivalent).

2.2 Analysis of RVLPs by Transmission Electron Microscopy

1. DMEM supplemented with 2 % and 10 % FBS.
2. Cell culture 6-well plates.

2.2.1 Infection of Cells

2.2.2 Preparation of Total Cell Pellet

1. Cell scraper.
2. Micro Tubes, 0.5 ml and 50 mm in length.
3. Glass vials, 20 ml.
4. Razor blade.
5. 2.5 % glutaraldehyde (GA) (*see* **Note 2**) diluted in 0.1 M phosphate buffer from 25 % stock solution (purchased, store at –20 °C), store at 4 °C.
6. 2 % osmium tetroxide (OsO_4) aqueous solution (purchased, store at 4 °C). Working solution: 1 % in 0.1 M phosphate buffer, store at 4 °C in aliquots (sealed glass vials).
7. Phosphate buffer, 0.1 M Na/K-phosphate buffer, pH 7.4: Solution A: Dissolve 13.6 g of KH_2PO_4 (MW 163.09) in 1000 ml dH_2O. Solution B: Dissolve 14.2 g of Na_2HPO_4 (MW 141.96) in 1000 ml dH_2O. For pH 7.4: Mix 19.7 ml of

solution A with 80.3 ml of solution B (100 ml end volume). For other pH, *see* Table 1.

8. Ethanol for analysis: 70, 80, 96, 100 % (absolute).
9. Acetone for analysis.
10. Acetone–Epon 1:1 mix, make fresh, avoid any air bubbles or smears. Remove frozen epon 30 min before use.
11. Preparation of epon stocks: Add 122 g of Epon 812 (Epoxy embedding resin, Fluka 45345), 80 g of DDSA (Hardener, Fluka 45346), and 54 g of MNA (Hardener, Fluka 45347) into a glass cylinder wrapped in aluminum foil to protect from light and mix on magnetic stirrer for 60 min at room temperature. Be careful not to make any air bubbles. Add 3.84 ml of DMP30 (Accelerator, Fluka 45348) and mix on magnetic stirrer for 60 min, protect from light. No air bubbles and no smears should be visible! Fill the prepared epon into 20 ml syringes, close them with Parafilm and store at −20 °C wrapped in aluminum foil.
12. Easy molds, polyethylene.
13. Small paper labels with printed block numbers.
14. Wooden sticks.
15. Desiccator.
16. Oven for polymerization, set to 60 °C.

2.2.3 Staining of Ultrathin Sections

1. Iso-butanol saturated water [16], 9 % iso-butanol in water (V/V).
2. Uranyl acetate (UA) 6 % in iso-butanol saturated water, dissolve 1.5 g of Mg-UA in 25 ml of iso-butanol saturated water. Filter through a 0.22 μm single use filter. Store as aliquots in small tubes at 4 °C protected from light.
3. Lead staining solution [17], dissolve 0.67 g lead citrate in 7 ml dH_2O and 1.0 g sodium citrate in 7 ml dH_2O and combine the two solutions. Let the mixture stand for 20 min while gently rocking 2–3 times. Add 4 ml of a freshly prepared 1 N NaOH solution and mix well. The solution should become clear, then add 25 ml dH_2O. The lead citrate is mixed 3:2 with iso-butanol saturated water. Before use, sterile filter the solution through a Minisart RC4 single use syringe filter. Store as aliquots at 4 °C.

Table 1

Preparation of 0.1 M Na/K phosphate buffer. Add Solution B to final volume of 100 ml according to the table

pH	5.0	5.2	5.4	5.6	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8	8.0
A	99.2	98.4	97.3	95.5	92.8	88.9	83.0	75.4	65.3	53.4	41.3	29.6	19.7	12.8	7.4	3.7

4. 75/300 mesh/inch copper grids.
5. 300 ml beakers, filled with Millipore (0.22 μm filtered) dH_2O.
6. Transmission electron microscope equipped with a camera.

2.3 Immune Response in Laboratory Animals

1. Mouse restrainer.
2. 23–25 G injection needle.
3. Microcaps capillary tube with a bulb dispenser.

2.3.1 Collecting and Preparing Serum Samples

2.3.2 Collecting and Preparing Fecal Samples

1. TNC buffer: 10 mM tris(hydroxymethyl)aminomethane (use stock solution with pH 7.4), 100 mM NaCl, and 5 mM $CaCl_2$.
2. TNC-T: TNC buffer with 0.05 % Tween 20 and protease inhibitor (protease inhibitor cocktail tablets complete, EDTA free).

2.3.3 Collecting and Preparing Milk Samples

1. Heating pad.
2. Isoflurane and isoflurane vaporizer (with an O_2 gas bottle).
3. Vitamin A eye ointment.
4. Oxytocin (10 IU/ml).
5. Syringe with an appropriate injection needle for subcutaneous injection.
6. Microcaps capillary tube with a bulb dispenser.

2.3.4 Antigen ELISA

1. ELISA 96-microwell plate.
2. Coating buffer (carbonate–bicarbonate buffer): 0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6.
3. Capture antibody targeting the antigen.
4. Humidified chamber.
5. PBS-T: PBS containing 0.05 % Tween 20.
6. Dilution buffer: PBS containing 0.05 % Tween 20 and 1 % casein.
7. Biotin labeled detection antibody targeting the antigen.
8. Streptavidin-HRP.
9. Peroxidase (HRP) substrate.
10. Stop solution: 2 M H_2SO_4.
11. ELISA microplate reader.

2.3.5 IgG ELISA

1. ELISA 96-microwell plate.
2. Coating buffer (carbonate–bicarbonate buffer): 0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6.

3. Concentrated or purified antigen.
4. Humidified chamber.
5. PBS-T: PBS containing 0.05 % Tween 20.
6. Dilution buffer: PBS containing 0.05 % Tween 20 and 1 % casein.
7. HRP conjugated anti-IgG detection antibody.
8. Peroxidase (HRP) substrate.
9. Stop solution: 2 M H_2SO_4.
10. ELISA microplate reader.

2.3.6 IgA ELISA

1. ELISA 96-microwell plate.
2. Coating buffer (carbonate–bicarbonate buffer): 0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6.
3. Anti-IgA capture antibody.
4. Humidified chamber.
5. PBS-T: PBS containing 0.05 % Tween 20.
6. Dilution buffer: PBS containing 0.05 % Tween 20 and 1 % casein.
7. Concentrated or purified antigen (e.g., virus suspension).
8. Biotin conjugated antibody targeting the antigen.
9. Streptavidin-HRP.
10. Peroxidase (HRP) substrate.
11. Stop solution: 2 M H_2SO_4.
12. ELISA microplate reader.

3 Methods

3.1 Design and Optimization of Polycistronic Amplicon Vectors

Interestingly, viral codon usage does not necessarily reflect the codon usage of the virus's primary host [18, 19]. Therefore, the level of translation due to expression of a native viral nucleotide sequence may significantly vary not only with the specific host cell used for transient expression but also with the context of expression, i.e., in the presence or absence of other viral products. We and others have experienced that the level of translated protein may be dramatically enhanced by adapting the codon usage in its genetic template. Accordingly, the first question that has to be considered is about the animal species in which the immunizing protein should be synthesized and about the context, i.e., which other viral proteins are to be expressed simultaneously, what amount of protein will be desirable, for example in view of particle formation, which will be more preferable, producing a stable protein or one to be soon degraded by the proteasome, etc.

These considerations will affect the selection of promoters to be used, the codon usage in synthetic genes, the sequence order of individual open reading frames, and the addition or omission of signal sequences, nuclear translocation signals or sequences to facilitate proteasomal degradation.

Codon optimization. At least the following issues should be considered: (1) Frequency of optimal or least optimal codons for translation of a protein in a mammal (as opposed to translation in the context of its native virus, in insect cells, or in prokaryotes). (2) Average G + C percent (optimum is usually around 50 %; desirably between 40 and 60 %; remove G + C peaks from 60 bp windows). (3) Removal of undesired *cis*-acting elements, i.e., cryptic splice donor sites or internal transcription termination sites; repeat elements; polymerase slippage. (4) Factors affecting mRNA stability and translation, i.e., stem-loop structures, internal ribosome entry sites, RNA-instability motifs, etc. (5) Restriction enzyme sites that may interfere with cloning steps. For example, free codon analysis is provided by GenScript's OptimumGene™ software (www.genscript.com/codon_opt.html).

Selection of promoter. An overview of relative promoter strength in various cell types has recently been published [20]. As a rule of thumb, the more protein is synthesized during immunization, the better immune response is achieved. Therefore, strong promoters, active in a variety of cell types are generally preferred, for example the simian virus 40 early promoter (SV40). The cytomegalovirus immediate-early promoter (CMV) may also provide strong gene expression but this is very much cell type-dependent. On the other side of the scale, the human Ubiquitin C promoter (UBC) may be selected if relatively low gene expression is desired. Internal ribosomal entry sites (IRES) provide translation from polycistronic mRNAs but the translation efficiency of the more 3′ located open reading frame is usually attenuated.

Subcellular localization. Particle formation for immunizing purposes may present complex problems because different strategies are being used by different viruses and this relevant strategy has to be kept in mind for the purpose of particle formation. For example, nuclear import of the influenza A virus protein M1 is first required in order to facilitate binding of the nuclear export protein (NS2, NEP) to ribonucleoproteins (N), which then allows the export of this complex together with viral RNA to the cytoplasm to initiate particle formation at the external cellular membranes [21]. Particle assembly in the rotavirus system is much less well understood, a fact that is underscored by our own attempts to generate triple-layered rotavirus particles, while double-layered particles were formed just as a consequence of co-expression of the two relevant components. Targeting newly synthesized proteins to the proteasome may be instrumental for generating a strong cellular immune response, whereas the same strategy may be

compromising the generation of desirable antibodies that may form only after particle assembly. Therefore, one may consider expressing the same protein from the same polycistronic vector in two forms, one with, the other without a proteinaceous destabilizing domain (DD) [22]. Typically, addition of a DD will result in rapid proteasomal degradation of a protein of interest.

3.2 Preparation of HSV-1 BAC DNA and HSV-1 Amplicon Vector Stocks

HSV-1 BAC DNA is a bacterial artificial chromosome (BAC) encoding the entire HSV-1 genome but with deleted packaging signal (*pac*) and deleted ICP27, which is an essential gene in HSV-1 replication [23, 24]. Deletion of the packaging signal inhibits that BAC DNA is packaged and the further deletion of ICP27 essentially eliminates the risk of contamination with replication competent helper virus. To enable that the BAC DNA encoded genes provide their helper functions, ICP27 is provided in *trans* on a separate plasmid [24].

Preparation of HSV-1 BAC DNA as well as the generation of HSV-1 amplicon vector stocks are described in detail in Chapter 7 within the book "Herpes Simplex Virus: Methods and Protocols, Methods in Molecular Biology" [9].

3.3 Transient Expression of Amplicon Vectors in Cell Cultures

3.3.1 Selection of the Appropriate Cell Line

Transduction of different cell types with the same HSV-1 amplicon vector stocks might result in different experimental outcomes. Therefore, it is certainly elusive to compare the amount of synthetized proteins and/ or the intracellular localization between different cell lines. We recommend selecting the cell lines in regard to your final experimental target (target species). In addition, it is certainly useful to test a cell line known to be well transducable with HSV-1 amplicon vectors (e.g., Vero 2-2, HepG2, HEK-293).

3.3.2 Maintenance of Cells and Preparation of Cells for Transduction

Here, detailed instructions for maintenance of Vero 2-2 cells and their preparation for transduction are described. Vero 2-2 cells are derived from Vero cells, thus epithelial African green monkey kidney cells but with stably expressing ICP27 [15]. Necessary adjustments for some other cell lines can be found in **Note 3**.

Vero 2-2 cells are maintained in DMEM (with 10 % FBS and G418) and propagated by splitting 1/5 twice a week (e.g., Mo and Thu) or 1/10 once a week.

1. For splitting wash the confluent Vero 2-2 monolayer in the 75 cm^2 tissue culture flask with 5 ml PBS.
2. Add 2 ml trypsin–EDTA and incubate at 37 °C for about 10 min. After incubation check if all cells are detached using a microscope (*see* **Note 4**).
3. Add 8 ml DMEM (with 10 % FBS) and bring the cells in suspension by pipetting up and down for several times, thereby rinsing the tissue flask bottom.

4. For a 1/5 split, transfer 2 ml of the cell suspension into a 75 cm^2 tissue flask. For a 1/10 split, transfer 1 ml. The remaining cell suspension might be used for experiments.
5. Add 10 ml DMEM (with 10 % FBS and G418) and let the cells grow in a humid incubator at 37 °C and 5 % CO_2.
6. About 24 h before transduction, cells have to be trypsinized and transferred into the appropriate dishes. For this purpose, the cell concentration in the cell suspension (obtained during splitting) is calculated using a hemocytometer.
7. Dilute the cell suspension with DMEM (with 10 % FBS) to the concentration of 1×10^5 cells per 0.75 ml.
8. Transfer 0.75 ml of the diluted cell suspension into each well of a 24-well tissue culture plate and incubate the cells in a humid incubator at 37 °C and 5 % CO_2 for 24 h.

3.3.3 Characterization of Transgene Protein Synthesis by Western Analysis

Western analysis of whole cell lysates represents a simple method to evaluate, if the desired proteins are synthetized in the targeted cell line as well as to roughly compare the quantity of protein synthesis between different cell lines. For a more precise analysis of the protein expression level, methods, which are more accurate in quantitative gene expression analysis (e.g., qRT PCR), might be used.

Concentrated virus like particles (VLPs) (*see* Subheading 3.3.6) can as well be examined using Western analysis and compared to the results of whole cell lysates as some transient expressed proteins might not incorporate into the particles and remain in the cell pellet during VLP purification.

1. Culture 1×10^5 cells per well in a 24-well tissue culture plate in a total volume of 0.5 ml DMEM (with 10 % FBS) and incubate for 24 h in a humid incubator at 37 °C and 5 % CO_2.
2. Dilute the HSV-1 amplicon vector stocks in a total volume of 0.25 ml DMEM (with 2 % FBS) per well to a multiplicity of infection of 2 (MOI = 2) (*see* **Note 5**).
3. Aspirate the medium from the cultured cells and add 0.25 ml of the diluted HSV-1 amplicon solution. Incubate for 1 h. Remove the amplicon solution and add 0.5 ml of DMEM (with 2 % FBS) and incubate for 24 h in a humid incubator at 37 °C and 5 % CO_2. Incubation time might be adjusted.
4. Scrape the cells into media using pipette tips (cut the tips, where necessary). Transfer the cell suspension of each well into separate 1.5 ml tubes. Wash each well with 0.2 ml PBS and transfer the PBS wash into the corresponding 1.5 ml tube. Check if all cells are harvested using a light microscope. If not, add again 0.2 ml PBS, scrape again and transfer the remaining cells into the 1.5 ml tube and centrifuge at maximum speed in

a tabletop centrifuge for 5 min. Discard the supernatant and dissolve the pellet in 25 µl 1× PLB. Boil the sample for 10 min.

5. Now the sample is ready for Western analysis using standard protocols.

3.3.4 Characterization of Transgene Protein Synthesis by Immune Fluorescence

1. Culture $0.8–1 \times 10^5$ cells per well on 12 mm coverslips in a 24-well tissue culture plate in a total volume of 750 µl DMEM (with 10 % FBS) and incubate for 24 h in a humid incubator at 37 °C and 5 % CO_2.
2. Dilute the HSV-1 amplicon vector stocks in a total volume of 0.25 ml DMEM (with 2 % FBS) per well to a multiplicity of infection of 1 (MOI = 1) (*see* **Note 5**). According to your experimental setup, the MOI might be adjusted.
3. Aspirate the medium from the cultured cells and add 0.25 ml of the diluted HSV-1 amplicon solution to each well. Incubate for 1 h. Remove the amplicon solution and add 0.5 ml of DMEM (with 2 % FBS) and incubate for 24 h in a humid incubator at 37 °C and 5 % CO_2.
4. The samples are now ready for immune fluorescence staining using standard protocols.

3.3.5 Kinetics of Antigen Production

Time course analysis of protein synthesis can be approached via tagging the protein of interest with a luciferase and measuring the intensity of the luciferase signal, correlating with the quantity of your protein of interest. We have chosen NanoLuciferase (NLuc) (*see* **Note 6**) as a C terminal protein tag because with its 19 kDa it is much smaller than other luciferase tags and the luminescent signal is longer lasting as well as more intense compared to other luciferase reactions [25]. Before adding a tag to the protein of interest, it always has to be considered that the tag might interfere with protein function. Here, the procedure for a time course analysis in cell culture is described in detail.

Transduction of Cells with HSV-1 Amplicon Vectors

1. Culture 25,000 Vero 2-2 cells per well in a total volume of 50 µl DMEM (with 10 % FBS) in a white or black 96-well tissue culture plate and incubate the plate in a humid incubator for 24 h at 37° and 5 % CO_2 (*see* **Note 1**).
2. Remove the media and transduce the cells with the desired MOI in a total volume of 40 µl. Incubate for 1 h in a humid incubator with 37 °C and 5 % CO_2 and replace the amplicons with 40 µl of DMEM (2 % FBS). Incubate the transduced cells in a humid incubator at 37 °C and 5 % CO_2 for the desired period of time. For a time course experiment you might incubate the transduced cells on different tissue culture plates for 8, 12, 24, 48, and 72 h post transduction.

Luciferase Assay

Quantitative signal measurement should be done with a luminometer. Alternatively the plate might be placed into an IVIS reader as a preliminary trial to in vivo experiments (*see* Fig. 1).

1. Let the sample tissue culture plate, the NLuc substrate and NLuc buffer equilibrate to room temperature.
2. Meanwhile start the luminometer and set the parameters (total measuring time, period between each measurement, time of substrate injection and volume of the substrate). Set the volume of the injected substrate to 40 µl (*see* **Note 7**).
3. Premix 1 part of NLuc substrate with 50 parts of NLuc buffer. Connect the luminometer injection tube to the mixed substrate and do not forget to wash the tubing and to prime before initiating the measurements (*see* **Note 7**).
4. Place the tissue culture plate into the luminometer and start measuring.

Data Analysis

For data evaluation the open source statistical computing program "R", which can be downloaded for free on http://www.r-project.org/, was used. The R script, which was used for the generation of the shown diagram (Fig. 2), can be found on http://www.vetvir.uzh.ch/aboutus/publikationen/supplements/rscript.html.

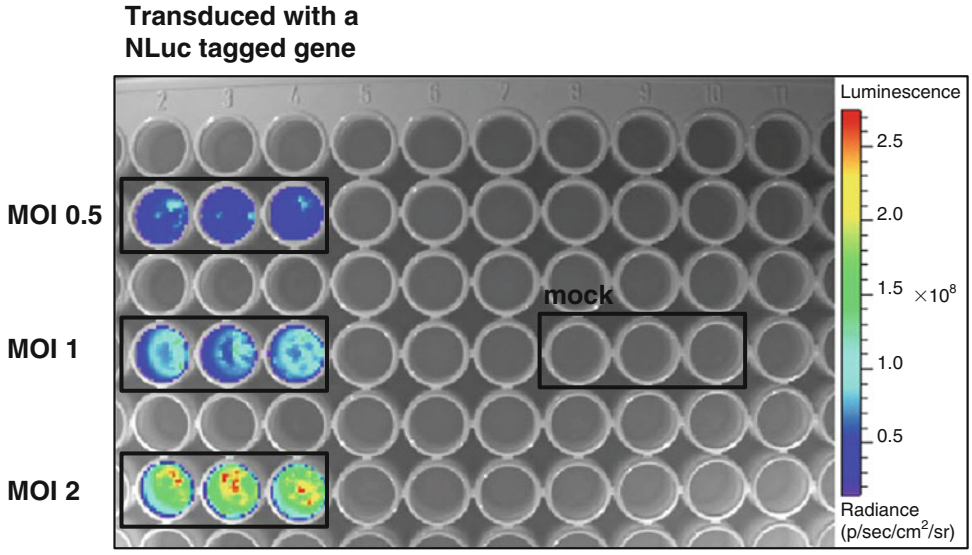

Fig. 1 Visualization of NanoLuc activity in transduced cells using an IVIS reader. Vero 2-2 cells were transduced in triplicates with HSV-1 amplicon vectors coding for the rotavirus proteins VP2, VP6, and a NLuc tagged VP7 at the indicated MOI. Substrate was added 24 hpt and light emission was recorded in radiance 30 min after substrate addition using an in vivo imaging system (IVIS Xenogen imaging system 100, living image software). As control, substrate was also added to non-transduced Vero 2-2 cells (mock). The colors indicate the measured luminescence signal ranging from low (*blue*) to high (*red*) number of radiance

Added comments should help understanding various steps of the diagram generation. The used data for the shown diagram can be downloaded on the same homepage.

3.3.6 Harvesting of HSV-1 Vector-Encoded Virus Like Particles (VLPs)

To examine eventual formation of virus like particles (VLPs), cells are transduced with HSV-1 amplicon vector stocks followed by harvesting and concentration steps of the total cell lysate.

1. 1.2×10^6 Vero 2-2 cells are grown in each 6 cm diameter tissue culture dish in a total volume of 3 ml DMEM (with 10 % FBS). Cells are cultured 24 h before transduction. It is recommended to use at least two 6 cm diameter tissue culture dishes for each HSV-1 amplicon construct. Otherwise only few particles might be harvested, which possibly might not be detected by Western analysis or electron microscopy.

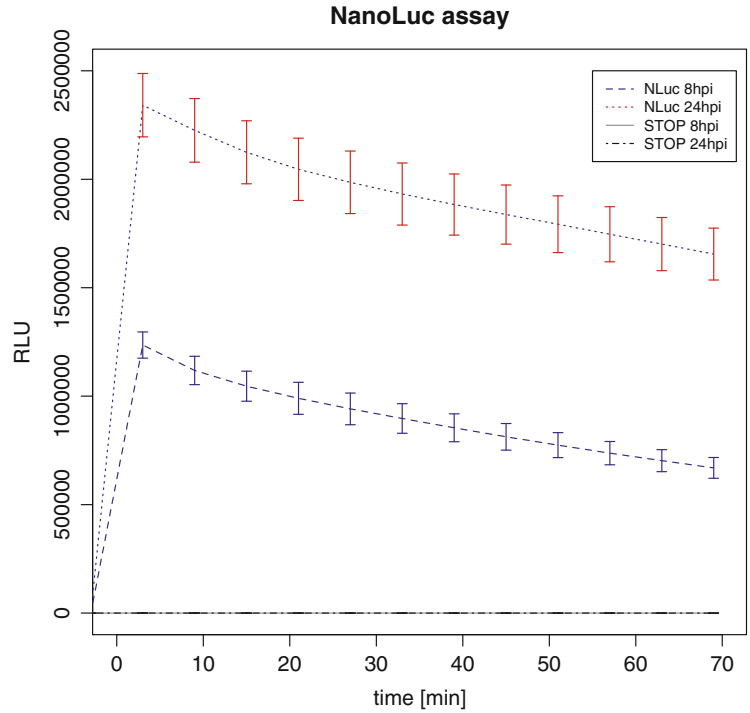

Fig. 2 NanoLuc activity over time. Vero 2-2 cells were transduced with HSV-1 amplicon vectors (MOI = 1) coding for the rotavirus proteins VP2, VP6, and either an untagged VP7 ("STOP") or a NLuc tagged VP7 ("NLuc"). Substrate was added after 8 or 24 hpt. Light emission was measured in relative light units (RLU) starting from 9 min before until 70 min after substrate addition using the MicroLumat Plus luminometer. Each measurement was done in triplicates. Data was analyzed and plotted using the open source statistical computing program R. Note that the measurements for the used negative controls ("STOP") were near to zero and appear as two overlapping straight lines at RLU = 0 in the diagram

2. HSV-1 amplicon vector stocks are diluted in 1.5 ml of DMEM (with 2 % FBS) to an MOI of 2 TU/cell (MOI=2) (*see* **Note 5**).
3. Aspirate the growth medium from the cells and add the diluted vector stocks to the cell culture plates. Incubate for 1–2 h in a humid incubator with 37 °C and 5 % CO_2 and subsequently replace the inoculum with 2 ml of DMEM (with 2 % FBS) and incubate the cells in a humid incubator with 37 °C and 5 % CO_2 for 2 days.
4. Scrape the cells into the media using a cell scraper. Transfer the suspension to a conical centrifuge tube. During each step, the tubes containing the cell lysates are kept on ice.
5. Wash each plate with 1 ml of PBS and transfer the resulting solution into the corresponding tube containing the cell lysate from **step 4**.
6. Perform three freeze/thaw cycles using liquid nitrogen and a 37 °C water bath.
7. Remove the cell debris by centrifugation for 10 min at 2600 × *g* and 4 °C (*see* **Note 8**).
8. Filter the supernatant through a 0.22 μm syringe tip filter into a new conical centrifuge tube. The pore size of the filter might be adjusted if the virus diameter exceeds 0.22 μm.
9. Prepare Beckman Ultra-Clear 14 × 95 mm centrifuge tubes by adding 5 ml of 10 % sucrose (in PBS) to each tube. Overlay the sucrose cushion with 1 ml PBS-PI.
10. Carefully transfer the filtrate from **step 8** on top of the prepared cushion (**step 9**). Equilibrate the tubes to 0.001 g with PBS.
11. Centrifuge for 2 h at 100,000 × *g* and 16 °C using a Beckman SW40 rotor.
12. Carefully aspirate the supernatant using a Pasteur pipette and resuspend the pellet in a small volume (e.g., 20 μl per plate) of PBS-PI. Seal the tubes with Parafilm and let the pellet redissolve overnight at 4 °C.
13. Transfer the suspended VLPs into an appropriate collection tube and store at 4 °C. The samples are now ready to be analyzed by negative stain electron microscopy and by immune electron microscopy. In order to examine the protein composition of the harvested VLPs, Western analysis might be performed and compared to Western analysis of the whole cell lysates of transduced cells and to the cell debris pellet from **step 7** (*see* **Note 8**).

3.4 Analysis of RVLPs by Transmission Electron Microscopy

This subchapter describes the ultrastructural examination of the HSV-1 amplicon vector encoded structural proteins assembled into virus-like particles in the transduced cells. The total cell pellet is fixed 24 h post transduction, embedded in epon and ultrathin

sections (Fig. 3) are prepared for transmission electron microscopy (TEM). The described protocol is based on standard protocols widely used in the field of electron microscopy. The cell pellet is chemically fixed with glutaraldehyde, which preserves the ultrastructure well. Postfixation is done by osmium tetroxide, which preserves and stains the phospholipids to some extent (of the cell membrane). Before embedding in epoxy resin (epon), the cell pellets are dehydrated through series of solvents. Finally, ultrathin sections are stained with uranyl-acetate and lead citrate.

3.4.1 Infection of Cells with HSV-1 Amplicon Vectors

1. Seed cells at a density of 4×10^5 cells (confluent cell monolayer, *see* **Note 9**) in 6-well tissue culture plates in 2 ml of DMEM (with 10 % FBS). Incubate overnight at 37 °C and 5 % CO_2.
2. Dilute the vector stocks in 1 ml of DMEM (with 2 % FBS) for a MOI of 5 TU per cell.
3. Aspirate the growth medium and add vector dilutions to the cells. Incubate for 1–2 h, then remove the inoculum. Add 2 ml of DMEM (with 2 % FBS) and incubate for 24 h at 37 °C and 5 % CO_2.

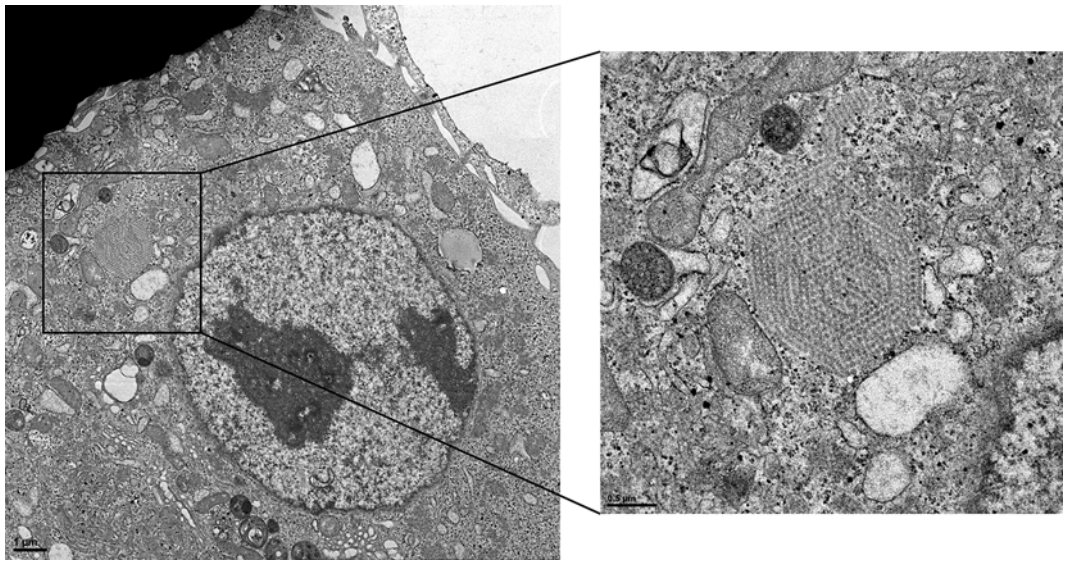

Fig. 3 Electron micrographs of ultrathin sections of amplicon vector-transduced cells. HepG2 cells were transduced (MOI = 5) with a HSV-1 amplicon vector which delivers a DNA cassette encoding a single polycistronic mRNA containing the coding sequences for the three rotavirus capsid proteins VP2, VP6, and VP7. The cells were harvested 24 hpt from monolayers by pelleting and fixation with glutaraldehyde and osmium tetroxide and embedded in Epon. Ultrathin sections are stained with uranyl-acetate and lead citrate and analyzed in a transmission electron microscope equipped with a CCD camera. Rotavirus-like particles (RVLPs) assembled in viroplasm-like structures within the cytoplasm of the transduced cells

3.4.2 Preparation of Total Cell Pellet: Fixation, Dehydration and Embedding

1. Scrape cells into the medium using a cell scraper. Transfer the suspension to a 15-ml conical centrifuge tube.
2. Centrifuge for 5 min at 300 × *g* to pellet the cells, remove supernatant.
3. Fixation: resuspend cell pellet in 2.5 % GA in phosphate buffer, transfer to 0.5 ml Micro Tube and centrifuge for 20 min at 3400 × *g*. Cut the tip of the tube using a razor blade, carefully rinse the cell pellet out of the tube with phosphate buffer into 20 ml glass vials and keep the cell pellet in phosphate buffer at 4 °C overnight. The pellets can be stored in phosphate buffer for weeks.
4. Post-fix the cell pellet with 1 % OsO_4 in phosphate buffer for 1 h at 4 °C.
5. Remove the 1 % OsO_4 solution (discard appropriately!; **Note 2**).
6. Dehydration of the cell pellet is done with ascending ethanol series starting at 70 %, followed by 80 %, 96 % for 10 min each; followed by three times 10 min in absolute ethanol. After this, ethanol is exchanged with acetone (solvent for epon) in two steps each lasting for 15 min. Be careful that the pellet never "dries" while changing the solvent solution.
7. Epon infiltration: add a 1:1 mix of acetone and epon to the pellet, incubate overnight in the hood at room temperature, keep tubes open to allow evaporation of residual acetone.
8. Embedding in epon: transfer the pellet carefully into Easy Molds using a wooden stick. Include in each mold a label describing its content. Fill each mold with epon and place the molds in a desiccator for 6 h at room temperature.
9. Polymerization: incubate for 2.5 days at 60 °C in an oven. Once polymerized, let the molds cool down, extract epon blocks using a capsule press and store them at room temperature.
10. Sectioning (*see* for example: Principles and Techniques of Electron Microscopy, Hayat [26]): ultrathin sections are cut (60–80 nm sections) using an ultramicrotome, the sections are collected on grids (**Note 10**) for examination.

3.4.3 Staining Ultrathin Sections with Uranyl-Acetate and Lead Citrate

These steps should is performed with grids containing ultrathin sections from **step 10 (**subheading 3.4.2).

1. Put drops (10–20 µl) of uranyl-acetate (UA) solution on a strip of Parafilm mounted on a smooth surface. Transfer the grids with the sections facing down onto the drop of UA. Cover with dark lid and incubate for 15 min.
2. Wash the grids by dipping them ten times into three 300 ml beaker containing dH_2O. It is important to dip the grids vertically

through the surface of the water. Let them dry under cover on Whatman filter paper, sections facing up.

3. Put drops of lead citrate solution (filtered 0.22 μm) on Parafilm and transfer grids onto the drops (section side down). Incubate for 15 min.
4. Wash the grids like in **step 2** by dipping them into three beakers containing dH_2O. Let the grids dry on Whatman filter paper.
5. Store the grids in appropriate storage box and let them dry fully.
6. Carbon-coating (4 nm) of the sections using a carbon coater (*see* **Note 11**).
7. The specimen can now be examined by electron microscopy.

3.5 Immune Response in Laboratory Animals

3.5.1 Challenge Infection

To evaluate if the developed vaccine against a particular virus is efficient, in vivo experiments are usually performed assessing the protection from infection or disease symptoms. Therefore, a challenge experiment is necessary. In order to simulate the natural infection, the route of experimental infection should be chosen carefully. For example, to simulate a rotavirus infection, virus challenge via oral gavage is appropriate because natural infection occurs via the oral route.

3.5.2 Collecting and Preparing Samples

Serum Samples

In experiments where daily observation of antibody titer is needed, only very small amounts of blood samples (5–20 μl) can be taken to avoid hypovolemia. Multiple blood collection can be performed via tail vein punctuation. Keep in mind that the collected blood volumes have to be adjusted to the animal weight as well as to the period of time of blood sample collection.

1. The mouse is either fixed in a restrainer or might be placed onto the wire cage without fixation. The tail vein is pricked using an injection needle (e.g., 23–25 G) near to the tip of the tail.
2. Blood is collected using a 5, 10, or 20 μl microcaps capillary tube. The blood will enter by capillary force. Blood is transferred into a collection tube using the microcaps bulb dispenser. For serum collection non-coated collection tubes are used.
3. The blood sample is incubated for 1 h at room temperature to allow blood clotting.
4. Centrifuge the sample for 2 min at maximum speed in a tabletop centrifuge. The serum (supernatant) is now ready for further analysis such as ELISA. The serum can also be stored at −20 °C until analysis. Prevent multiple thawing and freezing. In order to prolong the stability of the serum compounds such as antibodies, mix it with glycerol in a 1:1 ratio.

Fecal Samples

Fecal samples are collected and analyzed (e.g., Antigen ELISA) as rotavirus replicates in cells of the small intestine and is subsequently excreted and shed via feces. Thus, rotavirus can be detected in the feces of an infected animal.

1. Fresh feces from the individual animals are collected. Samples might be frozen immediately and stored at –20 °C.
2. Dissolve the fecal samples as a 10 % w/v dilution in TNC-T. Centrifuge the dissolved sample at 2500 × *g* for 10 min. The supernatant is now ready for further analysis (e.g., antigen ELISA) or can be stored at –20 °C.

Milk Samples

In our vaccination approach, the vaccinated mice are expected to generate an immune response, including the production of antibodies of the IgA type, which may be transmitted to the pups via milk, particularly with colostrum. To assess if these antibodies are detectable and quantifiable in the milk, milk samples are collected from the vaccinated dams to be assessed by ELISA for their specific antibody concentration, including discrimination into IgG and IgA types. This part describes a simple method to collect milk samples from lactating mice.

1. Separate the dam from their pups for at least 2 h. During this time the pups should be placed on a heating pad (38 °C) or remain with their foster mother.
2. Induce anesthesia of the dam with 5 % isoflurane and 600–800 ml/min O_2 flow using an induction box until a stable breathing rate of 40–50 breaths/min is reached. Switch the isoflurane gas supply from the induction box to the gas tubing. Apply a drop of vitamin A eye ointment onto each eye to inhibit drying out. Place the dam onto a heating pad (38 °C). Maintain the anesthesia with 2–3 % isoflurane and 600–800 ml/min O_2. Now the gas is provided via the gas tubing directly to the nose of the animal. Always monitor the respiration of the animal and take care that the breathing rate stays steady at 50–60 breaths/min.
3. Inject 1 IU oxytocin (100 µl of 10 IU/ml) subcutaneous (s.c.).
4. Milk flow is initiated by stimulation of the nipples. Use your thumb and the index finger to gently grasp the skin around the nipple. Pull the nipple with the surrounding skin gently away from the dam's body. Repeat this movement several times until the milk flow is initiated. If milk flow cannot be initiated, another s.c. injection of oxytocin (1 IU, 100 µl of 10 IU/ ml) might be necessary. Collect the milk drops using a capillary tube and transfer the milk into an appropriate tube. Keep on milking until no more milk is ejected. Go on to the next nipple and proceed as described. You might milk each nipple several times. Milk can be stored at –20 °C and used for IgA ELISA (*see* **Note 12**).

5. After milking, remove the dam from the isoflurane flow and let the animal wake up completely before putting back into the cage.

3.5.3 Sample Analysis Using ELISA

Immunoglobulins as well as antigen (e.g., viral proteins) can be determined using ELISA assays. Depending on the location and the time-point of sampling, different antibodies are expected. During a primary antibody response, antibody generation follows a typical course: First, IgM is produced followed by IgA or IgG [27]. IgA is usually secreted and thus found in mucosal surfaces as well as in milk. IgA is found at a lower concentration in the blood and during a shorter period of time compared to IgG [27]. The described ELISA protocols (obtained from Laura E. Esteban, Laboratorio de Inmunología y Virología, Universidad Nacional de Quilmes, Argentina) were modified from ref. [28].

Antigen ELISA

Here, a capture ELISA assay for antigen detection is described in detail. This ELISA protocol was used to determine viral shedding in mouse feces. Preparation of the feces is described in detail in Subheading 3.5.2.

1. Appropriately dilute the capture antibody in coating buffer and pipette 0.1 ml into each well of an ELISA 96-microwell plate. Capture antibodies are usually plated at 0.2–10 μg/ml. Anti-rotavirus antibody was diluted 1:1000. Incubate the plate in a humidified chamber for 1 h at room temperature. Remove the solution and wash the wells three times with PBS-T.
2. Appropriately dilute the samples and controls in dilution buffer. Dissolved mouse fecal samples (*see* Subheading 3.5.2) are generally tested as 1:80 dilutions. As control samples, antigen (e.g., concentrated virus) is diluted ($1:10^1$–$1:10^4$). Add 0.1 ml into each well and incubate the plate in a humidified chamber at 37 °C for 1 h. Remove the solution and wash the wells three times with PBS-T.
3. Dilute the biotin conjugated detection antibody in dilution buffer. The optimal dilution should be determined using a titration assay. Biotin labeled anti RV antibody (in glycerol, 50 %) was diluted 1:1000. Add 0.1 ml into each well and incubate the plate in a humidified chamber at 37 °C for 1 h. Remove the solution and wash the wells three times with PBS-T.
4. Dilute streptavidin-HRP in dilution buffer. The optimal dilution should be determined using a titration assay. In our setup streptavidin-HRP was diluted 1:2000. Add 0.1 ml into each well and incubate the plate in a humidified chamber at 37 °C for 30 min. Remove the solution and wash the wells three times with PBS-T.

5. Add 0.1 ml of the freshly prepared substrate to each well. Allow the color to develop for 15–30 min and measure the absorption at $\lambda = 650$ nm in a microplate reader before stopping the reaction. Do not allow the signal to exceed the optical density (O.D.) of 0.6. Otherwise the substrate will form precipitates after addition of the stop solution. Stop the reaction by adding 0.1 ml stop solution to each well. Allow the reaction to develop for 10 min and measure the plate at $\lambda = 450$ nm (*see* **Note 13**).

IgG ELISA

Mouse serum was examined for antigen (e.g., virus) specific immunoglobulin G (IgG) with the following protocol for an indirect ELISA assay.

1. Appropriately dilute the antigen solution in coating buffer. Concentrated virus stocks (concentrated over a sucrose cushion) were diluted 1:100 and CsCl gradient purified virus 1:10. Add 0.1 ml of the dilution to each well of an ELISA 96-microwell plate. Incubate the plate in a humidified chamber for 1 h at room temperature or over night at 4 °C. Remove the solution and wash the wells three times with PBS-T.
2. Appropriately dilute the samples and controls in dilution buffer. Mouse serum samples were diluted 1:100 or serial dilutions were made. Apply 0.1 ml of the diluted samples into each well and incubate for 1 h at 37 °C in a humidified chamber. Remove the solution and wash the wells three times with PBS-T.
3. Dilute the HRP conjugated detection antibody in dilution buffer to an appropriate concentration. The optimal dilution should be determined using a titration assay. Peroxidase conjugated goat anti-mouse IgG (in glycerol, 50 %) was diluted 1:4000. Apply 0.1 ml of the diluted detection antibody and incubate for 1 h at 37 °C in a humidified chamber. Remove the solution and wash the wells three times with PBS-T.
4. Add 0.1 ml of the freshly prepared substrate to each well. Allow the color to develop for 15–30 min and measure the absorption at $\lambda = 650$ nm in a microplate reader before stopping the reaction. Do not allow the signal to exceed the optical density (O.D.) of 0.6. Otherwise the substrate will form precipitates after addition of the stop solution. Stop the reaction by adding 0.1 ml stop solution. Allow the reaction to develop for 10 min and measure the plate at $\lambda = 450$ nm (*see* **Note 13**).

IgA ELISA

Antigen (e.g., virus) specific IgA was determined in the mouse milk as well as in mouse serum by the described capture ELISA assay. For milk collection *see* Subheading 3.5.2.

1. Appropriately dilute the capture antibody in coating buffer and pipette 0.1 ml into each well of an ELISA 96-microwell plate. Capture antibodies are usually plated at 0.2–10 μg/ml. Goat anti-mouse IgA, α chain specific is used 1:50. Incubate the plate in a humidified chamber for 1 h at room temperature. Remove the solution and wash the wells three times with PBS-T.
2. Add 0.1 ml of the appropriately diluted samples and controls. Dissolved mouse fecal samples (*see* Subheading 3.5.2) are usually tested 1:5 in dilution buffer or serial dilutions are made. Mouse serum samples are usually tested 1:20 in dilution buffer or serial dilutions are made. For milk samples serial dilutions are made. Incubate the plate in a humidified chamber for 1 h at room temperature. Remove the solution and wash the wells three times with PBS-T.
3. Prepare the antigen solution in dilution buffer. Concentrated virus stocks were diluted 1:100 and purified virus 1:10. Add 0.1 ml of the dilution to each well. Incubate in a humidified chamber for 1 h at room temperature or over night at 4 °C. Remove the solution and wash the wells three times with PBS-T.
4. Dilute the biotin-conjugated antibody in dilution buffer to an appropriate concentration. The optimal dilution should be determined using a titration assay. Biotin labeled goat anti-RV (in glycerol, 50 %) was used 1:1000. Apply 0.1 ml of the diluted detection antibody and incubate for 1 h at 37 °C in a humidified chamber. Remove the solution and wash the wells three times with PBS-T.
5. Dilute streptavidin-HRP in dilution buffer. The optimal dilution should be determined using a titration assay. In our setup streptavidin-HRP is diluted 1:2000. Add 0.1 ml into each well and incubate the plate in a humidified chamber at 37 °C for 30 min. Remove the solution and wash the wells three times with PBS-T.
6. Add 0.1 ml of the freshly prepared substrate to each well. Allow the blue color to develop 15–30 min and measure the absorption at $\lambda = 650$ nm in a microplate reader before stopping the reaction. Do not allow the signal to exceed the optical density (O.D.) of 0.6. Otherwise the substrate will form precipitates after addition of the stop solution. Stop the reaction by adding 0.1 ml stop solution. Allow the reaction to develop for 10 min and measure the plate at $\lambda = 450$ nm (*see* **Note 13**).

3.6 Characterization of the Antibody Response in the Targeted Species

In general, the same approaches are to be used as in the model system. In the case of challenge infections, it may be necessary to use a different virus strain that is virulent for the targeted animal species. Accordingly, the protective level as well as the antibody response may vary from the expectations raised in the model

system. In the case of indirect ELISA systems, one will have to adjust for the species-specific conjugate, whereas this will rarely be necessary in the case of competition ELISAs. It is of considerable value to plan for comparable assays in view of licensing and batch characterization of the future vaccine. Finally, one should keep in mind that challenge infections may provide an excellent opportunity to test the DIVA character of the newly developed vaccine candidate.

4 Notes

1. The white tissue culture plate is necessary to reduce measurement of crosstalk. In addition, the light reflection in the white plate leads to a maximal signal-to-noise ratio. Black plates are excellent when high luminescence values are expected. Using black plates, the crosstalk is extremely low but the signal-to-noise ratio is lower compared to the usage of white plates. Usage of transparent tissue culture plates is not recommended as high crosstalk does occur [29].
2. Caution: many reagents are highly toxic! Wear protective equipment (lab coat, gloves) and work in a ventilated hood, discard waste appropriately. Fixatives like glutaraldehyde (GA) and osmium tetroxide (OsO_4) fix cells by cross-linking their proteins via the amine groups (GA) or their phospholipids (OsO_4). They are harmful to living cells and you should avoid exposure to them. Osmium tetroxide: OsO_4 is highly reactive, evaporates easily, and is extremely toxic. It can fix any tissue it contacts, always use in fume hood! All objects that come in contact with it have to be discarded appropriately, never use metal tools, only plastic or wood.

   Uranyl acetate: UA is radioactive, to be used only in dedicated laboratory.

   Epon: avoid skin contact prior to polymerization because it can cause cancer! The resins used to embed the tissue are more dangerous than the fixatives and many of the components have been shown to cause cancer in rats or mice. During the embedding process the resins are dissolved in solvent that can easily carry the resin into your tissues and through any plastic gloves you may wear. In contrast to fixatives, whose actions are immediate and apparent, the consequences of exposure to the resins are not apparent for years. Please be careful with the resins prior to polymerization into hard blocks.
3. Generally, all cell work is done in a laminar flow and cells are grown in a humid incubator with 5 % CO_2 at 37 °C if not indicated otherwise.

   HEK-293: These human embryonic kidney cells can be easily maintained in culture in DMEM (with 10 % FBS, 1 % P/S)

by splitting them 1/5 twice a week. In order to obtain a confluent cell monolayer in a 6 cm diameter tissue culture dish, 2×10^6 cells are cultured 24 h before use. HEK-293 cells are very susceptible to transfection. They also showed a high amount of protein synthesis upon transduction.

HeLa: This human epithelial cervix cancer cell line is easily maintained in culture in DMEM (with 10 % FBS, 1 % P/S) by splitting 1/5 twice a week. For a confluent monolayer in a 6 cm diameter tissue culture dish, culture 1.2×10^6 cells 24 h before use. HeLa cells are known to be hardly transducable with HSV-1 amplicon vectors, which is consistent with our observations.

MDBK: The Madin-Darby bovine kidney cells are maintained in DMEM (with 10 % FBS, 1 % P/S) by splitting 1/5 once to twice a week. 1/10 splitting is not recommended. Alternatively to DMEM, you might take EMEM (with 7 % FBS) and keep the cells in a CO_2 free humid incubator at 37 °C. For a confluent monolayer in a well of a 24-tissue culture well plate, 1.25×10^5 cells are cultured 24 h before use. MDBK demonstrated to be a cell line with low amount of protein synthesis upon transduction with HSV-1 amplicon vectors.

HepG2: This human hepatocyte cell line is an excellent expression cell line and is well transducable. HepG2 do not grow as fast as several other cell lines. They are maintained in DMEM (with 10 % FBS, 1 % P/S) and propagated by 1/3 splitting twice a week. As these cells seem to be less robust than other cell lines, trypsin should be removed from the culture media during splitting. In order to get rid of trypsin, the cell suspension should be centrifuged for 5 min at $300 \times g$ and the supernatant should be discarded and replaced by fresh media before passaging and further use of the cells.

MA-104: These African green monkey kidney cells can be maintained easily in DMEM (with 10 % FBS, 1 % P/S) and propagated by splitting 1/5 twice a week or 1/10 once a week. For a confluent monolayer in a 24-well tissue culture plate, culture 2.5×10^4 cells per well 24 h before use.

4. If some cells are not detached after 10 min of incubation, support the procedure by knocking on the flask wall with your palm. If still some cells remain attached, incubate them for additional 2 min on 37 °C. Check again if all cells are detached. It might be that after several passages some cells will hardly be detached. In this case continue with splitting and passage them into a fresh flask. Knocking on the flask might result in cell clumps, which can be dispersed by pipetting the cell suspension up and down.
5. If the titer of the HSV-1 amplicon stock is too low to reach the desired MOI in the total transduction volume, transduction

can be repeated. Therefore, add one volume of the vector stock to the cells and incubate for 1 h. Remove the HSV-1 amplicon solution and repeat the transduction step until the desired MOI is reached.

6. NanoLuciferase plasmid DNA as well as the substrate was supplied by Promega.
7. Note that some luminometers do not have an automated injector. Priming is needed to fill the luminometer tubing with the substrate. Otherwise, remaining washing solutions (e.g., water or ethanol) will be injected into the wells.
8. The obtained cell debris pellet from **step 4** (in Subheading 3.3.6) can be examined using Western analysis. This is of particular interest if not all of the expected proteins were incorporated into the observed VLPs. The missing protein might remain in the cell debris pellet as some viral proteins can act as transmembrane proteins and might therefore be pelleted with the corresponding cell organelle.
9. Depending on the cell line used, the cell pellet should not be too big (1–2 mm in size) because of incomplete dehydration and therefore incomplete resin infiltration. For a starting point, use 1×10^6 cells.
10. Grids are manufactured of various metals, e.g., copper, nickel, or gold, and are available in different designs including square mesh, hexagonal mesh and parallel bars. Copper is the most common choice for grids and may be used with or without support film. We use 75/300 mesh copper grids without support film (*see* **Note 11**).
11. Once the sections are stained, they are covered with a thin layer of carbon using an evaporation machine. Carbon-coating stabilizes the ultrathin epon-sections in the electron beam.
12. Keep in mind that milking of the dam can interfere with normal growth of the pups.
13. As ELISA HRP substrates do vary, the measured wavelengths as well as the stop solution might vary. We used the TMB substrate kit from Thermo Scientific.

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Chapter 14

Construction and Application of Newcastle Disease Virus-Based Vector Vaccines

Paul J. Wichgers Schreur

Abstract

Paramyxoviruses are able to stably express a wide-variety of heterologous antigens at relatively high levels in various species and are consequently considered as potent gene delivery vehicles. A single vaccination is frequently sufficient for the induction of robust humoral and cellular immune responses. Here we provide detailed methods for the construction and application of Newcastle disease virus (NDV)-based vector vaccines. The in silico design and in vitro rescue as well as the in vivo evaluation of NDV based vectors are described in this chapter.

Key words Newcastle disease virus, Vector, LaSota, Lentogenic, Paramyxovirus, Reverse genetics, Vaccine

1 Introduction

Paramyxoviruses are enveloped viruses which contain a single stranded RNA genome of negative polarity that encodes 6–10 genes. Due to a feature known as transcriptional polarity, genes closest to the 3′ end of the genome are transcribed in greater abundance than those towards the 5′ end. Remarkably, the total length of the paramyxovirus genome is almost always a multiple of six. This characteristic, which is referred to as the "rule of six", is explained by the association of each nucleocapsid monomer (NP) with exactly six nucleotides [1]. The polymerase (L) protein, together with the phosphoprotein (P), is responsible for the transcription and replication of the genomic RNA in the cytoplasm of the host cell. The matrix protein (M) subsequently assembles between the envelope and the nucleocapsid core and facilitates the formation of virus particles. Entry of the virions into new cells is mediated by the surface spikes which consist of the fusion (F) protein and a receptor binding

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protein, which is named G (glycoprotein), H (hemagglutinin), or HN (hemagglutinin-neuraminidase).

Paramyxoviruses are able to infect a broad variety of species and cell types and express foreign genes with remarkable stability. Well-known paramyxovirus vectors include the vesicular stomatitis virus (VSV), measles virus (MV) and Newcastle disease virus (NDV). Apathogenic vaccine strains that can easily be manipulated using robust reverse genetics systems are available for each of these viruses. Here, we describe detailed methods for the use of the NDV vector. We will particularly focus on the construction, rescue, characterization and application of a cDNA clone of the lentogenic LaSota vaccine strain [2].

The described method can be divided into five sections. In section 1, a universal method for the construction of LaSota-based full-length cDNA clones encoding any gene of interest (GOI), including glycoproteins of viral origin, is provided. The method combines standard PCR and cloning procedures with state-of-the-art gene synthesis. In section 2, procedures for the rescue of the cDNA clones is described. Briefly, a quail cell line (QM5) is infected with a T7 RNA polymerase-expressing fowlpox virus (FP-T7) and transfected with a transcription plasmid encoding the full-length DNA copy of the NDV genome and expression plasmids encoding the NDV helper proteins NP, P, and L. Sections 3 and 4 focus on the amplification of rescued virus in embryonated chicken eggs and on the evaluation of the expression of the protein of interest. Finally, in section 5, some guidelines are given for the use of NDV-based vector vaccines in mammals.

2 Materials

Prepare and store all reagents at room temperature (unless indicated otherwise). Virus rescue should be performed in a biosafety level-2 laboratory and waste should be disposed according to general (local) regulations. Animal experiments should also comply with national guidelines and rules.

2.1 Components for Construction of a Full-Length cDNA Clone Encoding a GOI

1. DNA cloning and sequence analysis software (e.g., Clone Manager, DNAStar).
2. Parent NDV (lentogenic isolate, e.g., LaSota strain) infectious clone (pNDV) cDNA sequence.
3. GOI (c)DNA sequence.
4. DNAse and RNAse free ddH_2O.
5. pNDV in ddH_2O. Store at −20 °C (*see* **Note 1**).
6. Plasmid encoding GOI (pGOI) in ddH_2O. Store at −20 °C.
7. Plasmid miniprep and midiprep kits.
8. Restriction enzymes and buffers. Store at −20 °C.

9. PCR reaction mix containing DNA polymerase with proof-reading activity. Store at –20 °C.
10. Agarose gel chromatography reagents and equipment.
11. LB agar and medium. Store at 4–12 °C.
12. Kanamycin × 1000 stock (15 mg/ml). Store at –20 °C.
13. Sanger or next generation sequencing (NGS) reagents and equipment.
14. DNA quality and quantity analysis equipment (e.g., NanoDrop).

2.2 Components for Rescue of NDV from cDNA

1. QM5 cell line [3, 4] (*see* **Note 2**).
2. Complete QT35 medium: QT35 medium (Invitrogen) supplemented with 5 % fetal bovine serum (FBS), 1 % penicillin and streptomycin or alternatively: M199 medium supplemented with 10 % tryptose phosphate broth, 10 % fetal calf serum (FCS), 1 % penicillin and streptomycin. Store at 4 °C.
3. Trypsin–EDTA. Store at 4 °C.
4. CO_2 incubator at 5 % CO_2 and 37 °C.
5. Laminar flow-cabinet.
6. Cell culture flasks: 75 or 150 cm^2.
7. Cell culture plates: 6- and 96-wells.
8. Cell counter (Bürker-Türk or automatic).
9. Fowlpox-T7 (FP-T7) virus stock [5] (originally obtained from Dr. Geoff Oldham, Institute for Animal health, 508 Compton, UK).
10. Opti-MEM transfection medium.
11. Transfection reagents (jetPEI, Polyplus).
12. NDV expression plasmids: pCIneo-NP, pCIneo-P, pCIneo-L. Originally obtained from Olav de Leeuw, Central Veterinary Institute, part of Wageningen UR, 8221 RA Lelystad, The Netherlands (*see* **Note 3**).
13. Full-length cDNA clone (pNDV-GOI) in ddH_2O as constructed under Subheading 3.1.
14. Allantoic fluid.

2.3 Components for Amplification of Virus

1. Egg incubator at 37 °C and 35–40 % humidity.
2. 8–10 day-old embryonated chicken eggs.
3. Egg candler.
4. 1 ml syringe with needle, preferably 25 G, 16 mm.
5. Spatula, scissors and forceps.
6. Adhesive tape.
7. Sterile collection tubes.

2.4 Components for Evaluation of Expression of Protein of Interest

1. Cell culture plates: 96 wells.
2. Paraformaldehyde: 4 % in PBS. Store at 4 °C.
3. Methanol: 100 %. Store at −20 °C.
4. Blocking buffer: 5 % horse serum in PBS or 5 % bovine serum albumin (BSA) in PBS.
5. Primary monoclonal or polyclonal antibody recognizing the protein of interest.
6. PBS-T: PBS, 0.05 % v/v Tween 20.
7. HRP-conjugated secondary antibody recognizing the primary antibody.
8. Peroxidase substrate: 0.05 % 3-amino-9-ethylcarbazole (AEC) (stock; 1 mg/ml in dimethyl sulfoxide), 0.015 % H_2O_2 in 0.05 M acetate buffer, pH 5.5. For 20 ml substrate; (always make fresh) add 1 ml AEC stock to 19 ml acetate buffer, mix and add 100 μl 3 % H_2O_2 stock.
9. Standard light microscope.

2.5 Evaluation of Immune Responses Against Protein of Interest in Mammals

1. Animals, preferably target animals.
2. Inoculum: allantoic fluid diluted in complete culture medium (*see* **Note 4**).
3. Syringe suitable for intramuscular inoculation of target animals.
4. EDTA and serum blood tubes.
5. Viral RNA/DNA isolation kit.
6. Quantitative real-time (qRT)PCR reagents and equipment.
7. Virus neutralization test reagents.

3 Methods

3.1 Construction of Full-Length cDNA Clones Containing GOI

1. Determine the complete nucleotide sequence of your GOI from start to stop codon (*see* **Note 5**).
2. Option 1: Codon optimize and synthesize your GOI for optimal expression in the vaccine target species (*see* **Note 6**).
3. Option 2: Amplify the gene using standard high-fidelity PCR methods and confirm correct sequence by Sanger sequencing.
4. Add an eukaryotic Kozak consensus sequence (gccRcc**ATG**) before the start codon of the GOI (R = a/g) (*see* **Note 7**).
5. Add upstream of the Kozak consensus sequence the complete sequence of the intergenic region (IGR) of the P and M gene (sequence between stop codon P and start codon M).

This sequence contains the signals for transcription termination of the P gene and signals for initiation of transcription of the GOI.

6. *In silico* design a full-length cDNA copy of a lentogenic NDV by inserting your (optimized) GOI (including Kozak consensus sequence and upstream IGR) directly after the stop codon of the P gene in the full-length cDNA according Fig. 1a (*see* **Note 8**) generating pNDV-GOI.
7. Make sure that the total number of nucleotides of the virus encoded by pNDV-GOI complies with the rule-of-six (*see* **Note 9**) by adding 0–5 random nucleotides directly downstream the stop codon of the GOI.
8. Identify unique restriction enzyme sites in the regions flanking the GOI in pNDV-GOI (*see* **Note 10**).
9. Purchase the GOI including the flanking regions containing the unique restriction enzyme sites from a gene synthesis company (e.g., GenScript) (*see* **Note 11**). The gene synthesis company will provide you with a plasmid encoding your GOI. Hereafter referred to as pGOI.
10. Amplify pGOI in *E. coli* and purify the plasmid using a miniprep or midiprep kit.
11. Digest pNDV and pGOI with the unique restriction enzymes identified in the *in silico* analysis of point 3.7 according the restriction enzymes manufacturers' instructions.
12. Gel purify the digested pNDV and the GOI and subsequently ligate the two fragments together to generate pNDV-GOI (*see* **Note 12**).
13. Introduce the ligation mixture into highly competent *E. coli* using a convenient transformation procedure. Plate all bacteria on LB-agar plates containing 15 μg/ml kanamycin and subsequently incubate for 16–24 h at 37 °C.
14. Amplify >10 colonies in 10–50 ml LB-medium containing 15 μg/ml kanamycin under agitation at 37 °C for approximately 16 h.
15. Isolate pNDV-GOI plasmids using minipreps or midipreps according the manufacturers' instructions. Check the concentration and purity of the plasmids (e.g., NanoDrop).
16. Check all the potential pNDV-GOI plasmids by restriction enzyme analysis (*see* **Note 13**).
17. Check the complete sequence of 1–4 clones containing the correct restriction enzyme pattern by Sanger or next generation sequencing.
18. Purify a larger amount of a plasmid (>50 μg) containing the correct nucleotide sequence (*see* **Note 14**)

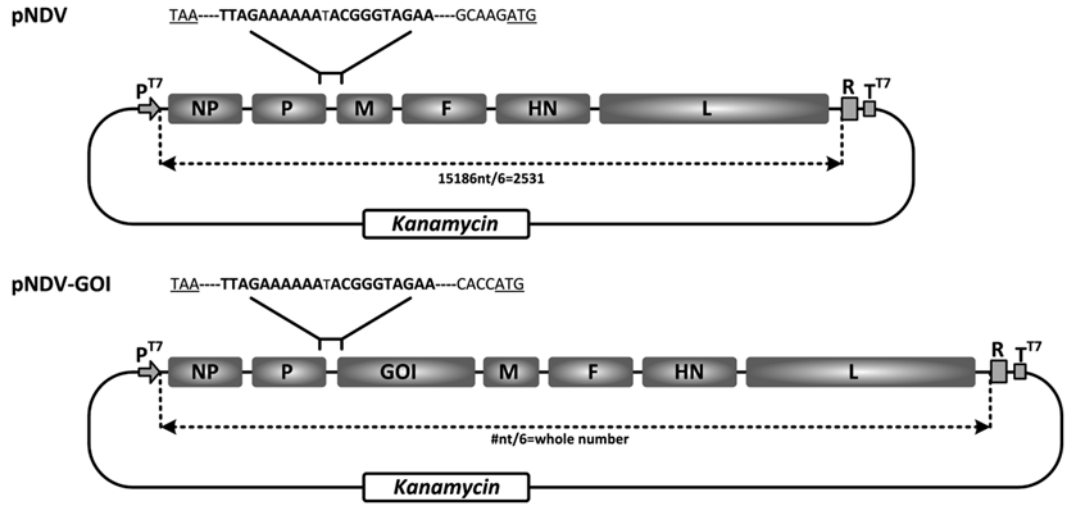

Fig. 1 Schematic presentation of pNDV and pNDV-GOI. The NDV and NDV-GOI antigenome is transcribed by a T7 polymerase recognizing the T7 promoter (P^{T7}) and the T7 terminator (T^{T7}). An encoded ribozyme (R) ensures that the resulting transcript is cleaved exactly at the end of the virus specific sequence. The intergenic regions contain a transcription termination and a transcription start sequence respectively (indicated in *bold*). A Kozak consensus sequence (CACC) is added before the start (*underlined*) codon of the GOI. The stop codon of the P gene is also underlined. The total amount (#) of nucleotides (nt) encoding for the virus and divided by 6 should result in a whole number

3.2 Rescue of NDV-GOI

(Infections and transfections are performed at 37 °C.)

1. Day −3: Subculture QM5 cells in a 75 cm^2 flask in complete QT-35 medium to reach confluence in 3 days.
2. Day 0: Wash the 75 cm^2 confluent monolayer of QM5 cells with PBS and dissociate cells using trypsin–EDTA. Subsequently, seed 500,000–700,000 cells per well of a six wells plate in 2.5 ml complete QT-35 medium (*see* **Notes 15** and **16**). N.B. It is strongly preferred to use 6-well plates.
3. Day 1: Remove medium and infect cells with FP-T7 (multiplicity of infection 1–5) in 1 ml Opti-MEM supplemented with 1 % FBS (*see* **Note 17**).
4. After 1 h incubation remove FP-T7 containing medium and add 1 ml Opti-MEM containing 1 % FBS.
5. After 30 min recovery, transfect cells with a transfection mixture containing the pCIneo-NP, pCIneo-P, and pCIneo-L expression plasmids and the pNDV-GOI transcription plasmid according to the transfection reagents manufacturer's instructions (*see* **Note 18**) (Fig. 2a). Preferably use a total of 3 μg DNA per well with a 1.5:1:1:2 ratio in μg of pCIneo-NP, pCIneo-P, pCIneo-L, and pNDV-GOI respectively. Include a positive control based on wild-type NDV and a negative control by omitting FP-T7 infection.

6. Remove the transfection mixture after 4 h and add 2.5 ml complete QT-35 medium containing 5% allantoic fluid to each well.
7. Incubate the cells for 3–5 days or until extensive cytopathic effect (CPE) is detected.
8. Filtrate the culture supernatant containing NDV-GOI with a 0.2 μm filter to remove residual FP-T7 virus and cell fragments. Use supernatants directly for virus amplification or store at −80 °C.

3.3 Amplification of Virus

1. Mark the interface of the air sac and the allantoic cavity of 8–10 day old embryonated chicken eggs using a candle flashlight. Place the eggs in an egg rack with the inoculation site uppermost. Spray the eggs with 70 % ethanol to sterilize the shell, allow to evaporate, and pierce gently a ≈2 mm^2 hole at the marked location. Now inoculate the eggs in the allantoic cavity (Fig. 2b) with 0.1 ml of culture medium containing the rescued virus using the syringe and needle in vertical position.
2. Cover the hole in the egg with a piece of adhesive tape.
3. Incubate the eggs for 2–4 days in the egg incubator. Make sure to check the temperature and humidity of the incubator each day. In addition check the viability of the embryo's each day with the egg candler.
4. Incubate the eggs for at least 4 h at 4 °C to kill the embryos.
5. Spay the eggs with 70 % ethanol and expose the allantoic membrane underneath the air sac by cracking the eggshell with a spatula (*see* **Note 19**).
6. Make an incision in the allantoic membrane using forceps and scissors.
7. Harvest the allantoic fluid, aseptically, with a 10 ml pipet and clarify the fluid by low speed centrifugation (≈5 min, 1.500 rpm).
8. Freeze allantoic fluid at −80 °C.

3.4 Determination of Viral Titer and Confirmation of Expression of Protein of Interest by Immunoperoxidase Monolayer Assay (IPMA) (See Note 20)

(Infections and transfections are performed at 37 °C in a CO_2 incubator.)

1. Seed 100 μl QM5 cells/well of a 96 wells plate (≈40,000 cells/well) in complete culture medium and allow the cells to attach for 3–4 h. Add 50 μl serial dilutions in complete culture medium of NDV or NDV-GOI. Transfect some addition wells with an expression plasmid expressing your GOI as a positive control.
2. Incubate the infected and transfected cells for 48 h.
3. Fixate cells with 4 % paraformaldehyde for 10 min (*see* **Note 21**).

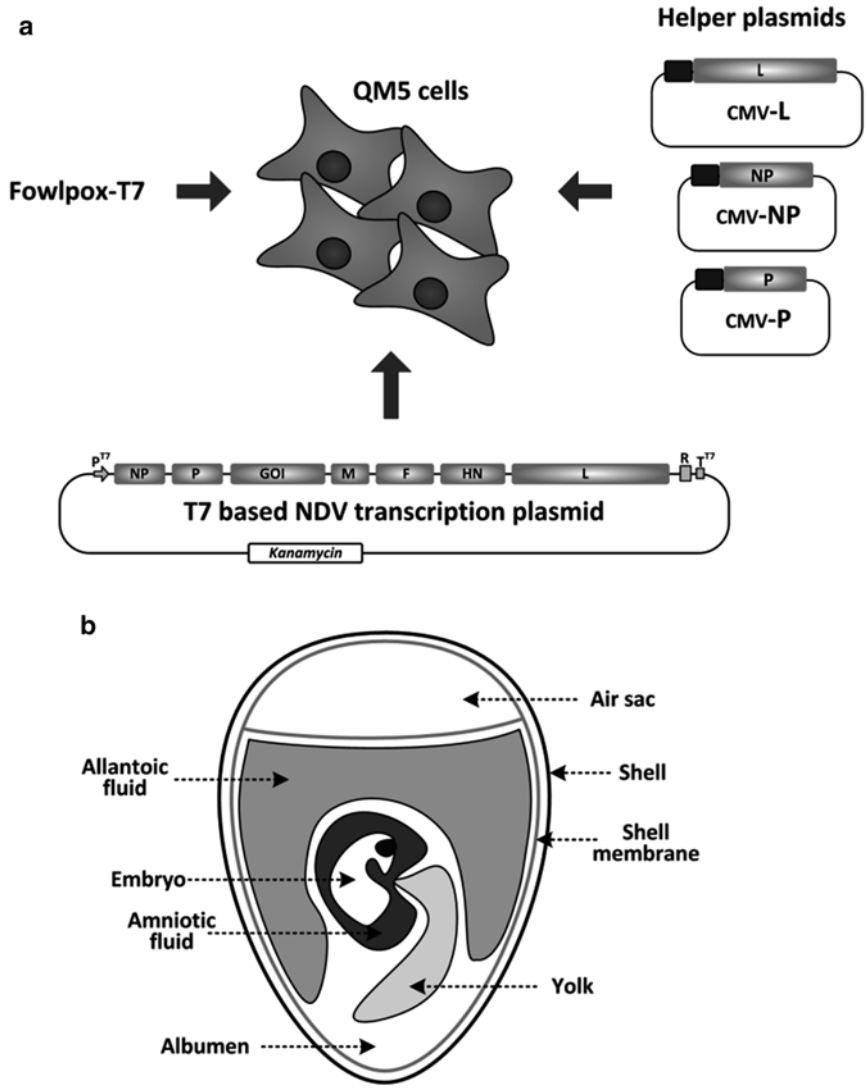

Fig. 2 Schematic presentation of NDV-GOI rescue procedure. (**a**) QM-5 cells infected with a fowlpox expressing T7 polymerase (fowlpox-T7) are transfected with three CMV promoter based expression plasmids and a T7 promoter based transcription plasmid. Approximately 3–5 days post transfection cell supernatants are injected in the (**b**) allantoic cavity of 8–10-day-old embryonated eggs. After 2–4 days incubation allantoic fluid containing NDV-GOI can be collected

4. Wash cells with PBS for approximately 1 min.
5. Permeabilize cells with ice-cold 100 % methanol for 5 min.
6. Wash cells with PBS for approximately 1 min.
7. Block cells with blocking buffer for at least 30 min at 37 °C.
8. Incubate cells with primary antibody recognizing the NDV-F protein or the protein of interest in blocking buffer for 1 h at 37 °C. Empirically determine the optimal antibody dilution.

9. Wash cells with PBS-T, three times 5 min.
10. Incubate the cells with secondary HRP-conjugated antibody in blocking buffer for 1 h at 37 °C. Use the antibody at the dilution factor indicated by the manufacturers. Alternatively determine the best antibody dilution factor empirically.
11. Wash cells with PBS, three times 5 min.
12. Incubate the cells with freshly prepared substrate buffer for 5–20 min depending on the level of color development. Monitor the color development under a light microscope at regular intervals.
13. Remove and discard the substrate buffer and subsequently wash cells with PBS.
14. Leave cells with PBS and determine the 50 % tissue culture infective dose ($TCID_{50}$) of NDV and NDV-GOI according the Spearman-Karber algorithm.

3.5 Evaluation of Immune Responses Against the Protein of Interest in (Target) Animals

1. Day −7; obtain target animals from breeding facility or from the field, divide them into equally numbered experimental groups and let them acclimatize for 1 week. Preferably, groups should contain 6–10 animals (*see* **Note 22**).
2. Day −1, obtain EDTA-blood and serum-blood samples of each animal and store plasma and serum at −20 °C.
3. Day 0, prepare NDV-GOI vaccine. In a first vaccination attempt a vaccine containing 10^7 $TCID_{50}$ values/ml will be appropriate.
4. Transport the vaccine to the animal facilities applying a cold chain.
5. Immunize the animals intramuscularly with 1 ml of vaccine (*see* **Note 23**).
6. Titrate the vaccine that comes back from the animal facilities to determine the actual vaccination titer according to the procedures described under Subheading 3.4.
7. Monitor temperature and body weight of each animal daily in the first week after vaccination.
8. Obtain serum samples weekly during the entire course of the experiment for the analysis of humoral immune responses.
9. Challenge all animals at 3 or 4 weeks post vaccination with the pathogen of interest.
10. Starting on the day of challenge, collect additional EDTA-blood samples daily for the evaluation of the presence of virus in plasma.
11. Stop the experiment typically 2–3 weeks post challenge by humanely euthanizing surviving animals. Collect organs

and tissues for immunohistochemistry and virus isolation (*see* **Note 24**).

12. Isolate viral RNA/DNA of the plasma samples and determine levels of viral RNA/DNA by quantitative real-time (qRT) PCR.
13. Make 10 % w/v tissue homogenates of organ material, isolate RNA/DNA and determine the level of virus by (qRT)PCR.
14. In addition to analysis by (qRT)PCR, perform virus isolations of selected samples (*see* **Note 25**). Briefly, make serial dilutions of the plasma and 10 % w/v tissue homogenates and add these dilutions to cells susceptible to the virus. Determine titers based on CPE or, alternatively, based on an IPMA staining.
15. Determine the presence of a virus neutralizing immune response before and after challenge using a virus neutralization test (VNT). Very briefly, incubate 30–300 infectious particles with serial dilutions of sera and add after 1–3 h incubation a standard amount of virus susceptible cells. After 2–5 days CPE can be evaluated as a read-out. Alternatively, once CPE is less apparent an IPMA can be performed. The level of neutralization can be calculated using the Spearman-Karber algorithm.

4 Notes

1. Instead of using NDV-LaSota other T7 polymerase based lentogenic NDV vaccine infectious clones can be used.
2. The QM5 cell line is thus far not commercially available; however, the cell line is used in many laboratories.
3. The helper plasmids encoding NP, P and L do not have to be of pCIneo origin. Any other eukaryotic expression plasmid containing the genes under a polymerase-II promoter may be suitable.
4. For stability of the virus it is important that the diluent of the virus contains protein. Complete culture medium containing FBS is recommended. Dilution in PBS is not recommended.
5. Expression of genes larger than 2 kb has been shown to significantly reduce virus titers. It is therefore recommended not to introduce genes larger than 2 kb and large genes (>1 kb) should preferably not be combined with a second additional expression cassette.
6. Codon optimization increases the expression of your GOI. Gene-synthesis companies can codon-optimize and synthesize your GOI.

7. Adding the eukaryotic consensus Kozak sequence increases translation efficiency of your GOI.
8. Preferably, genes are incorporated between the P and M gene ensuring relatively high expression of your GOI with minimal impairment of NDV specific protein expression required for efficient virus growth.
9. The total length of the NDV genome is a multiple of six nucleotides. Viruses that do not comply with the rule-of-six cannot be rescued.
10. Restriction enzyme digestion should preferably result in sticky overhangs facilitating the downstream ligation reactions.
11. Alternatively amplify the GOI and the flanking regions with unique restriction sites by fusion extension PCR using overlapping primer sequences. Very briefly, obtain three different cDNA fragments with >20 nucleotide overlap: one containing the left flanking region of the GOI, one containing the GOI and one containing the right flanking region of the GOI. Mix the three fragments at a 1:1:1 M ratio, perform ten PCR cycli to obtain a few fused DNA molecules, amplify the correctly fused DNA molecules by performing an additional 35 PCR cycles using the outermost left flanking forward and the outermost right flanking reverse primer. Digest the gel-purified fragment with the unique restriction enzymes and use this fragment to replace the corresponding fragment of the full-length clone.
12. Efficient ligation requires highly pure DNA fragments in a 1:1 M ratio of fragments. In general we use around 50–100 ng total DNA per reaction. For increased efficiencies ligation reactions can be performed at low temperature (4 °C) for a longer period (overnight).
13. Choose restriction enzymes that can clearly discriminate between clones containing the GOI and clones that lack this insert. Also include a double digest with the enzymes used in the cloning procedure. The latter reaction will indicate whether the sticky overhangs were prone to endonuclease activity.
14. For efficient transfection, plasmid stocks should contain >300 ng DNA per µl and should be highly pure. Spectra of $A260/A280 > 1.8$ and $A260/A230 > 2$ are recommended.
15. Recently we constructed QM5 cells expressing a velogenic F protein named QM5-F^{Herts}, which facilitates spread of lentogenic variants in cell culture. These cells can be provided upon request.

16. The cell density is a critical determinant in rescue experiments and it is important that cells are handled with care. Do not let cell monolayers overgrow.
17. FP-T7 stocks can be produced on QM5 cells. Briefly, 50 % confluent monolayers are infected at an MOI of 0.01. Four days after infection, cells are subjected to two freeze thaw cycles after which supernatants are collected and stored at −80 °C. Optimal stocks should contain $>10^7$ $TCID_{50}$/ml.
18. The efficiency of various transfection reagents for the use in reverse genetics systems strongly varies. A preferred transfection reagent is jetPEI (Polyplus).
19. There are several commercial tools available that facilitate the removal of egg shells and opening of the allantoic cavity.
20. Expression of the GOI can also be evaluated using Western blot. Very briefly, harvest supernatant and/or cells, homogenize or lyse cells and subsequently separate proteins under native of denaturating conditions in a 4–12 % acrylamide gel. Transfer the proteins to nitrocellulose and visualize your protein of interest using a primary antibody recognizing a linear epitope.
21. Completely submerge the plate in 4 % paraformaldehyde without a previous wash. This rapid procedure ensures inactivation of all intra- and extracellular virus.
22. Always include a mock vaccinated control group. Equalize group sizes of vaccinated and control groups to facilitate statistical analysis.
23. Although other vaccination routes might work as well, for initial experiments in mammals we advise to use the intramuscular route.
24. The post challenge period largely depends on the pathogen and animal model you are using. Before starting a vaccination experiment you always should perform a challenge experiment in which you monitor all the parameters as described for the post challenge period.
25. In general, (qRT)PCR analysis is more sensitive than virus isolation.

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Chapter 15

Chimeric Pestivirus Experimental Vaccines

Ilona Reimann, Sandra Blome, and Martin Beer

Abstract

Chimeric pestiviruses have shown great potential as marker vaccine candidates against pestiviral infections. Exemplarily, we describe here the construction and testing of the most promising classical swine fever vaccine candidate "CP7_E2alf" in detail. The description is focused on classical cloning technologies in combination with reverse genetics.

Key words Chimeric pestivirus, Reverse genetics, CP7_E2alf, Construction, Cloning, Marker vaccine candidate

1 Introduction

Classical swine fever (CSF) is among the most important viral disease of domestic and feral pigs and has a serious impact on animal health and pig industry. In most countries with industrialized pig production, prophylactic vaccination against CSF is banned, and all efforts are directed towards eradication of the disease, e.g., by culling of infected herds and animal movement restrictions [1]. Nevertheless, emergency vaccination of domestic pigs and wild boar remain an option to minimize both the spread and the socio-economic impact of outbreaks [2]. For this application, potent vaccines are needed that allow differentiation of infected from vaccinated animals. Among the promising next-generation marker vaccines, the chimeric pestivirus "CP7_E2alf" is the most far developed and characterized candidate. The chimeric "CP7_E2alf" virus is constructed using the full-length infectious cDNA clone "pA/BVDV" of Bovine viral diarrhea virus (BVDV) strain CP7 (GenBank accession numbers U63479.1 and AF220247.1). The parental virus was first described by Corapi et al. [3], and the generation of the cDNA construct is described by Meyers et al. [4]. In brief, total RNA from CP7-infected MDBK-cells was used to generate corresponding cDNA. In a first step, libraries were established in λZAPII (Stratagene) following the manufacturer's

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instructions. After screening of the derived fragments, sub-cloning into pBluescript plasmids (Stratagene) was done by in vivo excision following the instructions provided by the supplier. Assembly of the genome was subsequently done in several steps using clone intermediates (intermediates spanning different parts of the genome and rational supplementation of missing sequence stretches at the 5′- and 3′-ends). The final version of the full-length clone was based on the different fragments and cloned into the low-copy-number plasmid pACYC177 (New England Biolabs, GenBank accession X06402, L08776) including a T7 RNA polymerase promoter in front of 5′ end of the viral genome as well as a cleavage site (SmaI) at the 3′ end for linearizing the plasmid DNA. For in vitro transcription, the cloned full-length cDNA construct was linearized, purified, and transcribed into RNA using T7 RNA polymerase (New England Biolabs).

In vitro-transcribed positive-stranded RNA of "pa/BVDV" was finally transfected into bovine cells by using electroporation and the resulting recombinant virus progeny was further characterized as identical to parental BVDV CP7.

For the generation of the chimeric BVDV/CSFV pestivirus "CP7_E2alf", the envelope protein E2-encoding region, which is the most immunogenic protein in the structure of *Pestiviruses*, was replaced in the BVDV CP7 backbone with the respective coding sequence of Classical swine fever virus (CSFV) strain "Alfort 187" [5]. Therefore, in the following, an overview is given on its construction as an example for a chimeric pestivirus. Critical steps are described in detail and supplemented by methodological notes.

2 Materials

1. Porcine kidney cells (PK15, RIE0005-1, CCLV).
2. Diploid bovine esophageal cells (KOP-R, RIE244, CCLV).
3. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % BVDV-free fetal bovine serum (FBS).
4. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.
5. Trypsin buffer: 136 mM NaCl, 2.6 mM KCl, 8 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 3.3 mM EDTA, 0.125 % trypsin.
6. CSFV Alfort 187 strain.
7. *Escherichia coli* TOP10F′ cells (Invitrogen).
8. Plasmid purification kits (Mini, Midi, or Maxiprep).
9. Full-length cDNA clone "pA/BVDV" (kindly provided by G. Meyers, FLI) [4].

10. Restriction endonuclease enzymes KpnI, PacI, RsrII, SnaBI, and SmaI. T4 DNA ligase, Klenow enzyme for end repairing.
11. Polylinker containing sequences for PacI, RsrII, and SnaBI, restriction endonucleases generated by PCR with p7_PacI primer: 5′-CAAGGGTACCCATTAATTAACGGTCCTACG TAGTCCAGTATGGGGCAGGTGA-3′ (+sense) and p7R_KpnI P7R primer: 5′-GCTCTAGGTACCCCTGGGCA-3′- (–sense).
12. "E2Alf_PacI (5′-GCATTAATTAACCAGCTAGCCTGC AAGGAAGATT-3′, +sense)" and "E2AlfR_SnaBI (5′-GACCTACGTAACCAGCGGCGAGTTGTTCTGTT-3′, –sense)".
13. RNA extraction and purification kit for cultured cells.
14. RT-PCR system for cDNA generation and equipment for PCR amplification.
15. QIAquick Nucleotide Removal Kit (Qiagen).
16. T7 RiboMax Large-Scale RNA Production System (Promega).
17. Ethanol, RNAse-free water, agarose gel electrophoresis system, ethidium bromide.
18. Gene Pulser Xcell Electroporation System and electroporation cuvettes with 0.4 cm gap width (Bio-Rad).
19. 3 % paraformaldehyde, acetone, primary antibodies specific to CSFV proteins E2 and NS2/NS3. Anti-mouse IgG Alexa Fluor 488 conjugated.
20. Fluorescence microscope.

3 Methods

3.1 Cell Culture

Porcine kidney cells (PK15) and diploid bovine esophageal cells (KOP-R, RIE244, CCLV), are propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% BVDV-free fetal bovine serum (FBS). After a short washing step with PBS cells are detached with trypsin buffer. The cells are routinely passaged twice weekly at a split ratio of 1:6–1:8.

3.2 Generation of cDNA Constructs

All plasmids are propagated in *Escherichia coli* TOP10F′ cells (Invitrogen). Restriction enzyme digestion and cloning procedures are performed according to standard protocols. Plasmid DNA is purified by Qiagen Plasmid Mini, Midi, or Maxi kits according to the manufacturer's instructions. The E2-deleted cDNA construct of the original infectious CP7-clone "pA/CP7_ ΔE2PacI" and the E2-exchanged chimeric cDNA clone "pA/ CP7_E2alf", underlying the CP7_E2alf vaccine virus, are all constructed based on the above mentioned full-length cDNA clone "pA/BVDV".

1. Digest plasmid "pA/BVDV" with restriction enzyme KpnI, to excise the E2-gene to the end of the p7-encoding region.
2. Purify and religate the digested plasmid to obtain the intermediate cDNA construct "pA/CP7_ΔE2p7" which shows an in-frame deletion of E2 and p7 of the pA/BVDV.
3. Subsequently, the deleted p7-encoding region has to be repaired and a small polylinker with the restriction cleavage sites for PacI, RsrII, and SnaBI inserted. To this means, a PCR-fragment was amplified using plasmid DNA of pA/BVDV as template and primers "p7_PacI (CAAGGGTAC C C A T T A A T T A A C G G T C C T A C G T A G T C C A G T ATGGGGCAGGTGA, +sense)", which contains recognition sites for the restriction enzymes KpnI, PacI, RsrII, and SnaBI, and "p7R_KpnI P7R (GCTCTAGGTA CCCCTGGGCA, –sense)". The PCR-fragment is subsequently digested with KpnI and cloned into the KpnI site of plasmid "pA/CP7_ΔE2p7" (see above). The resulting construct was named "pA/CP7_ΔE2PacI" (Fig. 1).
4. In order to obtain the final construct "pA/CP7_E2alf" (Fig. 1), digest plasmid "pA/CP7_ΔE2PacI" with restriction enzymes PacI and SnaBI.
5. To amplify the E2Alf cDNA, perform a RT-PCR using RNA derived from CSFV Alfort 187 infected PK15 cells using primers "E2Alf_PacI (GCATTAATTAACCAGCTAGCCTGCAAGGAAGATT, +sense)" and "E2AlfR_SnaBI (GACCTACGTAACCAGC GGCGAGTTGTTCTGTT, –sense)", containing PacI and SnaBI restriction sites, respectively.
6. Purify the PCR fragment, covering the complete E2-encoding sequence of CSFV Alfort 187, and digest with PacI and SnaBI.
7. Ligate the digested PCR fragment into the plasmid "pA/CP7_ΔE2PacI". This results in the final plasmid "pA/CP7_E2alf".

3.3 Recovery of Chimeric Pestivirus "CP7_E2alf" from Recombinant cDNA Construct "pA/CP7_E2alf"

The final vaccine virus CP7_E2alf was obtained through transfection (electroporation) of in vitro-transcribed RNA from the linearized chimeric plasmid "pA/CP7_E2alf" into porcine kidney and bovine esophageal cells.

3.3.1 In Vitro Transcription

1. Linearize the full-length cDNA construct "pA/CP7_E2alf" with SmaI (*see* **Note 1**).
2. Purify the digested DNA by using the QIAquick Nucleotide Removal Kit (Qiagen) and precipitate the product with ethanol. Resuspend the dried DNA in RNase-free water and use it as template for in vitro-transcription (*see* **Note 2**).
3. Perform an in vitro transcription reaction by using the T7 RiboMax Large-Scale RNA Production System (Promega)

according to the manufacturer's instructions. Prototype T7 reaction mixture is: 1 µg linearized DNA, 4 µl T7 5X transcription buffer, 6 µl 25 mM rNTPs, 2 µl enzyme mix, and nuclease-free water to a final volume of 20 µl.

4. Mix gently and incubate the reaction at 37°C for 2 hours.
5. Estimate the amount of RNA by ethidium bromide staining after agarose gel electrophoresis (*see* **Note 3**).

3.3.2 Electroporation

1. Prepare a semi-confluent cell culture of KOP-R or PK15 cells at the time of harvest (*see* **Note 4**).
2. Detach about 1×10^7 cells using trypsin solution and wash cells twice with PBS.
3. Suspend the cells in 1 ml PBS and mix them with 1–5 µg of in vitro-transcribed RNA by gently pipetting up and down.
4. Transfer the DNA-cell mixture into electroporation cuvettes with 0.4 cm gap width (Bio-Rad).
5. Electroporate cells with two pulses at 850 V, 25 µF, and 156 ω, using the Gene Pulser Xcell Electroporation System (Bio-Rad) (*see* **Note 5**).
6. Seed cells immediately in culture vessels according to the experimental requirements and incubate them at 37°C for 72h

3.3.3 IF Staining

At the day of supernatant collection, virus replication is monitored by standard immunofluorescence (IF)-staining using a fluorescence microscope. For the detection of BVDV and CSFV proteins,

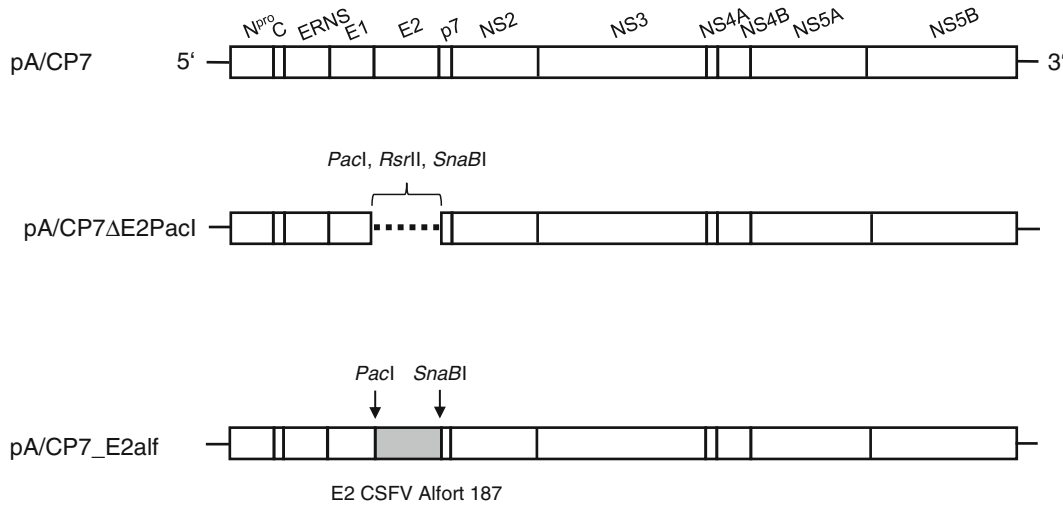

Fig. 1 Schematic representation of the engineered constructs. NTR, nontranslated region; C, E^{RNS}, E1, E2, coding sequences for the structural proteins; NS2, NS3, NS4A, NS4B, NS5A, NS5B, sequences encoding nonstructural proteins. The *horizontal dotted line* shows the deleted region, *arrows* indicate restriction enzyme sites. The *shaded box* represents the inserted sequence encoding the CSFV E2 protein of the CSFV isolate Alfort 187

the monoclonal antibodies (mab) WB210 (anti-E^{RNS} BVDV, CVL Weybridge), 01–03 (anti-E^{RNS} panpesti, Schelp), WB215 (anti-E2 BVDV, CVL Weybridge), CA3 (anti-E2 BVDV, Institute for Virology, University of Veterinary Medicine, Hannover), HC/TC50 (anti-E2 CSFV, Institute for Virology, University of Veterinary Medicine, Hannover), HC34 (anti-E2 CSFV, Institute for Virology, University of Veterinary Medicine, Hannover), mab-mix WB103/105(anti-NS3 panpesti, CVL Weybridge), and C16 (anti-NS3 panpesti, Institute for Virology, University of Veterinary Medicine, Hannover) were used. Standard IF-analysis using a fluorescence microscope (Olympus) were performed as previously described [6, 7].

1. For NS2/3-staining wash cells twice with PBS and fix them with 3% paraformaldehyde on ice for 15 min.
2. Wash cells with PBS, and permeabilize for 5 min with 0.0025% digitonin in PBS at room temperature.
3. For E^{rns} and E2 protein staining, fix cells and permeabilize for 10 min with ice-cold 80% acetone.
4. Wash cells with PBS and incubate them with the first antibody for 15 min. After washing the cells twice with PBS, incubate cells for 15 min with the second antibody (anti-mouse-Ig Alexa 488). Rinse cells with PBS again twice and investigate by using a fluorescence microscope.
5. The rescued virus preparations are further passaged using PK15 or KOP-R cells, and all virus titers determined as $TCID_{50}$. In all cases, the whole virus population is passaged to avoid negative selection that would be caused by biologically cloned virus. The virus is subsequently subjected to in vitro and in vivo characterization and stability tests (*see* **Note 6**).

4 Notes

1. Depending on the way the 3′ end of the pestivirus genome is generated, the plasmid must be digested with appropriate restriction enzymes. Because the extreme 3′ end of the pestiviruses usually consists of three or more cytosines, blunt end cutter like the restriction enzyme SmaI are used for linearization of DNA templates prior to in vitro transcription to ensure the production of RNA of correct length. It is important to avoid the use of restriction enzymes which produce protruding ends (overhangs). Extraneous transcripts can appear in addi-

tion to the expected transcript when such templates are transcribed. Furthermore, extra bases will be added to the transcripts, which may interfere with RNA replication.

2. Clean-up of digested DNA before in vitro transcription is possible with commercially available systems (e.g., QIAquick Nucleotide Removal Kit, Qiagen). For complete elution of bound DNA from the spin columns, DNA can be eluted twice. To elute the DNA, RNase-free water should be used instead of elution buffer. However, store eluted DNA at −70 °C, as DNA may degrade in the absence of a buffering agent.
3. The purified linear DNA should be examined by agarose gel electrophoresis prior to in vitro RNA-transcription to verify complete linearization, and to ensure the presence of a clean, non-degraded DNA-fragment of the expected size. It is useful to start with at least 30 % more DNA than is required for the transcription reactions to compensate for any RNA-loss during purification.
4. The appropriate number of cells depends on the general electroporation conditions and on the growth rate of the cells and must therefore be optimized. Cells should be seeded for 24 h before electroporation and should not be confluent before transfection.
5. For most purposes, it is not necessary to clean up the in vitro-transcribed RNA before electroporation of cells. However, unincorporated nucleotides can be removed by isopropanol precipitation. The DNA-template may be removed by digestion with DNase following the transcription reaction. It is crucial to use an RNase-free DNase that is qualified for the degradation of DNA while maintaining the integrity of RNA. After DNase digestion the in vitro transcripts have to be cleaned up by phenol extraction or alternatively by commercially available spin-column-based systems for the purification of total RNA.
6. Novel pestivirus clones and technologies for manipulation are available today. Full-length sequences cloned into bacterial artificial chromosomes (BACs) have been described [8] which can be directly manipulated using bacterial recombination systems like Red/ET. Furthermore, full-length PCR and mutagenesis as well as fusion-PCR allow now a swift direct manipulation of plasmid-cloned pestivirus genomes [9].

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Chapter 16

Analysis of the Cellular Immune Responses to Vaccines

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Abstract

Flow cytometry, enzyme-linked immunospot (ELISpot) and cellular cytotoxicity assays are powerful tools for studying the cellular immune response towards intracellular pathogens and vaccines in livestock species. Lymphocytes from immunized animals can be purified using Ficoll-Paque density gradient centrifugation and evaluated for their antigen specificity or reactivity towards a vaccine. Here, we describe staining of bovine lymphocytes with peptide (p)-MHC class I tetramers and antibodies specific towards cellular activation markers for evaluation by multiparametric flow cytometry, as well as interferon (IFN)-γ ELISpot and cytotoxicity using chromium (^{51}Cr) release assays. A small component on the use of immunoinformatics for fine-tuning the identification of a minimal CTL epitope is included.

Key words ELISpot, Cytotoxicity assay, Flow cytometry, NetMHCpan, Peptide-MHC class I tetramers, CTL epitope

1 Introduction

The use of flow cytometry and fluorochrome-coupled monoclonal antibodies has revolutionized the field of immunology allowing for detailed characterization of lymphocyte subsets and their role in immunity as well as for use in diagnostic assays in human and veterinary medicine [1, 2]. Cells isolated from vaccinated animals are labeled with antibodies specific for cluster of differentiation (CD) molecules, e.g., cellular subset markers such as the cytotoxic T lymphocytes (CTL) marker CD8 or activation markers such as CD69 or memory markers, e.g., CD45RO [3–7]. If available, the T lymphocytes can be labeled with peptide (p)-major histocompatibility complex (MHC) class I tetramers to monitor the appearance of antigen specific CTL [8] over a period of time post immunization. Lymphocytes can be fixed with paraformaldehyde and permeabilized by an amphipathic compound, e.g., saponin-containing buffer in order to stain and measure intracellular proteins [9, 10]. Intracellular staining allows for the quantification of immune

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mediators such as cytokines or proteins, e. g. perforin or granzyme B [11], which play an active role in cytotoxicity. The flow cytometers currently available on the market allow for staining with more than ten different fluorochromes, dependent on the number of lasers, which permits simultaneously quantification of several parameters in the same cell population. Another method for monitoring responsiveness of CTL, in the presence of an antigen, is IFN-γ release by individual cells using enzyme-linked immunospot (ELISpot) assay [12], which employs the sandwich enzyme-linked immunosorbent assay (ELISA) technique. Monoclonal or polyclonal antibodies specific for the desired cytokine can be used to coat polyvinylidene difluoride (PVDF) membrane in a 96-well plate format. Lymphocytes are then transferred onto these plates and cultured in the presence of an antigen, usually in the form of peptides, in a humidified atmosphere with 5 % CO_2 at 37 °C. During this period, cytokine released by individual cells binds to the immobilized antibody in close proximity. After a washing step, a polyclonal antibody, or another monoclonal antibody with a different epitope specificity but specific for the cytokine of interest is added to the wells. Following a second washing step, a secondary antibody which is conjugated to, e.g., alkaline-phosphatase (AP) is added to each well. Finally, after unbound antibodies are removed by washing, the presence of the cytokine is revealed by the addition of the AP substrate solution (BCIP/NBT). A dark precipitate forms and appears as spots where the cytokine is present (Fig. 1).

These spots can be counted with an ELISpot reader. An advantage of the ELISpot method is that it quantifies the number of responding cells (percentage of spots per cells added). IFN-γ ELISpot is often used as a surrogate assay for detecting a CTL

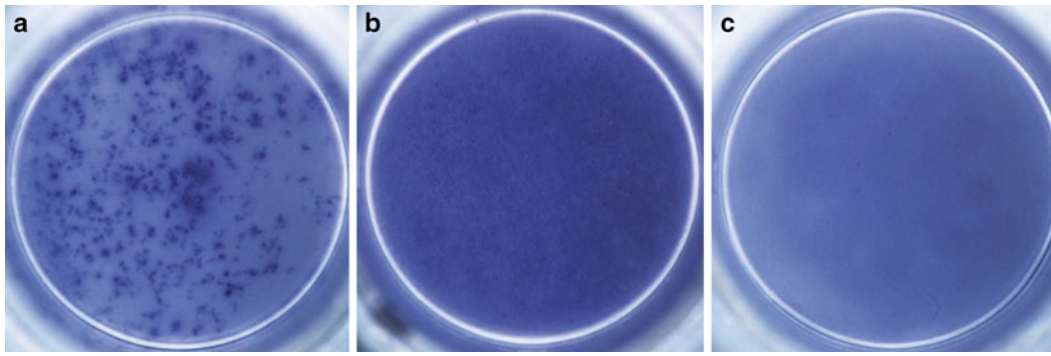

Fig. 1 ELISpot results as observed on a PVDF membrane in an individual well of a 96-well plate. (**a**) A positive well. *Dark blue spots* over a *light blue background* is indicative of the presence of positive cells secreting the cytokine of interest (in this example IFN-γ is measured). Spots can be counted and presented as spot-forming units (SFU) per 10^6 cells. (**b**) A saturated well. When too many cells are used in the assay, a uniform *dark blue color* will form and cover the well (in this case, titration of the number of cells to find the optimal number of cells to put in a well is required). (**c**) A negative well. A well with the absence of cytokine-secreting cells will appear *light-blue colored*

response because there are various difficulties associated with establishing CTL assays. However, IFN-γ release alone might not always correlate with protection [12, 13]. Cytotoxicity assays demonstrate a better correlation with protection in several vaccine studies [14–20], probably because it employs the direct lysis of autologous target cells, which clears infected cells. Target cells are incubated with for example radioactive chromium (^{51}Cr), washed and incubated with effector cells. Then the supernatant is collected and lysis is evaluated by measuring released ^{51}Cr. A convenient way to do this is to use the LumaPlates, which contain a scintillator making it possible to read it in a Top Counter machine (beta-counter).

ELISpot and cytotoxicity assays have been used in the past to identify minimal epitopes that induce immunity [15]. However, the use of programs that predict peptide binding to major histocompatibility complex (MHC) class I molecules has revealed that minimal CTL epitopes can be included in longer peptides that have been shown to be positive in such assays [21, 22] and concomitant use of immunoinformatics can help fine tune minimal epitope identification [23, 24].

Earlier versions of some of these methods have been described in the past [25] and have been more recently modified along with the emergence of new technologies. This has allowed us to successfully analyze the response to a commercial and experimental vaccine against *Theileria parva* in cattle [14, 15, 21, 22, 26]. However, these methods can be readily adapted to evaluate the immune responses toward vaccines in other livestock species.

2 Materials

Prepare all solutions with ultrapure water. All reagents are kept at room temperature unless it is indicated otherwise.

2.1 Flow Cytometry

1. Flow cytometer: we use BD FACSCanto™ II instrument (BD Biosciences).
2. Dulbecco's PBS 1×: 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4{\cdot}2H_2O$, 0.2 g of KH_2PO_4 in 1 l of water. Adjust pH to 7.2–7.4 with NaOH or HCl.
3. PBS-1×-saponin: Dulbecco's PBS, 0.1 % (w/v) saponin, 0.2 % sodium azide (NaN_3), 10 mM Hepes, 10 % fetal bovine serum (FBS). For 500 ml: 0.5 g of saponin, 10 ml of NaN_3 (10 %), 1.2 g of Hepes (MW: 238.3 g/mol), 51 ml of FBS. Buffer is kept at 4 °C.
4. Round (or V-shaped)-bottom 96-well plates.
5. Ficoll-Paque (GE Healthcare Life Sciences).

6. Tris-ammonium chloride buffer: for 500 ml, mix 4.15 g of ammonium chloride (NH_4Cl), 50 ml of 0.1 M Tris–HCl; adjust pH to 7.2.
7. Anti-perforin antibody ready-to-use therefore no dilutions are required (BD Pharmingen).
8. Anti-Fas-L (sc-957) (Santa Cruz Biotech).

2.2 ELISpot

1. ELISpot plates: Millipore MAIP S45. 96-well ELISpot plate (Millipore).
2. RPMI 1640 complete: RPMI-1640 supplemented with 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 100 IU of penicillin/ml, 100 μg of streptomycin/ml, 50 μg of gentamicin/ml, and 10 % FBS.
3. FBS, heat-inactivated.
4. Coating buffer: Carbonate/bicarbonate coating buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$ (1.6 g Na_2CO_3, 2.93 g $NaHCO_3$ in 1 l of distilled water) adjusted to pH 9.6 and filter-sterilize. Store at −20 °C. Filter before storage (0.2 μm filter).
5. Coating antibody: Mouse anti-bovine IFN-γ mAb CC302 (Serotec). Dilute to 100 μg/ml in PBS, aliquot and store at −20 °C.
6. Washing medium: un-supplemented RPMI-1640 medium.
7. Blocking medium: RPMI-1640 medium supplemented with 10 % FBS (heat inactivated).
8. MACS goat anti-human CD14-beads, LS columns, and magnets/stand (Miltenyi Biotec).
9. MACS buffer: 2 mM EDTA in 2 % FBS/PBS.
10. PBS: *see* Subheading 2.1.
11. PBS-T (PBS/0.05 % Tween 20): add 0.5 ml Tween 20 to 1 l of PBS.
12. PBS-T/BSA (PBS-T/0.1 % BSA): add 100 mg BSA to 100 ml PBS-T, filter-sterilize (0.2 μm filter).
13. Conjugate: anti-rabbit IgG-alkaline phosphatase conjugate (clone R696, Sigma-Aldrich).
14. Substrate: Sigma Fast BCIP/NBT substrate tablets (Sigma-Aldrich). Dissolve one tablet/10 ml dH_2O at RT for 30 min (on roller) or for a few minutes on the vortex mixer and filter through a 0.2 μm filter.
15. Monoclonal anti-rabbit IgG-alkaline phosphatase conjugate, clone R696 (Sigma) diluted 1:2000 in PBS-T/BSA.

2.3 CTL Cytotoxicity Assay

1. Ficoll-Paque (GE Healthcare Life Sciences).
2. RPMI 1640 complete: *see* Subheading 2.2.

3. Cytotoxicity medium: as RPMI 1640 complete but with 5 % FBS instead.
4. PBS: *see* Subheading 2.1.
5. Tissue culture plates, sterile, 24-wells and 96-wells (Costar).
6. Plastic pipettes, sterile, 5, 10, 25 ml (Sarstedt)
7. Chromium 51 (Na_2CrO_4), aqueous sterile solution (American Radiolabeled Chemicals Inc.).
8. LumaPlates (PerkinElmer).
9. TopSeal (PerkinElmer).
10. TopCount machine (PerkinElmer). If the LumaPlate system is used it is mandatory to have access to a TopCount machine.

2.4 Immuno-informatics

1. Internet access.
2. NetMHCpan: http://www.cbs.dtu.dk/services/NetMHCpan/
3. Amino acid sequence of relevant pathogen antigens.
4. Amino acid sequence of the MHC class I of interest or knowledge about which of the MHC allele sequences in NetMHCpan are used for the given antigen.

3 Methods

3.1 Flow Cytometry

In the section below, we describe the simultaneous staining of CD8 cells with peptide (p)-MHC class I tetramer and intracellular staining with cytokines.

1. Collect lymphocytes by centrifugation on a Ficoll gradient in a 15 or 50 ml Falcon (polypropylene) tubes (depending on the volume of blood being handled).
2. The ratio of blood to Ficoll is 3:2 respectively. Centrifugation is performed at $1300 \times g_{AV}$ for 25 min on a benchtop centrifuge without brakes (*see* **Note 1**).
3. Collect PBMCs at interface and transfer to a 15 ml polypropylene tube.
4. Add PBS solution in order to fill the tube completely.
5. Centrifuge at $600 \times g_{AV}$ for 10 min with brakes on (maximum deceleration) (*see* **Note 2**).
6. Lyse red blood cells: resuspend pellet in 5 ml of Tris-Ammonium Chloride buffer. Incubate for 3 min (only) at room temperature.
7. Fill tube to the top with PBS solution.
8. Platelets are removed by spinning down at a lower speed $300 \times g_{AV}$ for 10 min.

9. A second wash is performed at $300 \times g_{AV}$ for 10 min.
10. Resuspend in Dubecco's PBS 1×.
11. Count cells and dispense $2–5 \times 10^5$ PBMC/well (*see* **Notes 3** and **4**).
12. Spin down the plate at $830 \times g_{AV}$ for 3 min and remove the supernatant.
13. Wash with 200 μl of PBS-0.5 % bovine serum albumin (BSA) (*see* **Note 5**) per well and spin again at $830 \times g_{AV.}$
14. Add 200 μl of PBS-2 % formalin (or PBS-1 % paraformaldehyde) to each well and incubate at room temperature for 10 min (*see* **Note 6**).
15. Spin down at $830 \times g_{AV}$ for 3 min and remove the supernatant.
16. Wash once with 200 μl of ice-cold PBS-0.5 % BSA.
17. Add 200 μl of PBS-saponin, if performing intracellular staining, and incubate for 30 min at room temperature (*see* **Notes 7** and **8**).
18. Spin down at $830 \times g_{AV}$ for 3 min and remove the supernatant.
19. Add the primary antibodies of interest (20 μl of each antibodies dilution) diluted in PBS-0.5 % BSA (or PBS-saponin if performing intracellular staining) (*see* **Notes 9** and **10**). If a tetramer with the desired MHC class I and CTL epitope specificity is available, use 40 nM of p-MHC class I tetramer concomitantly with the antibodies for the extracellular markers (*see* **Notes 11–13**). If available, additional antibodies directed toward activation and memory markers can be included if these type of responses are explored.
20. Incubate at 4 °C for 30 min.
21. Wash twice with PBS-0.5 % BSA (or PBS-saponin if using antibodies for intracellular markers) (*see* **Note 14**).
22. Add the secondary antibodies (if not using directly coupled primary antibodies). For staining with the primary anti-CD8 antibody (ILA51; IgG1) we use 1 μl per $2–5 \times 10^5$ cells of rat anti-mouse IgG1 PerCP (BD Pharmingen) (*see* **Note 15**). For staining with the primary anti-Fas-L antibody (rabbit polyclonal) we use goat anti-rabbit FITC (Sigma F-0382; diluted 1:200 in PBS-0.5 % BSA or PBS-saponin).
23. Incubate at 4 °C for 30 min.
24. Wash twice as described at **step 21** in the flow cytometry procedure.
25. Resuspend in 200 μl of PBS-0.5 % BSA (*see* **Note 16**) and transfer to flow cytometry tube (BD Pharmingen) containing 200–300 μl of PBS or saline.

26. Acquire data on BD FacsCanto II using appropriate compensation controls (*see* **Note 17**).
27. Analysis can be performed using any flow cytometry analysis software. Currently we use FlowJo. It is a good practice to stain sample for viability and single cells before analysis in order to gate out dead cells and cell aggregates (*see* **Note 18**).

3.2 ELISpot Assay for Detection of Bovine Interferon-Gamma (IFNγ)

Cells used in ELISpot assay can be either PBMC or purified cell subsets such as CD8 cells or CD4 cells. The MACS system is used for purifying such subsets. In general we refer to manufactures protocol for purification of cell subsets. The way we perform this is listed below.

3.2.1 MACS Sorting for CD8, CD4 and Monocytes from Ex Vivo PBMC or Bulks

1. Separate PBMC as described under the flow cytometry methods into a clean 20 ml Sterilin tubes.
2. Wash in PBS (without magnesium and calcium) containing 2 % FBS (PBS/FBS).
3. Pellet cells by centrifugation at $300 \times g_{AV}$.
4. Add 12.5 μl mAb diluted in PBS, 2 % FBS (or complete RPMI) per 10^6 cells (i.e., 125 μl at 1/500 per 10^7 cells). Use IL-A51 for CD8 or IL-A11 for CD4 (*see* **Note 19**).
5. Incubate for 30 min at 4 °C.
6. Wash 2× (6–8 min $300 \times g_{AV}$).
7. Pellet the cells at $300 \times g$.
8. Add 10 μl of anti-mouse IgG coupled MiniMACS beads per 10^7 cells, for CD8 or CD4 purification. Use anti-human CD14 microbeads for monocyte purification. Mix and incubate at 4 °C for 30 min. MiniMACS columns can purify up to approx. 10^8 cells in total population.
9. Wash cells 2× (6–8 min $300 \times g_{AV}$) in PBS/FBS.
10. Prepare the MiniMACS column—place on the magnet and flush through with 0.5 ml PBS/2 % FBS, 2 mM EDTA (MACS buffer, ice cold).
11. Resuspend cells in 0.5–1 ml MACS buffer and apply this to the column.
12. Wash the column with 3×0.5 ml MACS buffer.
13. Remove the column from the magnet and apply 2 ml RPMI/10 % FCS.
14. Elute the cells rapidly with the plunger into a tube containing complete RPMI 1640.
15. Spin cells down, resuspend in complete RPMI then count.
16. N.B Mini macs columns can purify up to approx. 10^8 cells.

3.2.2 ELISpot Procedure

1. Coat Millipore MAIP S45 plates overnight at 4 °C with 50 µl/well with anti-bovine IFN-γ capture monoclonal antibody CC302 diluted at 1 µg/ml in sterile coating buffer in a cell culture hood. Tap plate gently to remove bubbles and to spread the antibody solution over the well surface. Wrap plates in aluminum foil or Parafilm and place it at 4 °C overnight.
2. Flick off coating antibody and wash plates 2× with washing medium (in sterile hood). Avoid drying of wells. Use multipipette to add 200 µl sterile washing buffer. Block plate for 2 h at 37 °C with 200 µl/well sterile blocking medium.
3. Flick off blocking medium (in sterile hood). Replace with 50 µl/well antigens at appropriate concentrations, e.g., peptide at 1 µM diluted in complete RPMI. Include medium control (no antigen) and mitogen (e.g., Concanavalin A) control wells. Peptides can be tested in the range from 10^{-6} to 10^{-12} M.
4. When peptides are used as antigens, monocytes are purified from the respective animals with anti-human CD14 MACS beads using the MACS separation system as described in beginning of Subheading 3.2. CD14 cells are used at 10 % of added purified CD8 cells. For example, 2.5×10^4 cells/well are used with a concentration of 2.5×10^5 CD8 cells/well.
5. If *T. parva* infected cell lines are used as antigens, cells are first inactivated by radiation (caesium source, 5000 rad) or alternatively use mitomycin (test manufactures suggested concentrations) and rested overnight in the CO_2 incubator before use, due to production of IFN-γ by the cells. Always include these rested infected cells alone as controls.
6. Prepare cells at appropriate dilutions in complete RPMI. If cell dilutions (PBMC) are tested: make cell dilution starting from for example 1×10^7 cells/ml in 96-well plate and transfer 50 µl/well in to the ELISPOT plate. Include control wells with no cells. If ex vivo purified CD8 cells are used, a start concentration of 2.5×10^5 cell/well/50 µl is used. If CTL lines are used a start concentration between 5×10^3–10^4 cells/well is used. Put the plate on shaker for 2 min to shake and distribute cells evenly.
7. Incubate the ELISPOT plate for 20 h at 37 °C in humidified incubator with 5 % CO_2. Ensure that plates are leveled for cells to be evenly distributed.
8. Flick off well contents, add 200 µl dH_2O-T/well and shake plates on shaker for 30 s. Repeat 3× (4× in all).
9. Repeat washing by adding 200 µl PBS-T and shake plates on shaker for 30 s. Repeat 3× (4× in all).
10. Remove excess PBS-T by tapping plates on paper towels, add 50 µl/well rabbit anti-bovine IFN-γ anti-sera (in-house, ILRI)

diluted 1/1500 in PBS-T/BSA and incubate for 1 h at RT. Instead of this antibody another polyclonal anti-bovine IFN-γ antibody can be used. Polyclonal anti-bovine IFN-γ antibodies are also commercially available.

11. Flick off well contents and wash by adding 200 μl/well PBS-T. Repeat 3× (4× in all).
12. Remove excess PBS-T by tapping plates on paper towels, add 50 μl/well of monoclonal anti-rabbit IgG-alkaline phosphatase conjugate and incubate for 1 h at RT.
13. Flick off well contents and wash by adding 200 μl/well PBS-T. Wash plates 6× in all with PBS-T (without shaking between washes).
14. Remove excess PBS-T by tapping plates on paper towels, add 50 μl/well BC-NIP substrate solution and incubate plates in the dark for 10 min at RT. To prepare the substrate, dissolve one tablet of Sigma Fast BCIP/NBT buffered substrate tablet in 10 ml distilled water by mixing for 2–3 min (Vortex or use a roller mixer). Pass the solution through 0.2 μm filter to remove un-dissolved substrate particles. Keep plate in the dark during spot development (10 min).
15. Flick off substrate and wash with copious amounts of tap water for 2 min/plate, also remove plastic manifold and wash back of wells.
16. Air-dry plates in the dark at RT and read using ELISpot reader.

3.3 Cytotoxicity

CTL assays are composed of a number of discrete methods, namely the generation of CTL (in this case from PBMC), which require two to three in vitro re-stimulations, labeling of target cells (here: 51-Chromium) and then the actual CTL assay.

3.3.1 Generation of CTL Lines to Theileria parva from PBMC in Cattle

Primary in vitro stimulation:

1. Obtain blood from an immunized animal and separate peripheral blood mononuclear cells (PBMC) using Ficoll density centrifugation as described under flow cytometry.
2. Resuspend PBMC at 4×10^6 cells/ml in complete RPMI 1640 (without HEPES).
3. Distribute 1 ml/well into 24-well tissue culture plates.
4. Irradiate a sufficient amount of autologous (or MHC matched) *T. parva* infected cells using a Caesium source for 30 min (expose to 5000 rads). If irradiation is not possible, mitomycin can be added to the infected cells in the recommended dose and time followed by washing before the cells are added to the PBMC. Resuspend cell at 2×10^5/ml.
5. Add 1 ml/well into the 24-well plate containing the PBMC.
6. Incubate the cells for 7 days in a humidified CO_2 incubator (5 %).

Secondary in vitro stimulation:

7. OPTIONAL: Harvest the cells and layer on Ficoll and centrifuge at $1300 \times g_{AV}$ for 20 min at RT to remove the dead cells (if many are dead). Harvest cells from the interface, dilute with an equal volume of cytotoxicity medium and pellet at $600 \times g_{AV}$ for 10 min. Resuspend the pellet and wash once at $300 \times g_{AV}$ for 10 min.
8. Resuspend the cells in complete RPMI at a concentration of 4×10^6 cells/ml and stimulate for a second time with irradiated autologous (or MHC matched) infected cells as described under "primary stimulation".
9. Incubate in the incubator for 7 days. At this stage, the CTL can be used in assay.

Tertiary in vitro re-stimulation:

10. OPTIONAL: purification of CD8 cells. Re-stimulation of the bulk cultures may propagate other cell subsets than CD8 cells. Therefore, we normally purify the CD8 cells before the tertiary re-stimulation. Use the MACS purification procedure described under the ELISpot procedure.
11. Harvest the cells from the plates, separate viable cells from the culture by centrifuging on Ficoll as described under secondary in vitro stimulation (*optional*) or harvest, wash and resuspend cells and stimulate for the third time as described above. However, for tertiary stimulation, the cell inputs are as follows: stimulated (responder) cells 2×10^6 per well with 4×10^5 autologous infected cells (stimulator) and 2×10^6 irradiated autologous PBMC as filler cells.
12. If there is a need to continue propagating the cultures after incubation for 7 days, separate viable cells as described above or harvest cells from the cultures as before and stimulate weekly using 1×10^6 responder cells per well, 4×10^5 stimulator cells and 3×10^6 filler cells.

3.3.2 Chromium-51 Release Assay Using Infected Cells or Peptide Pulsed PBMC as Targets

Cytotoxicity assays can be used to study the killing of target cells by, e.g., cytotoxic T-cells or NK cells. The principle is that the radioactive isotope Chromium-51 is incorporated in a target cell population. If the target cells are mixed with a CTL population that recognizes the target cells, these will release the chromium to the supernatant, which then can be measured in a scintillator.

The lysis of target cells is calculated as:

$$\%\,\text{specific release} = \frac{\text{Sample } {}^{51}\text{Cr release value} - \text{spontaneous release value}}{\text{Maximum } {}^{51}\text{Cr release value} - \text{spontaneous release value}} \times 100$$

Sample release: These values are obtained from samples where both CTL and targets are present in, e.g., various ratios.

Spontaneous release: This value is obtained from a control sample where ONLY the ^{51}Cr-labeled target cells are present.

Maximum release: This value is obtained from a control sample where the target cells are totally lysed by detergent.

Effector target ratio: Often, the effector cells (CTL) are titrated while the number of target cells are kept constant to give different ratios of effector to target cells (E:T ratios). For polyclonal CTL cultures, the effector–target ratio will normally be between 1 and 100. For clones less than 100.

Labeling of the Target Cells

Infected cell lines.

1. Harvest *T. parva* infected lymphoblast cells which are in exponential growth, centrifuge and resuspend at 2×10^7 cell/ml in cytotoxicity medium with HEPES.
2. Mix 50 µl of the target cells (10^6 cells) with ^{51}Cr, pre-diluted to 1 mCi/ml in a sterile 10 ml tube. Use 10 µl pre-diluted ^{51}Cr for infected cell lines and 20 µl for labeling of PBMC (*see* **Note 20**). Incubate for 1 h at 37 °C in a CO_2 incubator.
3. Add 10 ml of cytotoxicity medium to the cells and spin at $300 \times g_{AV}$ for 5–10 min at RT.
4. Break the cell pellet and wash 2× (10 ml) if a suction method is used to remove the supernatant and 3× if the supernatant is poured. Carefully, remove as much supernatant as possible.
5. Resuspend the labeled target cells in cytotoxicity medium at a concentration of 1×10^6 cells/ml.

Preparation of Cytotoxic T Cells (Effectors)

Peptide pulsed PBMC (or other target cells).

1. Count target cells and harvest, e.g., 10^6 cells, spin down and resuspend in 2–4 ml of complete RPMI.
2. Pulse with 1 µM of peptide of choice for 1 h at 37 °C.
3. Wash once with medium, resuspend cell pellet in 100 µl complete RPMI.
4. Add 10 µl of Chromium, incubate for 1 h *at* 37 °C and resuspend cells by shaking the vial two to three times during the incubation.
5. Wash 3× and adjust to 1×10^6 cells/ml in complete RPMI.
6. Harvest effector cells from 24-well plates (polyclonal CTL cultures or CTL clones).
7. OPTIONAL: Separate on Ficoll in order to remove dead cells and debris as described previously.
8. Resuspend in cytotoxicity medium, adjust cell concentration dependent on the desired effector/target ratio (E:T ratio), e.g., 1×10^7/ml will result in a E:T ratio of 40:1. Clones are normally used between 1 and 5×10^6/ml.

3.3.2.1 Cytotoxicity Assay

9. Distribute in duplicates or triplicates (100 µl/well) in two- or threefold dilutions of the effectors in 96-well culture plate (flat bottomed). Dilutions are done in the plate starting from the first row with 150 µl/well (threefold dilutions) or 200 µl/well (twofold dilutions).
10. *Threefold dilutions*: 50 µl is transferred to 100 µl medium in the row below which is resuspended and 50 µl from this is transferred to the next etc. Remember to throw out the last 50 µl. *Twofold dilutions*: 100 µl from each well is transferred from the upper row to the one below, mixed and so forth as for the threefold dilution. This can be done for the whole plate using a multichannel pipette.
11. Add to each well, containing effectors cells, 50 µl of labeled target cell (5×10^4) resulting in an effector to target cell ratio starting from 40:1.
12. Add in triplicate to separate wells, 50 µl of the target cell suspension to 100 µl of cytotoxicity medium, to measure *spontaneous release*.
13. Add also 50 µl in triplicates of target cells to empty wells for *maximum release*.
14. Incubate the plate(s) for 4 h at 37 °C in a CO_2 incubator.
15. After 4 h of incubation, mix the cells with a multichannel pipette and centrifuge the plate(s) for 5 min at RT $300 \times g_{AV}$.
16. Transfer maximal 50 µl of supernatants (or less) to a LumaPlate except for the maximum release wells. Be careful not to carry over cells in the supernatants from the bottom. This will result in a very high level of counts (*see* **Note 21**). Then, add 100 µl of 1 % Triton X-100 to each of the maximal release control wells. Resuspend and transfer same amount to the LumaPlate as for the other samples.
17. Check that the samples cover the bottom layer of the LumaPlate wells and leave the plate to dry overnight in the incubator or at RT or in an oven (not over 40 °C).
18. Put a piece of Topseal™ on top of the plate and count in a TopCounter on program for measuring ^{51}C.

3.4 Immunoinformatics

1. Access the NetMHC pan website. See link under Subheading 2.
2. Enter amino acid sequence into the first field in FASTA format.
3. Select the peptide length. Commonly, the selection of 8–11-mer peptides will give the most accurate results as it will select all possible peptides from your protein, or proteome, of interest with binding affinity to your MHC molecule under study.
4. Select species and MHC class I allele.

5. Submit and receive the results either online or by email.
6. With the list generated of potential peptide epitopes select the peptides among the top 2–3 % in the list and test them individually, or as longer overlapping peptides, with cells isolated from immunized/infected animals.

4 Notes

1. Layer the blood on Ficoll-Paque using 50 ml Falcon tubes. Add the blood by pouring it slowly onto the inner face of the Falcon tube when the tube is tilted to slow down the speed of blood reaching the surface of the Ficoll.
2. $600 \times g_{AV}$ is used because the first harvest of PBMC often will contain some Ficoll. The presence of Ficoll in the suspension makes it necessary to centrifuge at a higher g force to spin down the cells. Using $300 \times g_{AV}$ at this step may result in loss of cells.
3. Evaluation of tetramer positive cells sometimes requires 5×10^5–1×10^6 cells to quantify low frequency tetramer positive cells.
4. The number of wells to use will depend on the number of samples and different markers to evaluate in the assay.
5. Alternatively PBS with 2 % FBS can be used.
6. This step is important only when performing intracellular staining. When using p-MHC tetramers and intracellular staining, cells should be incubated first with the tetramers and subsequently washed before fixing and permeabilizing the cells; otherwise free fluorochrome-streptavidin from the tetramer preparation will enter the cells and all the cell population will appear positive at the analysis step. If only surface staining is performed, then fixing of cells is only required at the end of the assay before analysis with the flow cytometer (fixing is not necessary if the samples are analyzed the same day as the staining is performed).
7. Alternatively, this step can also be performed overnight at 4 °C.
8. If using p-MHC class I tetramers, this step should be done after incubating with the latter and washing/fixing the cells.
9. The set of antibodies to use for evaluating a cellular immune response towards a vaccine depends on the nature of the study. For analysis of CTL, relevant antibodies can include an antibody against the CTL marker CD8 [we use ILRI generated monoclonal antibody ILA51 diluted 1:250], in combination with for example antibodies against, interferon (IFN)-gamma

(γ), tumor-necrosis factor (TNF)-alpha (α), perforin and/or Fas-ligand (L) (Santa Cruz, sc-957; dilution 1:10).

10. Particular attention to the antibody isotype combination used is important if not handling directly fluorochrome-coupled antibodies in order to avoid cross reaction with the secondary antibodies used for labeling the primary antibodies.
11. If intracellular staining is performed, stain cells with the tetramers before staining with the antibodies for intracellular markers.
12. If using "one-pot mix and read" tetramers as described in [22], then the volume of tetramers resulting in optimal staining is 10 μl per $2–5 \times 10^5$ cells.
13. If staining of IL-2, IFN-γ, TNF-α, or other intracellular staining is performed, cells should be incubated with brefeldin A or monensin at 1.25 μg/ml and 2 μM, respectively concentration for 16 h before starting the staining process. This will block secretion of the cytokines and allow for accumulation of the intracellular protein of interest to sufficient amount to enable detection in flow cytometry.
14. For the first wash, use 150 μl of PBS-0.5 % BSA in each well, spin down at $830 \times g_{AV}$ for 3 min; flick off and add 200 μl of the PBS-0.5 % BSA and repeat the wash and spinning down.
15. The secondary antibody can be coupled to another fluorochrome, e.g., allophycocyanin (APC).
16. If samples will be evaluated the following day, samples should be resuspended in PBS/2 % formalin or PBS/1 % paraformaldehyde, kept at 4 °C and covered from light. Samples should not remain more than 2 days at 4 °C prior to analysis as cellular autofluorescence properties will change over time and this will result in having less defined populations.
17. As compensation controls, we include cells which are stained with either an anti-CD8 antibody or an anti-CD3 antibody followed by staining with a secondary antibody coupled to PE, FITC, PerCP, or any other fluorochrome that is used for automatic compensation by the BD FACS Diva software.
18. Dead cells can be stained with various fluorochromes, such as propidium iodide (PI), 7-aminoactinomycin D (7AAD), which emit in the same range as PE and PerCP, respectively. Alternatively, there are some commercially available products for use with a violet laser like BD Horizon Fixable Viability Stain 450 (BD, cat no. 562247). Single cells can be identified by plotting Forward Scatter Height (FSC-H) versus Forward Scatter Area (FSC-A) and/or Side Scatter Height (SSC-H) versus Side Scatter Area (SSC-A) followed by gating on the straight line.

19. In-house ILRI antibodies.
20. Chromium-51 is in general not a very toxic radioisotope but it is always good practice to reduce radiation as much as possible, e.g., keep the stock in a lead safe behind lead protection while taking the necessary aliquots and keep the cells in old lead containers when they incubate in the incubator. Radioisotope labs in different countries may have slightly different safety rules, so it is necessary to be aware of those.
21. In case cells are accidentally sucked into the pipette tips, they can be put back and the plate is spun again. This happens occasionally and it is mandatory to spin down the plate again.

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