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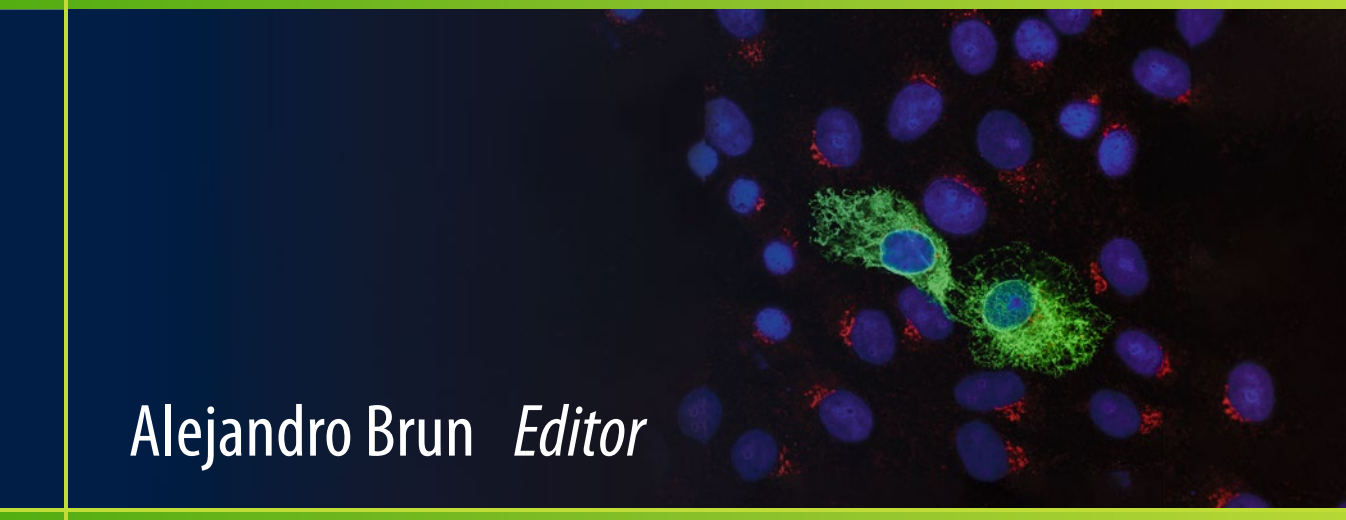

Alejandro Brun *Editor*

Vaccine Technologies for Veterinary Viral Diseases

Methods and Protocols

Second Edition

Humana Press

METHODS IN MOLECULAR BIOLOGY

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Edited by

Alejandro Brun

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Preface

This book presents a number of specific, useful protocols for antigen production, experimental antigen delivery, and the analysis of immune responses upon vaccination. Experimental vaccines for the prevention of animal diseases can often be tested in their natural hosts' or target species allowing a direct efficacy assessment in field settings. Since many human infectious diseases have an animal origin, this collection of techniques goes beyond the scope of animal's health and can provide very useful information for human vaccine development as well. This book edition complements a previous release including updated chapters as well as completely new ones. Subunit, genetic, or virally encoded, all of these antigen delivery techniques illustrate the many ways in which an antigen of interest or well-known immunogen can be provided through vaccination and the different immune mechanisms that can be triggered, constituting an open and highly interesting research field. Protocols have been carefully edited to provide the reader with an easy approach to different techniques that can be adapted to their particular needs.

Madrid, Spain *Alejandro Brun*

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Chapter 1

An Overview of Veterinary Viral Diseases and Vaccine Technologies

Alejandro Brun

Abstract

Ensuring the maximum standards of quality and welfare in animal production requires developing effective tools to halt and prevent the spread of the high number of infectious diseases affecting animal husbandry. Many of these diseases are caused by pathogens of viral etiology. To date, one of the best strategies is to implement preventive vaccination policies whenever possible. However, many of the currently manufactured animal vaccines still rely in classical vaccine technologies (killed or attenuated vaccines). Under some circumstances, these vaccines may not be optimal in terms of safety and immunogenicity, nor 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. In the context of viral diseases of veterinary interest, we overview current vaccine technologies that can be approached, with a brief insight in the type of immunity elicited.

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

1 Viral Diseases of Animals and the Need for Vaccination

One of the greatest landmarks in history occurred when mankind shifted from a hunter-gatherer to an agricultural lifestyle. During millenniums livestock and companion species (ruminants, swine, poultry, equids, cats, and dogs) were domesticated and raised, first for survival, then for profit and commerce. Since then, animal husbandry evolved as one of the most important activities for civilization and development. Today the importance of such activity is enormous since the proper management of land use and animal resources is necessary to avoid malnutrition and famine in underdeveloped countries or in countries where intensive subsistence farming is essential. The explosive 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 or insect protein will be more demanded, in view of the

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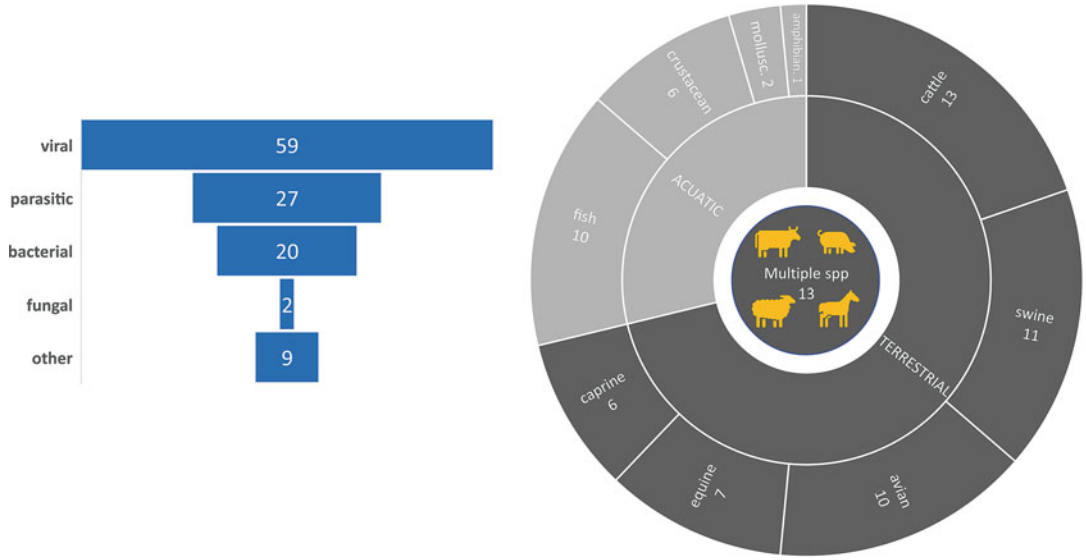

Fig. 1 Animal diseases of viral etiology are the most abundant. The OIE includes a list of animal diseases according to several criteria. More than half of the 117 listed OIE diseases of animals are caused by viruses. Currently, diseases listed by the OIE are 88 terrestrial and 29 aquatic diseases. Most of these diseases can be prevented or ameliorated by vaccination (with the exception of retroviral diseases) stressing the importance of vaccine technology research to maintain the highest standards of animal husbandry and production

significant impact of animal husbandry on world's environment [1]. On the other hand, the intensive farming of animal species inexorably leads to the onset of diseases caused by the 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 eradication should be available.

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, more than half of the 117 most important animal diseases are caused by viruses (Fig. 1), according to the OIE's list for terrestrial and aquatic notifiable animal diseases (*see* also Table 1). The listed viral diseases comprise viruses from 22 different families and four families of virus (*Herpes, Rhabdo, Pox, and Paramyxoviridae*) concentrate a high number of diseases (Fig. 2). Many other viral infections affecting wildlife pose also a threat to global health. 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.

Preventing transmission of infectious diseases at the animal–human interface is important for protecting the world population and ecosystems from both epizootics and pandemics, constituting

Table 1
The OIE's listed 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[b]	Reoviridae	Orbivirus	Live attenuated/inactivated
Crimean Congo hemorrhagic fever[a]	CCHFV[b]	Nairoviridae	Nairovirus	Not available
Equine encephalomyelitis (Eastern)[a]	EEEV[b]	Togaviridae	Alphavirus	Inactivated
Foot and mouth disease	FMDV	Picornaviridae	Aphthovirus	Inactivated
Infection with Aujeszky's disease virus (Pseudorabies)	SHV-1	Herpesviridae (*subfam.* α-herpesvirinae)	Suid Herpesvirus	Attenuated (deletion of glycoproteins gE, gC, gG)
Infection with rabies virus and other lyssavirus[a]	RABV	Rhabdoviridae	Lyssavirus	Inactivated/Attenuated/ Recombinant poxvirus; Adenovirus
Infection with rinderpest virus	RPV	Paramyxoviridae	Morbillivirus	Attenuated (Plowright and LA-AKO vaccines)
Japanese encephalitis[a]	JEV[b]	Flaviviridae	Flavivirus	Inactivated/Attenuated
Rift Valley fever[a]	RVFV[b]	Phenuiviridae	Phlebovirus	Attenuated/Inactivated
Vesicular stomatitis[a]	VSV[b]	Rhabdoviridae	Vesiculovirus	Inactivated/Attenuated
West Nile fever[a]	WNV[b]	Flaviviridae	Flavivirus	Inactivated/Attenuated/ Recombinant canarypox/DNA vaccine (USA)/Chimeric YF-WNV vaccine
Epizootic hemorrhagic disease	EHDV[b]	Reoviridae	Orbivirus	Inactivated/Attenuated (licensed USA, Japan)
Nipah and Hendra virus diseases[a]	NiV HeV	Paramyxoviridae	Henipaviruses	Soluble glycoprotein subunit (Australia)

(continued)

Table 1
(continued)

Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
Cattle diseases				
Bovine viral diarrhea	BVDV	Flaviviridae	Pestivirus	Inactivated/Attenuated
Enzootic bovine leukosis	BLV	Retroviridae	Lentivirus	Not available
Infectious bovine rhinotracheitis/ Infectious pustular vulvovaginitis	BoHV-1	Herpesviridae (α-herpesvirinae)	Varicellovirus	BoHV-1 gE-deleted either inactivated or attenuated (EU)
Lumpy skin disease	LSDV	Poxviridae	Orthopoxvirus	Live attenuated (LSDV/SPV)
Malignant catarrhal fever	(AlHV-1) (OvHV-2).	Herpesviridae	Gammaherpesvirus	Not available
Sheep and goat diseases				
Border disease	BDV	Flaviviridae	Pestivirus	Killed whole virus vaccine
Caprine arthritis/encephalitis	CAEV	Retroviridae	Lentivirus	Not available
Infection with peste des petits ruminants virus	PPRV	Paramyxoviridae	Morbillivirus	Attenuated Recombinant capripoxvirus or adenovirus (not validated)
Maedi-visna	MVV	Retroviridae	Lentivirus	Not available
Sheep pox and goat pox	SPV	Poxviridae	Orthopoxvirus	Inactivated/ Attenuated
Ovine pulmonary adenocarcinoma (infection with Jaagsiekte sheep retrovirus)	JSRV	Retroviridae	Beta retrovirus	Not available
Equine diseases				
Equine encephalomyelitis (Western)	WEEV[b]	Togaviridae	Alphavirus	Inactivated

Equine infectious anemia	EIAV	Retroviridae	Lentivirus	Not available
Equine influenza[a]	EIV	Orthomyxoviridae	Influenzavirus	Inactivated/Recombinant canarypox expr. HA
Equine rhinopneumonitis (Infection with equid herpesvirus-1/4)	EHV-1/4	Herpesviridae	Herpesvirus	Inactivated/Attenuated
Infection with equine arteritis virus	EAV	Arteriviridae	Arterivirus	Inactivated/Attenuated
Venezuelan equine encephalomyelitis[a]	VEEV[b]	Togaviridae	Alphavirus	Inactivated or Attenuated TC-83 strain
Infection with African horse sickness virus	AHSV[b]	Reoviridae	Orbivirus	Live attenuated (mono or polyvalent)
Swine diseases				
African swine fever	ASFV[b]	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
Influenza A virus of swine	IAV-S	Orthomyxoviridae	Orthomixovirus	Inactivated
Swine vesicular disease	SVDV	Picornaviridae	Enterovirus	Not available
Transmissible gastroenteritis/ porcine respiratory coronavirus infection	TGEV/PRCV	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

(continued)

Table 1
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Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
Duck virus hepatitis	DHV-1	Picornaviridae	Avihepatovirus	Inactivated/Attenuated
Duck virus enteritis	DEV-1	Herpesviridae (α-herpesvirinae)	Anatid herpesvirus-1	Attenuated
Avian Influenza (Infection with avian influenza viruses)[a]	AIV	Orthomyxoviridae	Influenzavirus A	LPAI inactivated/Recombinant fowlpox/Recombinant NDV/Recombinant DHV (China)
Fowl pox	FPV	Poxviridae	Avipoxvirus	Modified live attenuated
Infectious bursal disease (Gumboro disease)	IBDV	Birnaviridae	Avibirnavirus	Inactivated/Attenuated/ Recombinant herpesvirus/VP2-subunit vaccine
Newcastle disease[a]	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	Leporipoxvirus	Live attenuated
Rabbit hemorrhagic disease	RHDV	Caliciviridae	Lagovirus	Recombinant poxvirus
Other infections				

Camelpox	CPV	Poxviridae	Orthopoxvirus	Inactivated/Attenuated
Bunyaviral infections (*Akabane, Cache Valley, Schmallenberg and Nairobi sheep disease*)	AKAV[b] CVV[b] SBV[b] NSDV[b]	Peribunyaviridae/Nairoviridae	Orthobunyavirus Orthobunyavirus Orthobunyavirus Nairovirus	Inactivated (SBV and AKAV) Inactivated (NSDV, experimental) Not available (CVV)
Fish diseases				
Infection with HPR-deleted or HPR0 infectious salmon anemia virus	ISAV	Orthomyxoviridae	Isavirus	Inactivated
Infection with salmonid alphavirus	SAV	Togaviridae	Alphavirus	Inactivated
Infectious hematopoietic disease	IHNV	Rhabdoviridae	Novirhabdovirus	Inactivated,/DNA
Spring viraemia of carp	SVCV	Rhabdoviridae	Vesiculovirus	Not available
Viral hemorrhagic septicemia	VHSV	Rhabdoviridae	Novirhabdovirus	Not available
Oncorhynchus masou virus disease	OMDV	Alloherpesviridae	Salmonivirus	Formalin inactivated
Viral encephalopathy and retinopathy (viral nervous necrosis)	VNN	Nodaviridae	Beta-nodavirus	
Koi herpesvirus disease	KHV	Alloherpesviridae	Cyprinivirus	Live attenuated
Epizootic hematopoietic necrosis	EHNV	Iridoviridae	Ranavirus	Not available
Red sea bream iridoviral disease	RSIDV	Iridoviridae	Megalocytivirus	Formalin inactivated
Mollusc diseases				
Infection with Ostreid Herpesvirus 1 microvariants	OsHV-1	Herpesviridae (Malacoherpesviridae)	Ostreavirus	Not applicable
Abalone virus ganglioneuritis (Infection with abalone herpesvirus)	AbHV	Herpesviridae (Malacoherpesviridae)	Aurivirus	Not available

(continued)

Table 1
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Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
Crustacean diseases				
Infectious hypodermal and hematopoietic necrosis	IHHNV	Parvoviridae	Penstyldensovirus	Not developed
Infectious myonecrosis	IMNV	Totiviridae	Totivirus	Not developed
Taura syndrome	TSV	Dicistroviridae (Picornavirales)	Aparavirus	Not developed
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]Zoonotic disease
[b]Arthropod-borne virus (arbovirus)

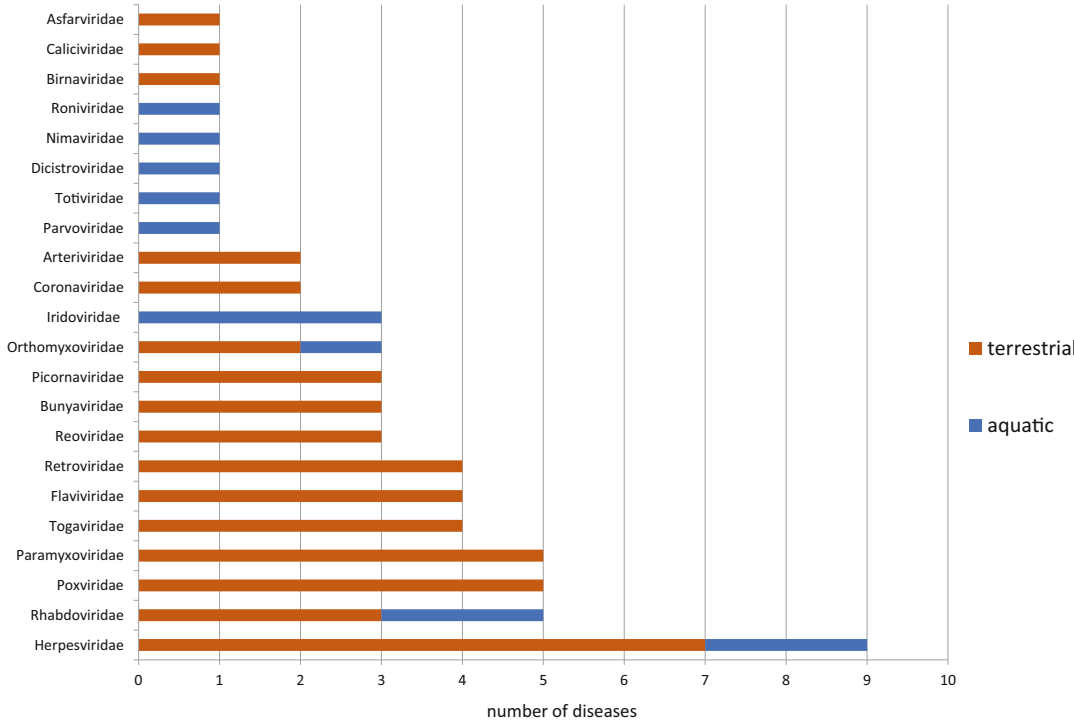

Fig. 2 Families of virus causing notifiable animal diseases. The figure depicts the viral families that include pathogenic viruses causing for terrestrial and aquatic animals

the basis for the "One Health" concept [2, 3]. In this sense, 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 "classic" 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 fish vaccines 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 [4] (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. It was frequently pointed out the convenience to support efforts to control emerging viral pathogens where they primarily occur, in order to avoid the rapid spread of pandemic viruses [5, 6]. It has been also demonstrated the importance of continuous research on technologies for vaccine design since they constitute powerful platforms to rapidly generate new experimental vaccines based on previous knowledge about protective host's immune responses: our previous knowledge in experimental adenovirus-based or RNA vaccines against animal virus infections has undoubtedly contributed to the rapid

deployment of COVID-19 vaccines. Apart from the need for efficacious vaccine approaches, the COVID-19 pandemic has been a "warning for surfers" or a "wake-up call" as it appears more necessary than ever to apply stricter measures to protect the ecosystems from human disruptive actions.

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 and, eventually, eliminate any intruder pathogen. This can be achieved providing the appropriate antigenic stimulus (the vaccine) to activate cellular mechanisms involved in recognition of the non-self. Thus, an efficient vaccine needs to be recognized as a non-self-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 naive B and T lymphocytes (Fig. 3).

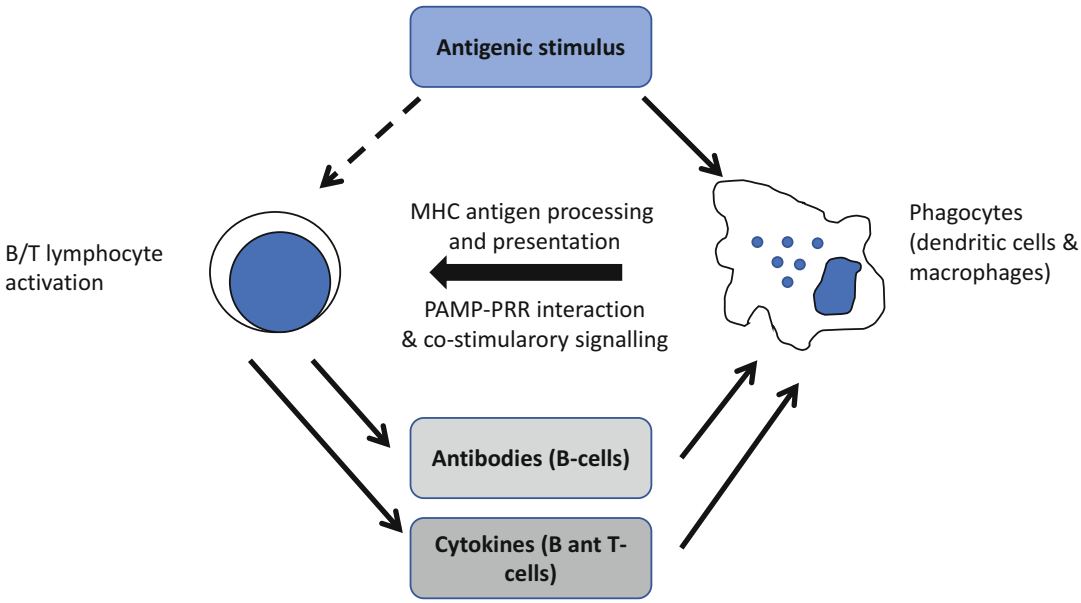

Fig. 3 The cellular cooperation in the immune response upon vaccination. Specialized phagocytes present the processed antigens to naive B- or T-cells that may become activated only if proper co-stimulatory signals are produced (derived from the interaction of PAMPs with cellular PRRs). Activation drives lymphocytes to secrete soluble mediators and antibodies initiating inflammatory responses (adapted from Roitt's Immunology second ed)

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 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 [7]. In particular, encountering of intracellular pathogenic virus ligands (such as single or double stranded RNA molecules) to PRRs activates the phagocytic cell 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 interferon regulatory factor (IRF)-mediated transcription of type I-interferons (IFNs) and other cytokines such as IL-1β and TNF [8]. Depending on the type of receptor involved in PAMP interaction and the tissue location in which occur, there will be a distinctive response of cytokine pattern that will dictate the type of immune response output. Apart from phagocytes other immune cell types such as innate lymphoid cells (ILCs) are present in epithelial barrier and mucosal tissues. ILCs do not carry T-cell receptors but can differentiate in subsets (mirroring those of CD4+ T lymphocytes) and are activated by proinflammatory cytokines [9]. They can be considered early immune-regulators and may also influence the shape of subsequent adaptive responses (see Chapter 8 in this volume). Also from the innate side, natural killer (NK) cells express functional TLRs specifically for detecting viral PAMPs and can be also activated by type I interferons [10]. 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 augments the quality and magnitude of the adaptive response in contrast to that elicited by vaccines based on inert antigens (inactivated virus or subunit vaccines) (Fig. 3). 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 [11, 12].

Upon naïve 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

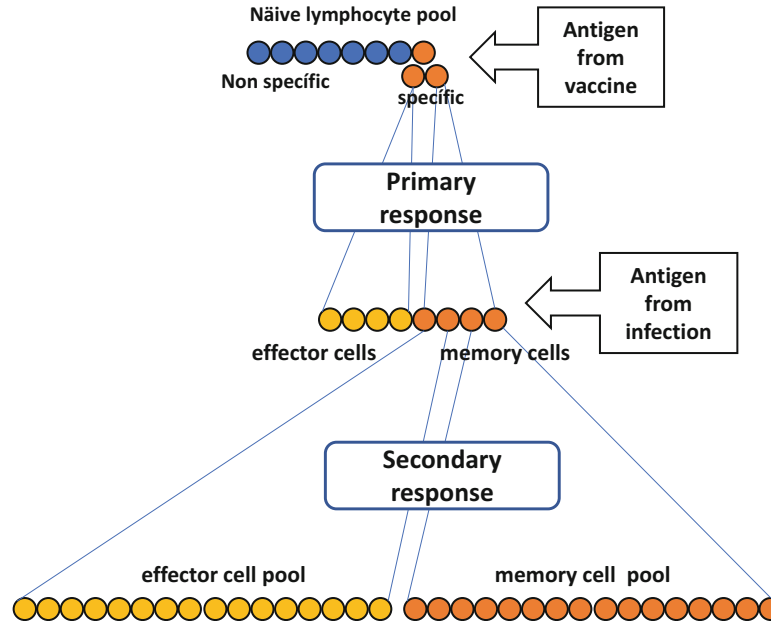

Fig. 4 Vaccination exploits the induction of specificity and immunological memory. A primary clonal expansion of lymphocytes is produced upon activation of naïve T-cells by phagocytes primed with specific vaccine antigens/stimulus. Both effector and memory cell pools are generated that upon encounter with pathogen (infection) will undergo a massive secondary expansion of both cell pools (adapted from Roitt's Immunology second ed)

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. 4). 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 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 CD4+ helper or CD8+ cytotoxic functions [13]. While humoral immunity neutralizes and eliminates extracellular virus, effector T-cell immunity enhances killing of viruses inside phagocytes and eliminates virus infected cells. Different populations of CD4 + T effector cells (i.e., Th1, Th2, or Th17) can be elicited upon vaccination. In general, while Th1 and Th17 cells play an important role in inflammation, Th2 cells are usually

involved in allergic reactions. This is why some vaccines that polarize toward an excessive Th2 response may trigger harmful adverse reactions. Ideally, the knowledge about immune correlates of protection should be derived from the pathogenesis of viral infection in the target species to which a vaccine is intended for but, unfortunately, these type of studies are often more difficult to perform in natural hots 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 viral immunopathogenesis in 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 vaccine 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 [14]. Methods for the analysis of cellular responses to vaccines can be found in Chapter 15 in this volume.

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 allows to categorize vaccines into four general classes or types (Table 2).

In our categorization type I vaccines include those produced by means of virus inactivating methodologies while type II vaccines include all attenuated virus used as vaccines, including those generated by reverse genetics. The 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, such as carrier micro/nanoparticle or virus-like particles (VLPs) vaccines produced by recombinant technologies in

Table 2
A proposed classification for the current vaccine technologies

Virus	Whole antigen	Fraction/component
Dead (no replicative)	**Type I** (inactivated, killed vaccines)	**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)

both prokaryotic and eukaryotic cells, and inactivated recombinant vectors expressing structurally heterologous vaccine antigens. In this category, both nucleic acid 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, synthetic RNA or self-replicating RNA molecules or 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 for replicate by generally chemical or physical (heat, radiation) 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

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 Adjuvanted	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 Animal tissue	Parenteral/ mucosal	none	Single. Repeated	Humoral, and cellular including CTL responses	+
IIIa. Subunit and carrier technologies, glycoconjugate and peptide vaccines, microparticle and nanoparticle formulations, virus-like particles	Adjuvanted	Prokaryotic cell culture, eukaryotic cell culture, Plant based. Chemical synthesis	Parenteral / mucosal	Chemical/Molecular	Repeated	Humoral and Th	++++
IIIb. Nucleic acid	VpG, delivery, liposome Self-amplifying RNA	Prokaryotic cell culture	Parenteral	Molecular	Repeated	Humoral and cellular	+++
IV. Viral vector based	Adjuvanted (molecular)	Mammalian, insect, plant cell culture	Parenteral	None/molecular	Single Repeated	Humoral and	+++

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 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, Fc-receptors bearing cells (i. e monocytes or macrophages) can be infected by virus complexed to non- or sub-neutralizing antibodies, a process well reported in dengue virus infections [15]. 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, thus preserving immunogenicity during the inactivation of viruses. However, it has been reported that BPL may also react to some amino acids including cysteine, methionine, and histidine so certain modification of proteins may occur affecting the immunogenicity of BPL vaccines (i.e., by altering the structure of viral capsids) [16, 17]. Similarly, BEI (a compound widely used for the inactivation of foot and mouth disease virus (FMDV) in the preparation of vaccines) has also been shown to react with proteins if used at high concentrations [18]. 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.

Other inactivation approaches are based in the use of hydrogen peroxide or protonating compounds, such as diethyl-pyrocarbonate (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 [19, 20]. Using a histidine-protonating agent such as DEPC it was reported the abolishment of vesicular stomatitis virus (VSV) infectivity and pathogenicity in mice [21]. These animals survived a further lethal challenge and this protection was associated to the induction of neutralizing antibodies although no further reports have arisen since the first description.

Other classical inactivation techniques by physical methods are based on direct exposure to several types of radiation: thermal (heat), electromagnetic (light) or ionizing (gamma rays). Short-wave ultraviolet light radiation (UV-C) has been one of the most used in the human vaccine manufacturing. More recently, ultra-short pulsed (USP) laser treatment has been reported to successfully inactivate an influenza virus strain without affecting its biological and immunological properties [22].

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.

5 Type II Vaccine Technologies

Live attenuated virus vaccines are among the most successful forms of vaccines particularly with regard 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 loss 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, the hepatotropic Rift Valley fever virus serially passaged in brain tissues failed to replicate efficiently in liver though acquired neurovirulence [23]. Other classical attenuation methods induce temperature sensitive (ts) mutants grown at lower temperatures rendering virus variants unable to replicate at normal temperatures in their hosts or random mutations using antiviral compounds with mutagenic properties such as nucleoside analogous.

The main advantages of attenuated vaccines over inactivated or killed vaccines or subunit vaccines can be related with a broader 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 viral peptides will be presented through MHC class-I restriction), and also with the possibility of administration by similar or natural routes of infection (i.e. nasal/mucosal route for influenza vaccines [see Chapter 13 in this volume]). The immune responses elicited should resemble those 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 hand, possible disadvantages of attenuated virus vaccines are the genetic instability, allowing reversion to virulence or loss of replicating phenotype, problems related with immunosuppressed individuals, or deleterious effects of some attenuated vaccines when used in pregnant 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

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, and 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 process	Herd immunity (most if vaccine spreads)	Storage limited Risk for pregnancy
		Single dose administration	Interference with live virus (pre-existing immunity). Presence of defective interfering particles
			Discrimination of vaccinates and infected animals more difficult
			Immunosuppression

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) [24] has allowed to develop novel attenuated vaccines with enhanced safety features (see Chapters 12, 13, and 14 in this volume). For DNA viruses defining virulence and/or immunomodulatory genes allowed its deletion by homologous recombination techniques [25].

In most of the cases the modification of these genomes introduces an important characteristic for veterinary vaccines: the possibility to differentiate infected from vaccinated animals [26]. 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, and 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 produce subunit vaccines are based on bacteria, yeast, insect or mammalian cells with continuous improvements being made using more sophisticated approaches (see Chapters 2, 3 and 4 in this volume). 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 (see Chapter 5 in this volume). In live insects (Lepidoptera) recombinant baculoviruses can be used to infect insect larvae and transgenic silkworms can be also generated (reviewed in [27]). 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, and 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 unilamellar liposomes, termed virosomes. These synthetic structures can be also complemented with immune-stimulatory conjugates or even heterologous molecules such as DNA, siRNA, and antibody fragments (reviewed in 27). 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 [28]. 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 [29] 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 [30]. 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 [31]. 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 class-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, allow differentiation of vaccinated and infected animals (DIVA), antigens 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 (see Chapter 3 in this volume). DNA vaccination has been so far successful in mice models of disease. However, experimental DNA vaccination in large animals against livestock viral diseases has been rather unsuccessful and 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 using stronger promotors, replicon-based plasmids (Alphavirus), increasing plasmid uptake efficiency or by co-delivery of immune-stimulatory molecules. The only DNA vaccines licensed to date have been against WNV in horses (now retired from market) and VHS in salmonids [27]. Nonetheless, it remains an 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 offering an undoubtedly attractive exploratory field to ascertain the suitability of vaccine antigen candidates (see Chapter 6 in this volume). As appealing as DNA vaccines, mRNA vaccines appear to be the preferred choice in view of the fast and successful generation of several COVID-19 vaccines. RNA vaccines proposed also 20 years ago were not developed faster due to the intrinsic lower stability of RNA over DNA. Thus, in comparison to DNA vaccines, fewer attempts have been made to provide immunity in experimental animal models [32, 33]. However, RNA offers different ways to develop prophylactic vaccines. RNA can be used also as a potent adjuvant (see Chapter 7 in this volume) for subunit vaccines. One of the more obvious advantages of RNA over DNA is the lack of a nuclear localization step, dodging potential integration or editing (splicing) events by host's nuclear enzymes. Provided stability, conservation, and cost of production issues are solved, mRNA vaccine technology might be further used to improve the quality of current vaccines in the veterinary field considering that many of the strategies used to immunomodulate DNA vaccines could be applied to RNA as well.

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 viruses by means of homologous recombination or CRISPR-Cas9 techniques (see Chapter 11 in this volume). Among the DNA viruses used to deliver vaccine antigens Poxvirus (from both orthopoxvirus (see Chapter 10) and parapoxvirus genus), Herpesvirus, Adenovirus (see Chapter 9), 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, 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 [34]. 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 [34]. 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 [34]. 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 [35, 36]. For some bunyavirus the identification of virulence genes non-essential for growth in vitro allowed replacement for reporter genes or other viral antigens ([37, 38] and Chapter 12 in this volume). As attenuated virus 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, and 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 [39] 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 usages or in silico epitope prediction algorithms, may allow development of platforms for experimental vaccine antigen candidates [40]. Though these approaches are far from being generalized they hold promise on the future of rationale vaccine design for some relevant viral diseases [41, 42].

9 Concluding Remarks

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. Nonetheless, a transition from successful experimental vaccines to industrial production and manufacturing may become a bottleneck 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.

The following chapters illustrate a number of different techniques to provide antigen delivery in order to develop vaccines against animal diseases. Though the number of techniques and protocols in this volume is not exhaustive, they show a varied number of technologies available that can be developed further for other veterinary diseases since most of the techniques can be virtually adapted for a particular antigen of interest. Among them, representative protocols for each of the broad categories for vaccine technologies discussed above. Some chapters are also included related to different techniques and protocols for analyses of the cellular immune responses or to the analysis of particular immune cell populations since vaccine technologies and the evaluation of the immune responses go necessarily hand in hand.

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Chapter 2

Production and Purification of Candidate Subunit Vaccines by IC-Tagging Protein Encapsulation

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Abstract

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

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

1 Introduction

We have developed a molecular tagging system that has different applications [1–6]. This method is particularly useful to generate particulate material that potentiates the host immune response for vaccination purposes [7–9]. Our method encompasses two components: (i) Protein muNS-Mi: this is a truncated version of avian reovirus (ARV) protein muNS that forms the matrix of the globular viroplasms produced by ARV in the infected avian cells [10]. Protein muNS-Mi forms close-to-spherical, ordered inclusions when being expressed either in insect cells with the baculovirus protein expression system, mammalian cells or bacteria (Fig. 1). The size of the baculovirus-produced inclusions range between 1 and 4 μm, so we named them "microspheres"or MS [7], while those produced in bacteria are 400 nm in diameter and thus named nanospheres or NS [5]. (ii) The second component of our method is a 66 residues long domain of muNS-Mi, called Intercoil or IC that we use to tag a foreign protein of interest either in its N or C terminus. The presence of the IC tag has no effect whatsoever in the distribution or activity of the tagged protein by itself. However, if muNS-Mi is

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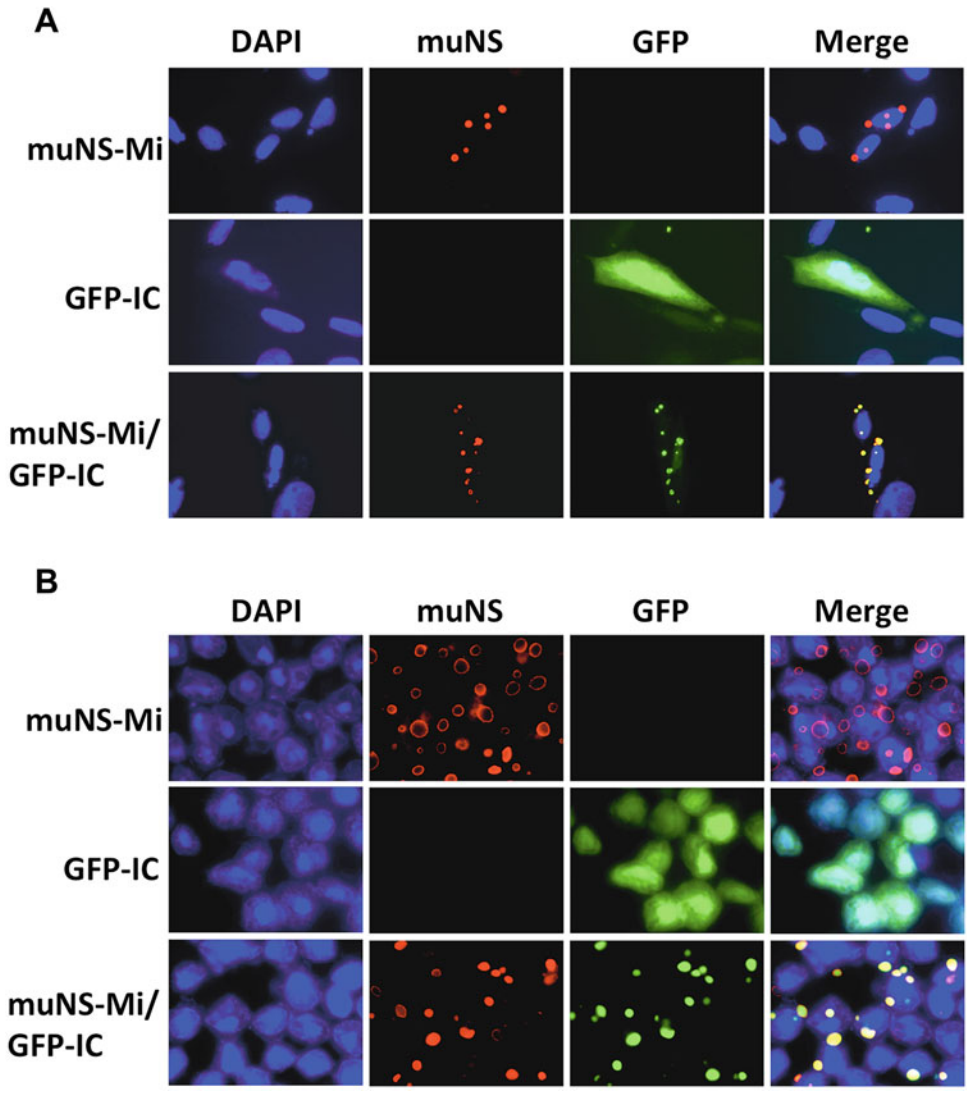

Fig. 1 Capture of IC-tagged proteins in muNS-Mi **microspheres.** (**a**) The figure shows CHO-K1 cells that express the proteins indicated at the left of the figure, after transfection with the corresponding expression plasmids (see text). In green is shown the autofluorescence of the GFP protein (GFP column). muNS-Mi microspheres are shown in red as detected by indirect immunofluorescence using primary antibodies directed again ARV protein muNS (column muNS), and Alexa Fluor 594 anti-rabbit secondary antibody. Nuclei are 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

co-expressed in the same cell, the tagged protein will be efficiently relocated to muNS-Mi MS or NS.

Thus, we can load such spheres with any foreign epitope and use them for immunization purposes. They will carry many copies of the tagged epitopes and be combined with other MS carrying

different epitopes to generate multivalent vaccines. Also, the different epitopes can be simultaneously loaded into the same spheres with identical purpose. The latter is particularly suited for complex epitopes formed by the interaction of several different proteins. We have previously shown that presentation of epitopes in muNS-Mi MS 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 [7]. The intrinsic adjuvancy of the MS was confirmed with a different virus, AHSV, where, used alone or in combination with MVA, produced also complete protection against viral challenge [9]. Here we describe how to generate and purify muNS-Mi MS (Fig. 2b) and NS (Fig. 2a) loaded with foreign IC-tagged antigens to use them for immunization.

2 Materials

2.1 Baculovirus Expression System (MS)

2.1.1 Cells and Tissue Culture

1. Sf9 cells.
2. Chinese Hamster Ovary cells (CHO-K1, ATCC-CCL-61).
3. Eukaryotic expression vector (pCDNA3.1 Zeo + or equivalent).
4. Fetal Bovine Serum (FBS).
5. 100× Penicillin-Streptomycin-Glutamine solution.
6. SF-900II serum-free medium (SFM) supplemented with 10% FBS and 1% Penicillin-Streptomycin-Glutamine solution (*see* **Note 1**).
7. Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FBS and 1% 100× Penicillin-Streptomycin-Glutamine solution.
8. 1.3× SF-900II medium.
9. 4% low melting point agarose: prepare in milli-Q water and sterilize by autoclaving.
10. 0.33% Neutral Red solution.
11. Phosphate Buffered Saline (PBS): 137 mM NaCl; 2.7 mM KCl; 8 mM Na_2PO_4; 1.5 mM KH_2PO_4. Adjust to pH 7.3 with NaOH and sterilize by autoclaving.
12. 0.25% Trypan Blue solution in PBS: sterilize by filtration.
13. Eukaryotic cell transfection reagent: i.e. Lipofectamine (Life Technologies).
14. Incubator (28 °C) for monolayer cultures of Sf9 cells.
15. Shaking incubator (28 °C) for suspension cultures of Sf9 cells.

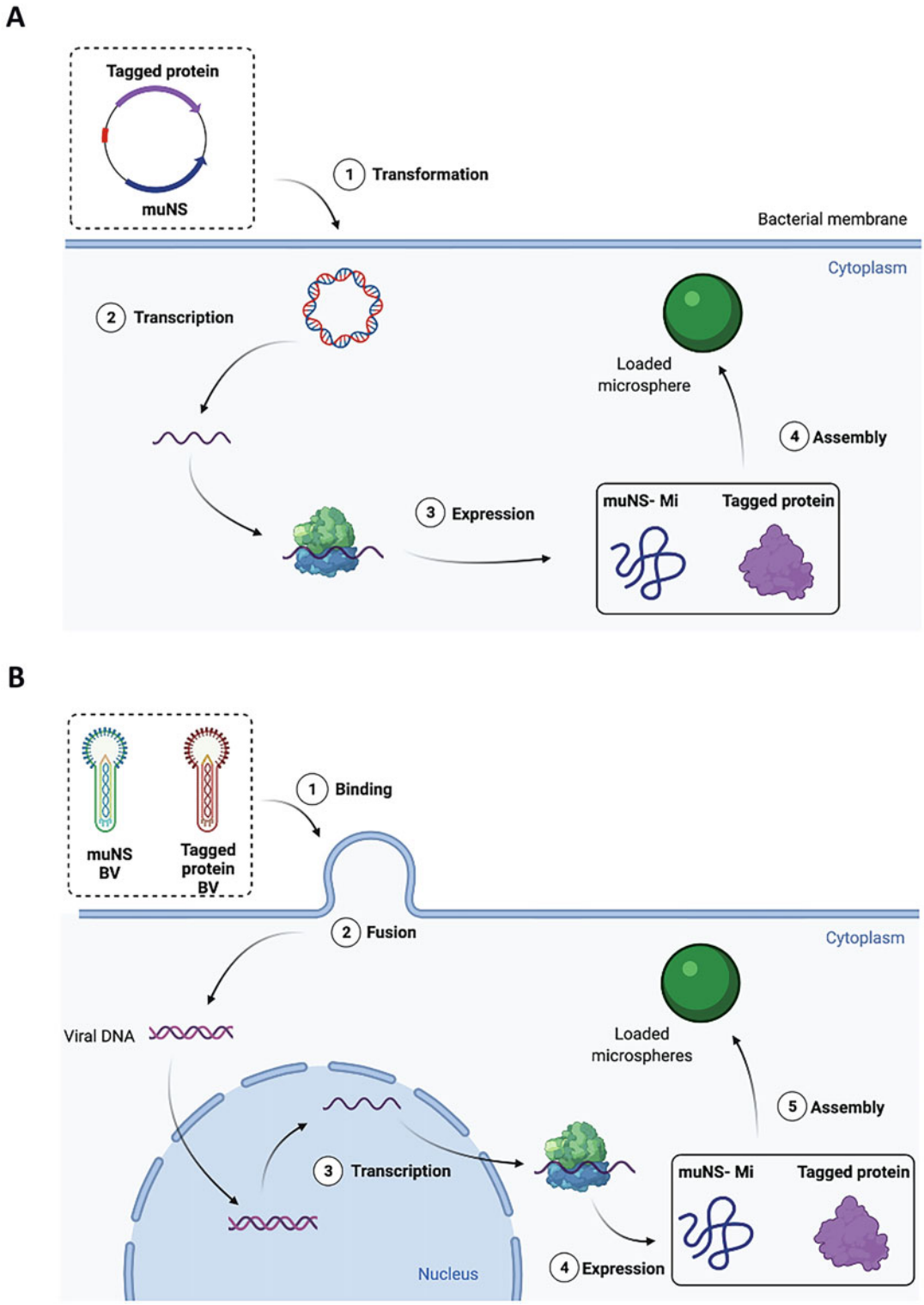

Fig. 2 Expression strategies for NS and MS production. (**a**) NS are produced in bacteria by co-expression of muNS-Mi and the IC-tagged epitope, both directed from the two polylinkers of a dual expression plasmid. (**b**) For producing MS, two recombinant baculoviruses, one for muNS-Mi and a second one for the IC-tagged epitope, are used to co-infect Sf9 insect cells

16. Incubator at 37 °C with 5% CO_2 atmosphere and humidity for culturing CHO-K1 cells.
17. Membrane filters (pore diameter 0.22 μm).
18. Autoclave for sterilization of materials and solutions.
19. Glass Erlenmeyer flasks (250 mL–1 L) for suspension cultures.
20. Plastic tissue culture (TC) flasks (75 and 150 cm^2) and multi-well plates for monolayer cultures.
21. Aluminum foil.
22. Protease Inhibitor Cocktail (we use a solution from Sigma Aldrich/Merck containing 23 mM AEBSF, 100 mM EDTA, 2 mM Bestatin, 0.3 mM Pepstatin A, and 0.3 mM E-64).
23. Neubauer chamber.
24. Eppendorf microfuge tubes; 15–250 mL centrifuge tubes.

2.1.2 Fluorescence Microscopy

1. Phosphate Buffered Saline (PBS).
2. Primary antibodies against ARV muNS protein were raised in our laboratory [6].
3. Alexa Fluor® 594 goat anti-rabbit IgG (H + L) antibody.
4. Fixing solution: 4% PFA in PBS. 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 pore-size membrane and store at 4 °C.
5. Blocking solution: 2% BSA in PBS. Add 2 g of BSA to 100 mL PBS, mix, filter through 0.22 μm pore-size membrane and store at 4 °C.
6. Permeabilization solution: 0.5% Triton X-100 in PBS. Add 0.5 mL of Triton X-100 to 100 mL PBS, mix and filter through 0.22 μm and store at 4 °C.
7. 1000× DAPI DNA staining solution: 0.1 mg/mL DAPI in water or PBS. Mix until completely dissolved and store at −20 °C. Keep a working aliquot in the dark at 4 °C.
8. Mowiol/DABCO solution: Mix 2.4 g of Mowiol (supplied by Calbiochem), 6 g of glycerol, and 6 mL of water and incubate on a shaker 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 2.5% DABCO (triethylenediamine, $N2[C_2H_4]3$) to final concentration of 0.1% (w/v). Store in 500 μL aliquots at −20 °C.
9. Microscope slides, round coverslips of 15 mm diameter and coverslips of 22 × 60 mm size.
10. Laminar flow hood.

2.1.3 Microsphere (MS) Purification and Analysis

1. Resuspension Buffer minus magnesium (RB-): 10 mM HEPES pH 7.9, 10 mM KCl.
2. Resuspension Buffer minus magnesium, plus Triton X-100 (RB-T): 10 mM HEPES pH 7.9, 10 mM KCl, 0.5% Triton X-100.
3. Resuspension Buffer plus magnesium (RB+):10 mM HEPES pH 7.9, 10 mM KCl, 5 mM $MgCl_2$.
4. Radioinmmunoprecipitation assay buffer (RIPA): 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 0.5% Sodium Deoxycholate, 0.1% SDS, 1% NP40, 5 mM $MgCl_2$.
5. Protease inhibitor cocktail.
6. Refrigerated centrifuge with adaptor for 15 mL and 50 mL conical centrifuge tubes.
7. Ultrasonic liquid processor or sonicator, with a small probe/tip able to be inserted in a 15 mL conic centrifuge tube.
8. 10% sodium dodecyl sulfate (SDS) in water.
9. Spectrophotometer (i.e., Nanodrop 2000 or similar).

2.2 Bacterial Expression System (NS)

1. *E. coli* strain XL1-Blue (Stratagene).
2. BL21-CodonPlus-RP (DE3) (Agilent Technologies) and Rosetta (DE3) Competent Cells (Novagen).

2.2.1 Bacterial Cell Cultures

3. Luria-Bertani (LB) broth culture media.
4. Ampicillin sodium salt.
5. Incubator (37 °C) for bacterial cells.
6. Shaking incubator (37 °C) for suspension bacterial cell cultures.
7. Glass Erlenmeyer flasks (250 mL to 2 L) for suspension cultures.
8. Petri dishes.
9. Aluminium foil.
10. Isopropyl-β-D-1-thyogalactopyranoside (IPTG).
11. Membrane filters (pore diameter 0.22 μm) and autoclave for sterilisation of materials and solutions.

2.2.2 Nanosphere (NS) Purification and Analysis

1. Lysis buffer: 0.25% Tween-20, 1 mM DTT, 200 mM NaCl, 20 mM Tris pH 7.5, 2 mM $MgCl_2$.
2. RB− buffer + EDTA: 10 mM HEPES pH 7.9; 10 mM KCl; 0,5 mM EDTA.
3. RB+ buffer: 10 mM HEPES pH 7.9; 10 mM KCl; 5 mM $MgCl_2$.
4. RB+ T buffer: 10 mM HEPES pH 7.9; 10 mM KCl; 5 mM $MgCl_2$; 0.5% Triton X-100.

5. Protease inhibitor cocktail.
6. Refrigerated centrifuge with adaptor for 15 mL and 50 mL conical centrifuge tubes.
7. Ultrasonic liquid processor or sonicator, with a small probe/tip able to be inserted in a 15 mL conic centrifuge tube.
8. 10% sodium dodecyl sulfate (SDS) in water.
9. RIPA Buffer: 150 mM NaCl; 50 mM Tris pH 7.5; 0,5% Sodium Deoxycholate; 0.1%.
10. Lysozyme (Sigma Aldrich).
11. French press homogeneizer (i.e., Avestin Emulsiflex C5 or similar).
12. Ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich).

3 Methods

3.1 Baculovirus Expression System (MS)

3.1.1 Growing Sf9 Cells and Baculoviruses

1. Put 50 mL of Sf9 cells in SF-900 II SFM medium supplemented with 10%FBS 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 28 °C with moderate shaking (130 rpm).
2. Three days later, take a small aliquot of the culture, mix with Trypan Blue solution (*see* **Note 3**) and count cells by placing a small amount of the mixture on a Neubauer chamber.
3. When cell concentration rises over 1–3 $\times$ 10^6 cells/mL (usually three days), dilute the culture 1/10 in fresh medium and repeat the incubation.
4. For amplifying baculovirus stocks, take 21 $\times$ 10^6 Sf9 cells from the suspension culture and place them on a 175 cm^2 TC flask. Wait until they stick 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 28 °C (*see* **Note 4**).
6. Decant the incubation medium into a 50 mL centrifuge tube and centrifuge at 500 $\times$ *g* for 5 min to remove cells and large debris. Store the supernatant it in the dark at 4 °C.
7. Before discard them, sterilize by autoclaving all the materials used.

3.1.2 Titration of Baculovirus Stocks

1. In 6-well plates, plate 1.2 $\times$ 10^6 cells/well and allow to settle at least 1 h.
2. While cells are settling, prepare serial dilutions of the baculovirus stocks in SF-900 II SFM medium without supplements.

We will start with the 10^{-2} dilution: put 10 μL of the virus stock in an Eppendorf tube containing 990 μL of SF-900 II medium. 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 obtain the next dilution and so on (*see* **Note 5**).

3. Replace medium from cells with 800 μL of SF-900 II medium 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 28 °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 SF-900 II SFM medium supplemented with 10% FBS and 1% glutamine-antibiotics on top of the solid medium and incubate at 28 °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 (and no longer than 12) (*see* **Note 7**).
9. Remove the staining solution and count plaques under a trans-illuminator by turning the 6-well plates upside-down.
10. Calculate virus titer: Mean plaque number in the duplicate samples x dilution order x inoculum dilution (*see* **Note 8**).

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

1. In a laminar flow hood, immerse round coverslips in methanol, sterilize them by flaming and put them in sterile, tissue culture 12 well plates.
2. Trypsinize monolayers of CHO-K1 cells (or the cells of your choice, *see* **Note 9**). Count them as above in the Neubauer chamber and place them on top of the sterilized coverslips from the previous step at a concentration of 1.5×10^5 cells/well (coverslip).
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 alone or using the following combinations: A + B and A + C.
4. Incubate cells 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 Incubate at 37 °C for at least 15 min.
6. Wash once with PBS, and permeabilize the cells by incubating 5 min at room temperature with permeabilization solution.

7. Wash once with PBS and incubate for 1 h with blocking solution on a rocking platform at room temperature.
8. Proceed to 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 corresponding just to the epitopes. For most antibodies, a dilution of 1/1000 in PBS or blocking solution will work.
9. Wash three times with PBS and add the secondary antibody of your choice following the manufacturer instructions (*see* **Note 11**).
10. Remove the coverslips with forceps and place them on a slide on top of a drop of mounting medium, cells facing down (*see* **Note 12**).
11. Let them dry for at least one 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**).

3.1.4 MS Production and Purification

1. Dilute Sf9 cells grown in suspension at least 10 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 28 °C for 6–7 days: take aliquots every few days 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 1. 700 × *g* and 4 °C (*see* **Note 15**).
5. Wash twice with 10 mL PBS in a 15 mL centrifuge tube, spinning as in 4 in each wash.
6. Resuspend the pellet in RIPA buffer (10 mL) containing the protease inhibitor cocktail and leave 5 min on ice.
7. Introduce the sonicator tip in the cell solution 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 once in 10 mL of RIPA buffer.
10. Wash the pellet twice in 10 mL of RB-T buffer.
11. Wash the pellet once in 10 mL of RB+ buffer and resuspend it finally in 1 mL of RB+ buffer for quantification.

12. 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**).
13. 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**).
14. Protein quantification is performed using the NanoDrop™ 2000/2000c spectrophotometer.

3.2 Bacterial Expression System (NS). Nanosphere Production and Purification

1. The whole desired construct containing the target epitope which will be loaded into the NS, is cloned into the MCS2 of the plasmid pET Duet-Mi to generate the pET Duet-Mi-IC-epitope (*see* **Note 19**).
2. Transform BL21 CodonPlus-RP (DE3) or Rosetta (DE3) competent bacteria with plasmid pET Duet-Mi-target-epitope, plate on ampicillin-containing LB-agar plates and incubate overnight. Pick a single colony in 5 mL of LB medium and incubate at 37 ° C in shaking conditions overnight.
3. The next day dilute the culture 1:25 an incubate at 37 °C in shaking conditions to reach a OD_{600} ~ 0.6–0.8.
4. Induce expression with 1 mM IPTG and incubate for 3 h at 37 °C with shaking at 210 rpm.
5. Stop shaking and pellet bacteria at 3200 × *g* for 30′ at 4 °C.
6. Wash the pellet twice in PBS and resuspend in 1/10 volume of lysis buffer with protease inhibitor and keep it frozen (*see* **Note 15**).
7. Incubate the thawed pellet for 30 min with 1 mg/mL lysozyme at 37 °C in the same lysis buffer.
8. Lyse bacteria by passing the suspension three times through a French Press homogenizer (*see* **Note 20**).
9. Centrifuge the NS at 2700 × *g*, 5 min at 4 °C.
10. Wash the pellet three times in RB + T buffer.
11. Wash the pellet three times in RB + buffer.
12. Resuspend the pellet in 1 mL of RB-buffer with 0.5 mM EDTA for quantification.
13. Check the presence of NS at the microscope at 1000× magnification (*see* **Note 21**).
14. 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**).
15. Protein quantification is performed by standard protein quantification techniques after NS disassemble (*see* **Note 18**).

4 Notes

1. We use SF-900 II because cells reach higher densities in this medium, but we add anyway serum on it to avoid proteolysis when cells break after the 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). The idea is to have an outer (dirty) lid that should be removed when the culture is taken into the laminar flow hood, where the inner (clean) double layer will serve as lid once conveniently loosen.
3. We dilute the cell suspension five times with Trypan blue solution (400 μL Trypan Blue solution 0.25% in PBS(1×) plus 100 μL of cell suspension), before loading 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 a 4% LMP agarose solution in the microwave. When completely melted, mix with cold 1.3× SF-900 medium: 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 200 μL of the corresponding dilution, plus 800 μL of medium, that makes a inoculum dilution factor of 5 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 it is difficult to decide if the IC-Tagging is working when observing baculovirus-infected Sf9 cells. This is because some proteins produce precipitates when overexpressing them in the baculovirus system, and also because insect cells are spherical and their nuclei increase in size upon baculovirus

infection. Thus, testing the system in a typically flat (adherent) mammalian cell line makes easier the observation of the possible capture of the IC-tagged protein by muNS-Mi MS.

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 according to 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 1000X solution to 1X.
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. An identical example, but performed in Sf9 cells was included in Fig. 1 for comparison. In this particular case, the recruiting of IC-tagged GFP to muNS-Mi microspheres is perfectly seen in both cell lines.
14. The microspheres can be obtained 3 days after infection with the recombinant baculoviruses [2], but we observed that the method is more reproducible, consistent, and less baculovirus is needed when leaving the infections 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 sonication was productive we use to check the cell solution at the microscope before and after sonication to be sure that nuclei were broken.
17. At this point, and when necessary, a single sonication pulse is usually enough.
18. In some particular preparations, the microspheres are difficult to dismantle and a variable amount of protein is not able to enter the separating (running) PAGE gel, accumulating in the concentrator (stacking) or in the bottom of the wells. Thus, previous to PAGE, take 50 μL, centrifuge at 10,000 × *g* for

5 min and discard supernatant. Resuspend the pellet in 50 μL of 10% SDS and incubate for 15 min at room temperature to dismantle the microspheres. Then, we mix them with SDS-PAGE loading buffer, boil them 5 min and proceed with the electrophoretic run.

19. Generation of plasmid pET Duet-Mi was previously described [5], and bears the sequence of muNS-Mi on the first polylinker of (Novagen's) pET Duet. Using dual plasmids guarantees a balanced co-expression of both proteins in the same cell.
20. A different homogenizer can be used but French press lysis produce very clean nanosphere preparations.
21. The NS are very small and should be observed without drying. Thus, a cloud of loose, moving particles should be seen, instead of patches of clumped material. It is also advisable to check dispersity by DLS analysis.

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Chapter 3

Elastin-like Polymers as Nanovaccines: Protein Engineering of Self-Assembled, Epitope-Exposing Nanoparticles

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Abstract

In this chapter we describe two unconventional strategies for the formulation of new nanovaccines. Both strategies are based on obtaining chimeric genes that code for proteins in which the major antigens of the pathogens are fused to an elastin-like recombinamer (ELR) as carrier. ELRs are a family of synthetic protein biopolymers obtained using DNA recombinant techniques. The ELRs employed in the present chapter are block copolymers that are able to assemble, under controlled conditions, into nanoparticles similar to virus-like particles and to provoke an immune response. We describe the biosynthesis of ELRs genetically fused to an antigenic sequence from *Mycobacterium tuberculosis* and a simple procedure for obtaining stable nanoparticles displaying the antigen in the first strategy. The second approach describes the production of a DNA vaccine library consisting of plasmids codifying for major antigens from Rift Valley fever virus fused to different ELR-based block copolymer architectures.

The procedures described can be adapted for the production of other chimeric DNA-protein vaccines based on protein polymer carriers.

Key words Recombinant protein polymers, Nanovaccines, Elastin-like polymers, Genetic engineering, Virus-like particles, DNA vaccination

1 Introduction

The current global pandemic has highlighted the importance of developing effective and safe vaccines using alternative and versatile procedures and has accelerated the scientific and social acceptance of historically unconventional vaccines. The series of vaccines developed using different genetic-engineering approaches clearly shows the possibilities of developing innovative, safe, and effective vaccines that comply with strict and rigorous authorization procedures and safety regulations at all times [1]. Apart from the antigen chosen, the design of the carrier or adjuvant molecule is also of

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great importance for the success of the prospective vaccine [2]. Some of the most interesting alternative carriers for vaccines include the recombinant production of protein backbones that spontaneously form the desired macrostructures and which can be genetically complemented with antigenic fragments [3, 4].

During the design of these recombinant protein devices, multicomponent chimeric genes with a careful balance between the gene encoding the structural part or carrier responsible for automatically forming virus-like particles, fused with the antigenic peptide that should be displayed on the device's surface, are constructed. The chimeric genes may be expressed in a heterologous expression system to obtain the multifunctional polypeptides for administration as protein nanoparticles [3] or cloned into a plasmid vector suitable for RNA-DNA vaccination in mammalian hosts [5].

Elastin-like polymers (ELPs) are bioinspired, synthetic biopolymers derived from selected motifs from one of the extracellular matrix proteins, namely natural elastin. Almost all of them comprise repeat motifs from the elastomeric domains of elastin, such as the pentapeptide Val–Pro–Gly–Xaa–Gly, as a monomeric component of their sequence (the guest residue (Xaa) can be any naturally occurring L-amino acid except proline) [6]. The monotonous molecular structure of ELPs confers a set of interesting properties, such as stimuli-responsiveness, biocompatibility, and self-assembling behavior [7], but also technical difficulties that hinder their synthesis [8, 9]. The recombinant production of elastin-like polymers has allowed the generation of large numbers of specific ELPs whose complexity at a molecular level and accuracy and reproducibility of production means that this technique has supplanted other synthetic methods. These biopolymers obtained using genetic-engineering techniques are commonly referred to as elastin-like recombinamers (ELRs) [10].

The potential of ELRs in the biomedical field as advanced biomaterials or therapeutic agents, including fusion partners with antigens for the development of nanovaccines that induce a similar or better immune response than that found for more traditional vaccines, has been widely explored. ELRs have been synthesized to combat many different diseases. The different ELR-based vaccines include the porcine circovirus type 2 antigen of the capsid protein-based virus-like particle (VLP) vaccine for porcine circovirus-associated disease [11, 12], which generates higher protection than commercial vaccines, or the M2e influenza antigen fusion ELR [13], both of which are produced in *Escherichia coli*. Furthermore, plants have also been developed as expression hosts, for example, the fusion of Ag85B/ESAT-6 antigens to ELRs for the development of a subunit vaccine against tuberculosis disease [14], or the fusion of ELRs to the beak and feather disease virus (BFDV) capsid protein for a subunit vaccine against Psittacine beak and

feather disease [15], both of which have been successfully produced in tobacco plants. ELRs have also been studied as cytotoxic T lymphocyte (CTL) vaccine carriers, with promising results [16, 17].

The purpose of this chapter is to describe two different vaccination strategies employing ELRs as vaccine carriers and the corresponding protocols.

2 Materials

2.1 Reagents

2.1.1 Materials for Genes Construction and Expression

- β-Mercaptoethanol.
- Acrylamide/Bis-acrylamide.
- Agarose Seakem (Cambrex).
- Agarose gel Extraction Kit or filter.
- Ampicillin.
- Ammonium persulphate (APS).
- Assay to analyze the protein endotoxin content.
- Bromophenol Blue.
- Chloridric acid.
- Copper chloride (CuCl).
- Coomassie Brilliant-blue R-250.
- D (+) glucose.
- Dimethyl sulfoxide (DMSO).
- Disodium hydrogen phosphate (Na_2HPO_4).
- dNTPs mix.
- DNA primers (Table 2).
- DNA Modifying Enzymes: FastAP phosphatase (Fermentas); Shrimp alkaline phosphatase (SAP), *Pfu* Turbo DNA Polymerase; T4 DNA ligase.
- Dialysis membranes.
- *E. coli* strains: -XL1-Blue (Stratagene): endA1 supE44 hsdR17 thi1 recA1 gyrA96 relA1 lac [F′ proAB lacIq ZΔM15 Tn (Tetr)] s; BLR(DE3) (Novagen): F-ompT hsdSB(rB- mB-) gal dcm Δ (srl-recA) 306:: Tn10 (Tetr) (DE3).
- Ethanol.
- Ethylenediaminetetraacetic acid (EDTA).
- EndoFree Plasmid Maxi Kit.
- Ethylenediaminetetraacetic Acid.
- General Labware: glassware (bottles, flasks, beakers, pipettes, etc.) is sterilized by autoclaving, plastic ware (tips, conical

tubes, microcentrifuge tubes, vials, serological pipette, cell culture flasks, 96 multiwell black clear bottom plate (Greiner CELLSTAR), multiwell Plate, 0.2 μm pore size filters, cell culture chamber slides (CellTreat)), is provided sterile and DNA/RNA free.

- Glycerol.
- Molecular weight markers: DNA and protein Ladders.
- Phenylmethylsulfonyl fluoride (PMSF).
- Plasmids: pDrive Cloning Vector (Qiagen); modified pET-25b (+) Expression plasmid (Novagen).
- Plasmid Mini and midi-prep Kit.
- Site-directed Mutagenesis Kit (i.e., Stratagene's QuickChange).
- Restriction enzymes: -*Dpn*I; *Ear*I (*Eam*l 104I); *Eco*RI; *Hind*III; *Sap*I (*Lgu*I) and buffers.
- Sodium dodecyl-sulfate polyacrylamide gel electrophoresis SDS-PAGE.
- Simply safe nucleic acid stain (Eurx).
- Sodium chloride (NaCl).
- Sodium dodecyl sulfate (SDS).
- Sodium hydroxide.
- Tris(hydroxymethyl)aminomethane (Tris).
- Tetramethylethylenediamine (TEMED).
- Ultrapure water type I.

2.1.2 Materials for Analysis of Gn Glycoprotein In Vitro Expression

- 293 T: Human embryonic kidney cells (ATCC-CRL 1573).
- Black-/clear-bottomed 96-well plates.
- Diamidino-2-phenylindole dihydrochloride (DAPI).
- Dulbecco's modified Eagle's medium (DMEM).
- Fetal bovine serum FBS.
- Glutamine.
- Goat anti-mouse Alexa Fluor 488-conjugated antibody.
- Transfection reagents (i.e., Lipofectamine® LTX Plus Reagent and Turbofect™).
- Milicell® EZ Slides (Merck Millipore).
- Paraformaldehyde (PFA): 4% Formaldehyde Solution in PBS.
- Penicillin/Streptomycin 10,000 units/mL of penicillin and 10,000 g/mL of streptomycin.

- RVFV-immunized mouse serum (Kindly given by Dr. Alejandro Brun).
- Triton X-100.

2.1.3 Materials for Analysis of In Vivo Biodistribution in Mice

- 293 T Human embryonic kidney cells (ATCC-CRL-1573).
- Anhydrous *N*,*N*-dimethylformamide (DMF).
- BALB/c mice aged 12–16 weeks.
- Ethanol.
- Isoflurane.
- Potassium acetate CH_3COOK.
- Psoralen-PEG_3-Biotin (Thermo Scientific).
- Qdot® 800 streptavidin conjugate.

2.2 Buffers and Media

All solutions are prepared with nuclease-free water and molecular biology grade reagents.

- Bacterial Media: *Luria-Bertani* (LB) medium: tryptone 10 g/L, NaCl 10 g/L, yeast extract 5 g/L pH 7.0. LB-Agar: LB, 1.5% (p/v) Agar pH 7.0. *Modified Terrific Broth* medium (mTB): tryptone 12 g/L, yeast extract 24 g/L, glycerol 4 mL/L, $(NH_4)_2$ SO_4 3.3 g/L, KH_2PO_4 6.8 g/L, Na_2HPO 7.1 g/L 4, glucose 0.5 g/L, lactose 2.0 g/L, $MgSO_4$ 0.15 g/L. *SOC* medium (Super Optimal broth + Catabolic repressor): Tryptone 20 g/L; 5 g/L yeast extract; 10 mM NaCl, 2,5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose. All the bacterial media are sterilized in an autoclave at 120 °C and 1 atmosphere for 20 min.
- DNA loading buffer 5×: 30% (v/v) glycerol, 0.1% (w/v) SDS, 0.05% (w/v) bromophenol blue, 50 mM Tris, 0.05 mM EDTA pH 8.
- Phosphate-buffered saline (PBS) pH 7.4: Na_2HPO_4 10 mmol/L, KH_2PO_4 1.8 mmol/L, NaCl 137 mmol/L, KCl 2.7 mmol/L.
- Protein loading buffer 5× pH 6.5: 0.3 M Tris, 10% (w/v) SDS, 50% (v/v) glycerol, 25% (v/v) β-mercaptoethanol, 2% (v/v) bromophenol blue in ultrapure water type I.
- Protein stain buffers: Copper chloride-based negative staining, 0.3 M $CuCl_2$; Coomassie stain 0.1% Coomassie blue, 40% methanol (v/v), 10% acetic acid (v/v).
- SDS-PAGE Running buffer (pH 8.3): 25 mM Tris-base pH 8.3, 192 mM glycine, and 0.1% (w/v) SDS.
- Sonication buffer, pH 8: 10 mM Tris-base 1 mM EDTA pH 8. Prior to its use 1 mM PMSF was added.

- TAE buffer pH 8: 40 mM Tris-acetate, 1 mM EDTA.
- TE buffer pH 8: 10 mM Tris, 1 mM EDTA.
- TBS pH 7.6: 20 mM Tris-base and 140 mM NaCl.

2.3 Equipment Required

For ELPs gene construction, bioproduction and purification: autoclave, refrigerated centrifuge, microcentrifuge, gel electrophoresis equipment for DNA and proteins analysis, UV light transilluminator, UV–vis spectrophotometer with a thermostatized sample chamber, static and orbital shaking incubators, mechanical stirrer, thermostatic water bath, refrigerators, sonicator, lyophilizer, −80 °C freezer and vortex. For in vitro cell culture: biosafety cabinet, CO_2 incubator, centrifuge, optical microscopy, microplate reader, confocal microscopy. For in vivo biodistribution assays: 15 W UV lamp, anesthesia induction chamber, and IVIS (In Vivo Imaging System).

3 Methods

3.1 ELR-Vaccine Device Synthesis

3.1.1 Molecular Design Guidelines

For the development of ELR nanovaccines in which the antigen is exposed on the outer part of the nanoparticle as a fusion protein with the ELR backbone, an ELR block copolymer based on an amphiphilic structure comprising two diblocks was chosen. The hydrophilic block is a glutamic acid (E)-based polar block, the γ-carboxyl groups in the $[(VPGVG)_2\text{-}(VPGEG)\text{-}(VPGVG)_2]_{10}$ hydrophobic block, undergo strong polarity changes between the protonated and deprotonated states. As a consequence of changes in pH around its effective p*K*a, the Tt values of this block are directly affected by the pH. The E-block does not show any phase transition at pH values above its p*K*a (4.1), but can affect the Tt of block copolymers associated with it. The hydrophobic block has the sequence $(VGIPG)_{60}$, and coacervates in water under physiologically relevant conditions (pH 7.4, 37 °C), thereby maximizing hydrophobic interactions that drive self-assembly, whereas under the same conditions, the incorporation of charged hydrophilic residues, such as glutamic acid, prevents block aggregation. The result of this combination is a block copolymer that self assembles into multimeric nanoparticles or hydrogels, depending on the concentration, at room temperature before reaching physiological conditions (*see* **Note 1**).

When building the ELR construct, certain aspects, such as the location of the blocks when the nanoparticle is formed, have to be taken into account. Thus, the antigenic sequences must join to the terminus of the hydrophilic block of the combined hydrophobic-hydrophilic blocks so that they are exposed at the surface of the nanoparticle after self-assembly (Fig. 1).

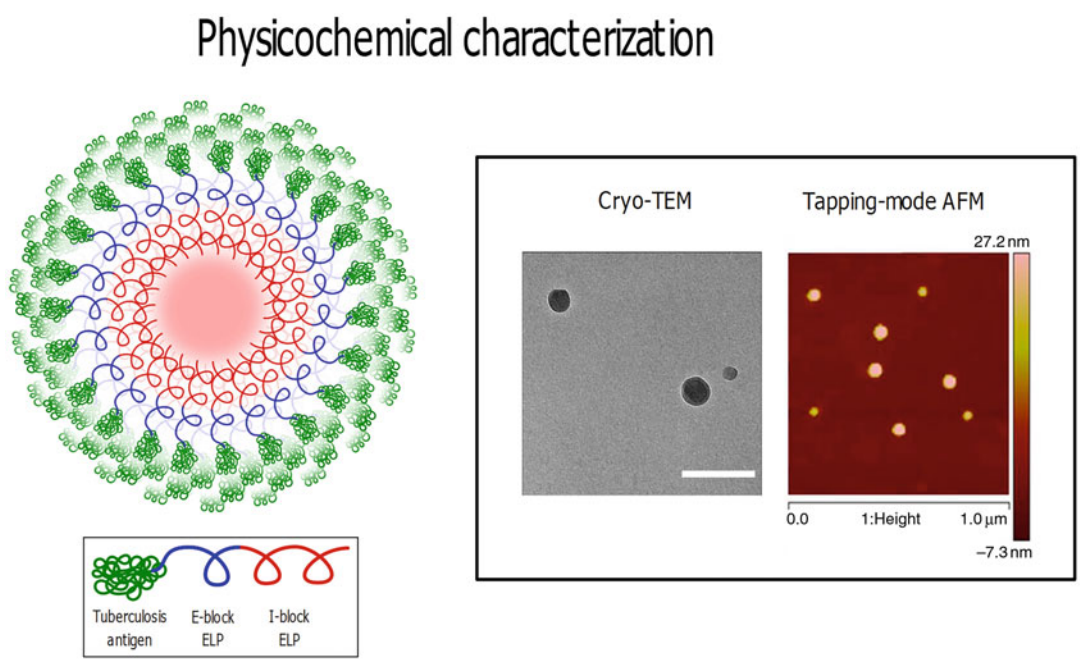

Fig. 1 Schematic representation of the chimeric protein nanoparticle comprising tuberculosis antigen, E-block and I-block. Physicochemical characterization of these self-assembled nanoparticles was carried out using cryo-TEM and tapping-mode AFM. The scale bar for the sample analyzed by cryo-TEM is 100 nm

3.1.2 Chimeric Gene Construction

Sequential introduction of repetitive ELR polypeptides codifying gene segments to form fusion genes with a fully controlled composition and chain length is carried out using the "recursive directional ligation" (RDL) strategy, which allows the monomer genes to polymerize in a seamless and unidirectional manner. This seamless cloning requires the use of type IIS restriction enzymes, which recognize asymmetric sequences and cleave DNA outside their recognition site. This feature makes them suitable for polymer biosynthesis by guaranteeing unidirectional ligation and avoiding the addition of extraneous amino acids that could alter the properties of the final polymer product. To enable application of the RDL to obtain ELR-based polymeric genes, the sequence has to be flanked by *Ear*I recognition sites at both ends and by one *Sap*I restriction site coinciding with the 5′*Ear*I site of the gene, which can be used for linearization of the cloning vector and as a source for gene insertion. In brief, the same plasmid can be opened with *Sap*I or the gene insert extracted by digesting with *Ear*I as the insert and plasmid have been designed to have compatible ends (*see* **Note 2**). Additionally, some considerations for optimization of *E. coli* expression may drastically improve protein polymers yield (*see* **Note 3**).

Construction starts with a pDrive cloning vector bearing the $E_{50}I_{60}$ block, which has been previously constructed by RDL.

1. Plasmid linearization is achieved by digestion of 3 μg of pDrive plasmid containing the $E_{50}I_{60}$ block with 3 U of *Sap*I in a final reaction volume of 50 μL under the suggested reaction conditions.
2. To ensure that the plasmid has been correctly and exhaustively digested, and to avoid future problems during cloning, we recommend to perform an analytic electrophoresis on 1% agarose in TAE buffer (*see* **Note 4**).
3. To avoid self-ligation of the linearized plasmid, the adequate amount of Shrimp Alkaline Phosphatase (SAP) phosphatase should be added, along with the corresponding buffer, incubating at 37 °C for 1 h. To calculate the amount of SAP needed, remember that 1 pmol of DNA ends is equivalent to about 1 μg of a 3 kb plasmid (*see* **Note 5**).
4. Inactivate the SAP enzyme by incubating at 70 °C for 15 min and then cool to 4 °C.
5. Add the corresponding volume of DNA loading buffer to the sample (*see* **Note 6**).
6. Separate the linearized plasmid by preparative DNA electrophoresis on 1% agarose in TAE buffer (*see* **Note 7**) at a fixed voltage of 60 V for approximately 90 min (*see* **Note 8**).
7. Isolate the linearized plasmid band visualized under the UV light transilluminator from the preparative agarose gel by cutting it and physically removing with a scalpel. Avoid unnecessary exposure of the DNA to the potential mutagenic action of UV light.
8. Extract the DNA of interest from agarose by melting in chaotropic buffer in a 50 °C water bath and apply to the spin-column of a commercial Gel extraction system.
9. Measure the DNA concentration using a UV–VIS spectrophotometer (*see* **Note 9**).
10. Select the antigen sequence and synthesize its sequence flanked by *Ear*I sites, then clone in a suitable plasmid.
11. To obtain a sufficient quantity of the cloning plasmid bearing the antigen sequence, it should be transformed into competent *Escherichia coli* for XL1-Blue cloning. The competent cells and the BD Falcon polypropylene tubes must be placed on ice before thawing so that the cells are aliquoted directly into the pre-chilled tubes. The highest efficiency for the transformation of XL1-Blue supercompetent cells is achieved after a heat pulse of 45 s (*see* **Note 10**). Heat pulses of less than 30 s or more than 45 s result in lower efficiencies. After the recovery incubation, plate in LB-agar with the corresponding antibiotic.

12. Incubate overnight at 37 °C for 16–18 h to allow the transformants to form isolate colonies. Higher incubation times can result in the development of satellite colonies.
13. To screen the clones bearing the correct plasmid, select the desired number of transformant colonies and grow each of them overnight in 5 mL LB medium supplemented with the appropriate antibiotic (250 rpm, 37 °C).
14. Extract the plasmids from the bacterial cultures using the alkaline lysis method.
15. Measure the plasmid concentration using a UV–VIS spectrophotometer.
16. To isolate the sequence of the antigen gene from the plasmid, digest 5 μg of plasmid containing the hapten (immunogenic sequence) with 5 U *Ear*I at 37 °C for 6 h in a final reaction volume of 50 μL (*see* **Note 11**). The amount of plasmid to be digested also depends on the size of the DNA encoding the hapten: shorter fragments are difficult to visualize.
17. Control an aliquot of the digestion mixture in an analytic gel of appropriate concentration to check for complete and correct digestion before performing preparative electrophoresis.
18. The hapten gene is isolated by preparative electrophoresis at 3 V/cm for approximately 90 min or until the band of interest is completely separated. The mobility of the loading buffer dyes helps to estimate the position of the DNA bands.
19. Isolate the band for the gene of interest from the preparative agarose gel by cutting it with a scalpel in an ultra-violet (UV) transilluminator and purify the DNA fragment of interest as described above.
20. Measure the insert concentration using a micro-volume UV-VIS Spectrophotometer.
21. Perform a ligation reaction with pDrive$E_{50}I_{60}$ *Sap*I linearized plasmid and *Ear*I digested insert (1:5 molar ratio) with at least 70 ng of plasmid and the corresponding quantity of insert, 1 U of T4 DNA ligase, 2 μL of T4 ligase buffer and a final volume of 20 μL (*see* **Note 12**). Incubate at 22 °C for 1 h or at 4 °C overnight.
22. Transform 5 μL of the ligation reaction mixture (up to 50 ng of the experimental DNA) into *Escherichia coli* strain XL1-Blue and plate in LB-agar with the appropriate antibiotic (*see* **Note 13**), incubating the transformants at 37 °C overnight or at room temperature for 48 h.
23. Once the colonies have grown, select the desired number of transformant colonies and grow each of them overnight in 5 mL LB medium supplemented with antibiotic (250 rpm, 37 °C).

24. Extract their plasmids using the alkaline lysis method.
25. Correct insertion of the gene fragment can be tested by restriction mapping using *Ear*I or *Eco*RI endonucleases and an analytical agarose gel. Restrictions with *Eco*RI produce two bands: a band of approximately 4000 bp corresponding to the pDrive plasmid and another corresponding to the gene. Digestion with *Ear*I leads to gene fragment liberation along with other bands from the pDrive vector.
26. After performing restriction mapping, verify the selected plasmids by DNA sequence analysis to confirm the correctness of their sequence.
27. When the desired complete sequence is achieved, subclone the final gene into a modified pET-25(+) expression vector. Briefly, extract the gene from pDrive using *Ear*I, as in previous steps, and ligate with *Sap*I linearized and dephosphorylated pET-25 (+), as described previously for pDrive, at a 1:1 molar ratio (*see* **Note 14**).

3.1.3 ELR Expression and Purification: Isolation of a Highly Productive Colony

Before starting production of the ELR of interest, it is necessary to determine the expression level of several clones of the expression bacterial strain containing the same final plasmid DNA to select the best producer. Colony selection is an important procedure to achieve a high-yield production of proteins. To that end, it is recommended to perform two small-scale production screenings consecutively to identify the most productive colony.

1. To obtain expression bacterial clones of the final plasmid DNA, transform the final construct into *E. coli* competent BLR(DE3) cells and culture on an LB-agar plate (*see* **Note 15**). Follow the appropriate transformation protocol (*see* **Note 16**).
2. Analyze the expression rate of the protein of interest with respect to the total protein fraction by performing a simple induction test. To that end, several colonies are analyzed, each one being used to inoculate 5 mL of auto-inducible modified TB medium (mTB) supplemented with the antibiotic of interest (*see* **Note 17**) until the appropriate final concentration of 1% (*see* **Note 18**).
3. Culture each inoculum at 37 °C with shaking (250 rpm) for 16 h (overnight). An appropriate amount of biomass is generated under these conditions and expression of the protein of interest is induced.
4. Analyze a 1 mL sample from the cultures grown for each colony by polyacrylamide gel electrophoresis, as described below:
5. Separate the bacteria from the culture medium by centrifuging the samples at 12,000 × *g* and a temperature of 4 °C for 1 min; discard the supernatant.

6. Wash the bacterial pellet by adding 1 mL of ultrapure water type I or 1× PBS (*see* **Note 19**) to the pellet and resuspend it with a vortex (*see* **Note 20**).
7. Repeat **steps 5** and **6** to obtain a pale pellet.
8. To observe a suitable concentrated band pattern, resuspend the pellet in 200 μL of ultrapure water type I with a vortex.
9. Remove a 20 μL aliquot and mix with 5 μL of 5× protein loading buffer to denature native proteins into unfolded rod-like structures with a uniform negative charge density per unit mass. This charge affects the electrophoretic mobility of each polypeptide formed (*see* **Note 21**).
10. Boil the mixture for 5 min and after 5 min spin-down, load 10 μL of the sample onto an SDS polyacrylamide gel (SDS-PAGE) with an appropriate %T (*see* **Note 22**). Heating the samples for a few minutes in the presence of a reducing agent such as 2-mercaptoethanol helps to denaturalize proteins completely by reduction of the disulfide bonds.
11. Perform the electrophoresis at 1 mA per cm and select the colony that produces the most intense band for the protein of interest with respect to the bacterial proteins; this is the most productive colony.
12. Increase the quality of the producer colony by performing a second screening between best-producer-colony "children." To that end, plate the most productive colony and grow it on an LB-agar.
13. Repeat **steps 2–11** and select the most productive colony again (*see* **Note 23**).

3.1.4 ELR Production

Once a highly productive colony for the polymer of interest has been obtained, the next step is to produce the ELR.

1. To ensure an appropriate quantity of bacterial biomass, the selected *E. coli* BLR(DE3) colony is cultured in sequential steps. Firstly, in a 50 mL tube, a pre-inoculum is obtained in 5 mL of LB supplemented with the required antibiotic and glucose, until a final concentration of 1% (v/v) is obtained (*see* **Note 24**). Culture overnight at 37 °C with shaking (250 rpm).
2. Subsequently, 100 μL of the resulting pre-inoculum culture is inoculated in 30 mL of fresh antibiotic/glucose-supplemented LB broth in a 150 mL Erlenmeyer flask (*see* **Note 25**). Culture at 37 °C and 250 rpm for 6 h until the exponential phase is reached (OD600 = 0.6–0.7) (*see* **Note 26**).
3. A high yield of recombinant protein expression is achieved with mTB medium supplemented with the appropriate antibiotic. Prepare the desired volume of medium for total production culture and inoculate with 1 mL of the last inoculum (**step 15**) per 500 mL of broth (*see* **Note 27**).

Culture at 37 °C and 250 rpm overnight until the stationary phase is reached. At that point induction has finished and the stationary phase of the bacterial growth curve has been reached (*see* **Note 28**).

4. Stop metabolism and growth by cooling the cells to 4 °C with the help of an ice bath.
5. Verify that the protein of interest has been expressed correctly by analyzing the total protein fraction composition during the induction time-course on a polyacrylamide electrophoresis gel.
6. Harvest the cells by centrifugation at 4 °C and 5000 × *g* for 15 min. Discard the supernatant and wash the cells from the pellet by resuspension in 100 mL of TBS wash buffer per liter of culture, shaking energetically. It is important to keep the temperature low with ice (*see* **Note 29**). The washing step should be repeated until the supernatant is colorless and the pellet is clear, which shows the absence of residual medium.
7. Prepare the bacteria for lysis. Firstly, resuspend the bacterial pellet in 25 mL of TE sonication buffer per liter of culture (*see* **Note 30**).
8. To avoid bacterial protease activity during the purification steps, add 2 mL of 10 μg/mL PMSF protease inhibitor to the cell suspension per liter of added TE buffer and maintain at 4 °C.
9. Bacteria are lysed by performing 7–14 sonication cycles, each cycle comprising pulses of 10 s each every 15 s at 100 W for 30 min (*see* **Note 31**).
10. Separate solid bacterial debris from the sonicated suspension by centrifugation at 4 °C and 15,000 × *g* for 90 min. Store the two phases, considering that the supernatant should contain the soluble polymer while the pellet is the insoluble phase (*see* **Note 32**).
11. Check which phase contains the protein of interest by SDS-PAGE: collect 1 mL of sample, centrifuge and test the protein content of each phase in an SDS-PAGE assay. Commence the purification process with the phase selected.

3.1.5 ELR Purification

ELR purification is based on the smart nature of these biomaterials, more specifically the inverse temperature transition phenomenon (ITT) typical of ELRs, which can be triggered by heat, changes in pH or addition of salt. This procedure involves a simple method called Inverse Transition Cycling (ITC) which allows recombinant proteins and peptides to be purified (Fig. 2). Although a standard ITC protocol has been established for chimeric proteins, each target protein requires some degree of prior optimization.

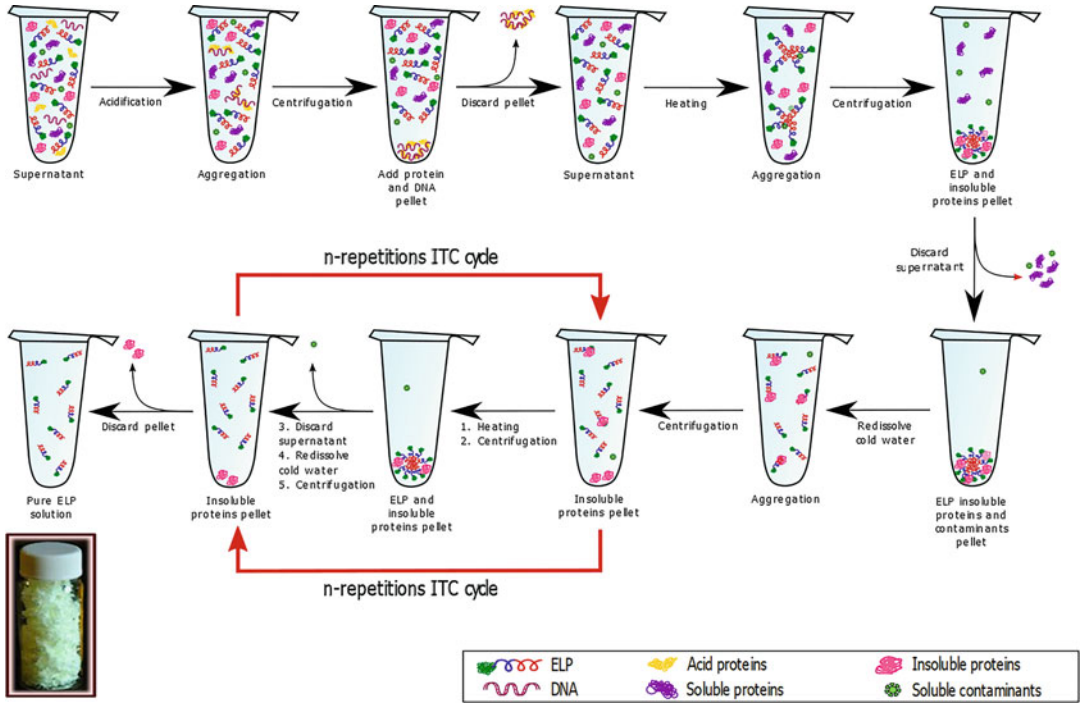

Fig. 2 Scheme showing the purification of recombinant proteins using the inverse transition cycling (ITC) method. In order to obtain completely pure recombinant proteins, *n* ITCs are carried out in such a way that the chimeric polymer is obtained free from contaminants

1. Facilitate the separation of soluble contaminants by acidification of the soluble fraction to pH 4 with dilute hydrochloric acid. Denatured acid proteins and DNA are removed by cold centrifugation at 4 °C and 15,000 × *g* for 20 min. Discard the pellet and keep the supernatant, which should contain the protein of interest.
2. Recover the polymer by triggering its phase transition by increasing the solution temperature to 40 °C (above the ELR inverse transition temperature) for 2 h in a warm water bath. At this temperature the ELR forms micrometer-sized aggregates, which can be separated from the soluble fraction of the cell lysate by centrifugation at 40 °C and 15,000 × *g* for 20 min. Decant and discard the supernatant (*see* **Note 33**).
3. Soluble contaminants and insoluble proteins can co-localize in the solid fraction with the ELR. As such, resuspend the pellet in 2 mL of cold ultrapure deionized water per liter of culture, and leave to stir at 4 °C for 12 h.
4. Insoluble contaminants trapped in the ELR aggregates are removed as precipitates by centrifugation at 15,000 × *g* for 30 min.

5. The ELR-containing soluble fraction is decanted and retained, thereby completing the ITC cycle.
6. Repeat the purification process (**steps 25–29**) several more times to increase the purity of the ELR protein (*see* **Note 34**).
7. Freeze sample, eliminate water, and dry by lyophilization.

3.1.6 Endotoxin Removal from ELRs

Endotoxins are complex lipopolysaccharide from the outer membrane of Gram-negative bacteria, such as *E. coli*, typically found as contaminants in recombinant proteins. Their structures comprise three subdomains covalently linked to each other, and they exhibit strong activity. As a result, endotoxin-contaminated nanomaterials have shown toxic effects in in vivo or in vitro transfection studies, thereby confounding the results [18]. Consequently, before starting in vivo or in vitro immunosafety assays, endotoxin levels must be evaluated and these substances removed, if present, by way of a secondary treatment with sterile sodium hydroxide (NaOH) and sodium chloride (NaCl).

1. Precipitate the ELRs from the last cold supernatant at 25 °C by adding sterile 10 N NaOH to a final concentration of 0.4 N and mixing vigorously.
2. Incubate the mixture on ice for 15 min, then add 5 M NaCl to a final concentration of 2 M.
3. Collect the protein by centrifugation at 25 °C and 8500 × *g* for 20 min. Discard the supernatant and resuspend the pellet in sterile PBS at approximately 50 mg per 20 mL.
4. Repeat **steps 33–35** three times. After the third treatment, adjust the solution pH to 6–8.
5. Dialyze the last cold supernatant with cold ultrapure water, using 0.2 mm filters, until the salts have been completely removed. The number of dialysis cycles required can be estimated by considering that two equilibria against a 1000-fold volume excess of buffer will decrease the salt concentration 10^6-fold; three dialyses under these conditions 10^9-fold, etc.
6. Freeze the sample, eliminate the water, and dry by lyophilization.
7. Weigh the ELR obtained, calculate the yield achieved, and store at −20 °C.
8. Determine endotoxin levels in the purified ELR by resuspending it at serial dilutions, from 1 mg/mL, in sterile molecular biology grade water.
9. Use a commercial assay to assay the protein endotoxin content. Levels of endotoxin should be typically ≤0.1 EU/mg of protein polymer (1 EU = 100 pg of endotoxin), although the purity requirement depends on the route of administration, residence time, and application (Table 1).

Table 1
Endotoxin limit defined in the EUROPEAN PHARMACOPOEIA – EDQM 5.1.10

Route de administration	
Intravenous	5.0 IU of endotoxin per kilogram of body mass
Intravenous for radiopharmaceuticals	2.5 IU of endotoxin per kilogram of body mass
Intrathecal	0.2 IU of endotoxin per kilogram of body mass
Parenteral formulations administered per square meter of body surface	100 IU of endotoxin/m^2

Guidelines for using the test for bacterial endotoxins
https://www.edqm.eu/sites/default/files/medias/fichiers/COVID-19/updated_covid-19_vaccines_package_oct_2020.pdf

10. Introduce any chemical post-translational modifications required (*see* **Note 35**) and verify your ELR by physicochemical characterization and ELR-device immune response (*see* **Note 36**).

3.2 ELR-Based DNA Vaccines

In this section we describe the protocols followed by Gonzalez-Valdivieso et al. to establish a gene library of DNA nanovaccines specifically designed against the RVFV glycoprotein Gn, including genes encoding for ELR blocks [4]. Six chimeric fusion genes are described in that work, all of them including the gene sequence codifying for the Gn glycoprotein from RVFV and ELR blocks of a different nature.

3.2.1 Plasmid Mutagenesis and Cloning

Starting from a pCMV plasmid containing the gene codifying for the glycoprotein Gn under the control of cytomegalovirus (CMV) promoter, which is a potent promoter for attaining high gene expression (kindly provided by Dr. Alejandro Brun of Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Centro de Investigación en Sanidad Animal (CISA), Madrid), PCR site-targeted mutagenesis is performed to prepare the plasmid and clone the genes codifying for different ELR blocks next to the gene encoding the viral Gn glycoprotein (*see* **Note 37**).

First, the endogenous *Sap*I recognition site (5′-GCTCTTC-3′) is removed by substitution of a cytosine for an adenine at position 3306 in the plasmid sequence (Tables 2 and 3, Fig. 3a).

Starting from a pCMV plasmid containing the gene codifying for Gn glycoprotein:

1. Prepare the PCR reactions in ice:
   - 5 μL of 10× reaction buffer.
   - X μL (50 ng) of dsDNA template (*see* **Note 38**).
   - X μL (125 ng) of oligonucleotide forward primer.

Table 2
Sequences of mutagenesis primers for the c.1295C>A mutation in the DNA sequence of pCMVNSmGn for removal of a *Sap*I restriction site, and the original and mutated sequences

Mutation	Primer	Sequence (5′–3′)	T_m (°C)
Point mutation	MutSappCMVFor	GCGTATTGGGCGCT**A**TTCCGCTTCCTCGAC	78.5
	MutSappCMVRev	GTCGAGGAAGCGGAA**T**AGCGCCCAATACGC	78.5
Mutation	**Sequence**	**DNA sequence (5′–3′)**	
Point mutation	Original	GCGTATTGGGC**GCTCTTC**CGCTTCCTCGCTC	
	Mutated	GCGTATTGGGCGCTATTCCGCTTCCTCGCTC	

Mutated nucleotides are depicted in red. The recognition site for *Sap*I is depicted in bold

Table 3
PCR settings for the c.1295C>A mutation

Segment	Cycles	Temperature (°C)	Time (s)
1	1	95	30
2	12	95	30
		55	60
		68	360

The recommended duration of the elongation step at 68 °C is 1 min per kb of plasmid length

- X μL (125 ng) of oligonucleotide reverse primer.
- 1 μL of dNTP mix (10 mM).
- 1 μL of *Pfu* Turbo DNA polymerase (2.5 U/μL).
- Ultrapure deionized water type I to a final volume of 50 μL.

The preparation of a negative-control PCR sample lacking DNA template (replaced by ultrapure deionized water type I) is highly recommended.

2. Put the reaction tubes in the thermocycler and select the parameters described in Table 3.
3. After the PCR reaction, place the reaction tubes on ice for 2 min to cool the reaction to ≤37 °C.
4. Add 1 μL (10 U) of the *Dpn*I restriction enzyme directly to each amplification reaction tube. The amplification mixture is digested with *Dpn*I restriction enzyme to eliminate the DNA template and used to transform XL1-Blue supercompetent cells.
5. Gently mix by pipetting up and down. Spin down the reaction mixture in a microcentrifuge for 1 min and immediately incubate at 37 °C for 1 h to digest the parental (non-mutated) DNA.

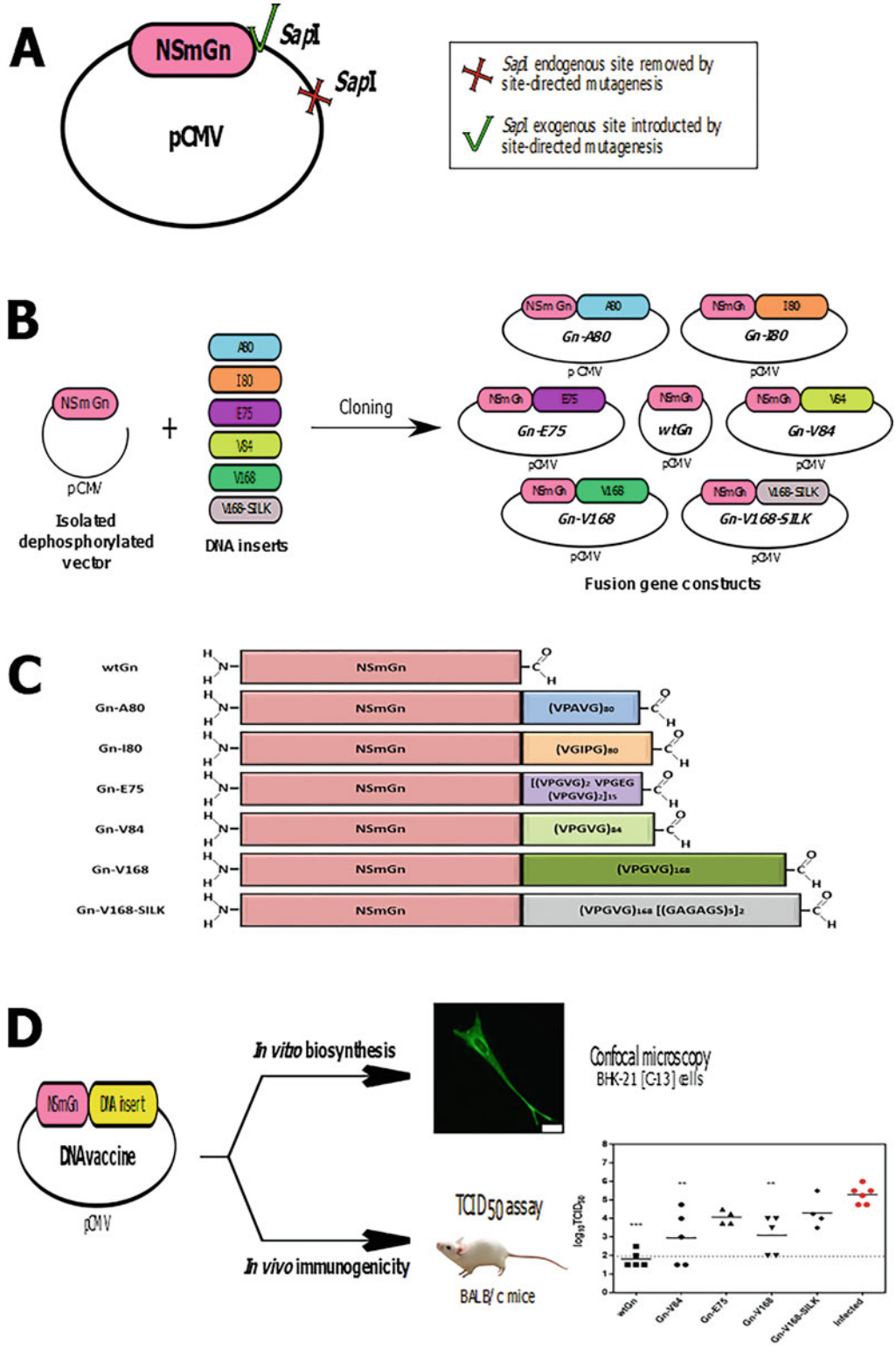

Fig. 3 (**a**) Site-directed mutagenesis of the cloning vector pCMV together with the gene insert encoding the NSmGn fusion protein, which results in the removal or introduction of the SapI enzyme restriction sites indicated. (**b**) Fusion gene constructs obtained from cloning the dephosphorylated vector pCMV-NSmGn and

6. Thaw the XL1-Blue supercompetent cells on ice. For each reaction, aliquot 50 μL of the supercompetent cells into a pre-chilled 14-mL round-bottomed polypropylene tube.
7. Add 1 μL of the *Dpn*I-digested DNA to the aliquots of the supercompetent cells.
8. Incubate the reaction mixture on ice for 30 min.
9. Heat the reaction mixture at 42 °C in a water bath for 45 s and then place on ice for 2 min.
10. Add 450 μL of SOC medium (preheated to 42 °C) and incubate the transformation reaction mixture at 37 °C and 250 rpm for 1 h.
11. Plate 50 μL of each transformation reaction on LB-Agar plates containing ampicillin for the plasmid vector.
12. Incubate the transformation plates at 37 °C overnight in the dark.
13. Select isolated colonies.
14. Pick a single colony from a freshly streaked selective plate and inoculate a culture of 5 mL LB medium containing ampicillin. Incubate overnight in an incubator at 37 °C and 250 rpm.
15. Extract the DNA using a commercial Plasmid Miniprep Kit according to the manufacturer's instructions and elute the DNA in 50 μL of elution buffer.
16. After DNA extraction, measure the absorbance at 260 nm to determine the DNA concentration and quality with a microvolume UV–VIS Spectrophotometer (*see* **Note 39**).
17. Correct mutagenesis of the plasmids is analyzed by restriction mapping with *Ear*I. Restriction digestions of non-mutated plasmid pCMV-NSmGn with *Ear*I produce six bands of 280, 488, 992, 1033, 1100, and 1804 bp. Due to the mutation c.1295C>A, an *Ear*I/*Sap*I recognition site is removed from position 3306 and the fragments of 1100 and 1804 bp are fused into one fragment of 2904 bp. Fragments of 280, 488, 992, and 1033 bp remain unaltered.
18. Among the colonies rendering the expected bands, verify the positive colonies by DNA sequencing, as described above. Table 4 lists the sequencing primers that can be used to verify transformants' DNA.

Fig. 3 (continued) the gene inserts encoding the ELR blocks. (**c**) Design and composition of the fusion proteins resulting from the fusion genes developed. The ELR blocks include their amino acid composition. Scaled scheme. The molecular weights are as followed: NSmGn, 73.4 kDa; A80, 30.6 kDa; I80, 34.0 kDa; E75, 31.3 kDa; V84, 34.5 kDa; V168, 69 kDa; V168-SILK, 73 kDa. (**d**) Results obtained after in vitro and in vivo experiments with DNA vaccines. In vitro biosynthesis was performed in BHK-21 [C-13] cells, while in vitro immunogenicity was tested in BALB/c mice

Table 4
Sequences of sequencing primers and their melting temperature

Primer	Sequence (5′–3′)	T_m (°C)
CtGn For	TTATAGGGTGCTTAAGTGC	42
XhoI For	ATCCGGTACTCGAGGAAC	46
pCMV Rev	CCTGAACCTGAAACATAAAATG	46
Mid1Gn For	CGTGATGAAGACACACTGTC	45
Mid2Gn For	GCCTTTATGTGTAGGGTATG	44

Table 5
Sequences of mutagenesis primers for c.2028_2029 ins GTATGAAGAGCTAGGC mutation and the original and mutated sequences

Mutation	Primer	Sequence (5′–3′)	T_m (°C)
Multiple nucleotides insertion	MutCterGnFor	GCCCCTATTCCTCGTCATGTATGAAGAGC TAGGCGGCCG	80.2
	MutCterGnRev	CGGCCGCCTAGCTCTTCATACA TGACGAGGAATAGGGGC	80.2
Mutation	**Sequence**	**DNA sequence (5′–3′)**	
Multiple nucleotides insertion	Original	TATTCCTCGTCATTAGGCGGCCGCGG	
	Mutated	TATTCCTCGTCATGTAT**GAAGAGC**TAGGCGG CCGCGG	

The second mutation involves the insertion c.2028_2029ins GTATGAAGAGCTAGGC into the Gn sequence to insert a *Sap*I restriction site and STOP codon. Mutated nucleotides are depicted in red. The recognition site for *Sap*I is depicted in bold

Table 6
PCR settings for c.2028_2029insGTATGAAGAGCTAGGC mutation

Segment	Cycles	Temperature (°C)	Time (s)
1	1	95	30
2	18	95	30
		55	60
		68	360

The recommended duration of the elongation step at 68 °C is 1 min per kb of plasmid length

Once successful removal of the *Sap*I recognition site is obtained, a new *Sap*I recognition site (5′-GCTCTTC-3′) is introduced after the Gn glycoprotein codifying gene by a second site-directed mutagenesis cycle (Tables 5 and 6 and Fig. 3a). ELR-encoding genes can therefore be introduced

next to the gene codifying for the RVFV glycoprotein Gn to form the final fusion gene constructs.

Starting from the pCMVNSMGn c.1295C>A mutated plasmid as described above:

19. Prepare the PCR reactions in ice:
    - 5 μL of 10× reaction buffer.
    - X μL (50 ng) of dsDNA template.
    - X μL (125 ng) of oligonucleotide forward primer.
    - X μL (125 ng) of oligonucleotide reverse primer.
    - 1 μL of dNTPs mix.
    - 1 μL of *Pfu* Turbo DNA polymerase (2.5 U/μL).
    - ultrapure deionized water type I to a final volume of 50 μL.
20. Put the reaction tubes in the thermocycler and select the parameters shown in Table 6.
21. After the PCR reaction, place the reaction tubes on ice for 2 min to cool the reaction to ≤37 °C.

After PCR site-directed mutagenesis, the parental template pDNA is digested with *Dpn*I and XL1-Blue supercompetent cells are transformed. The plasmid DNA of these cells is purified and analyzed by restriction digestion following the protocols described above for the first mutagenesis cycle.

The second site-targeted mutagenesis (c.2028_2029insGTATGAAGAGCTAGGC) involves insertion of the 16 nucleotides "GTATGAAGAGCTAGGC " at position 2826 in the plasmid sequence. This mutation creates a new *Ear*I/*Sap*I recognition site at position 2826. The theoretical fragments for the final plasmid after digestion with *Ear*I are 280, 488, 620, 992, 1033, and 2300 bp. As such, the fragment of 2904 bp is split into two fragments of 620 and 2300 bp, respectively. After this mutation, the pCMVNSmGn plasmid is therefore ready for introduction of the ELR-encoding genes from a gene library to develop different constructs, thereby resulting in DNA nanovaccines with enhanced expression of Gn glycoprotein from RVFV in eukaryotic systems.

Finally, verify the positive colonies giving the expected bands by DNA sequencing to corroborate the presence of the new *Ear*I/*Sap*I site.

3.2.2 Synthesis of the DNA Vaccine Library

Once the pCMV plasmid with the gene sequence encoding for the RVFV Gn glycoprotein has been successfully mutated to allow the introduction of ELR-encoding genes, sequential introduction of the repetitive polypeptide-coding gene segments to form fusion genes, with full control of composition and chain length, is carried out using the RDL (recursive directional ligation) technique (Fig. 3b). The genes encoding for the A80, I80, E75, V84,

V168, and SILK blocks are available from previous studies from the group and were constructed using the same procedure of DNA concatenation involving sequential rounds of RDL with the monomeric DNA sequences (Fig. 3c).

1. The mutated plasmids are linearized and dephosphorylated following **steps 1–9** in Subheading 3.1.3 "Chimeric gene construction."
2. During the cloning of DNA vaccines, different ELRs genes are isolated as described in **steps 14–21** in Subheading 3.1.3 "Chimeric gene construction." In this cloning, due to the size of the inserts, T4 ligation is performed in a 1:2 vector-to-insert ratio to ensure ligation of one insert molecule per vector.
3. Plate the transformed cells on LB-agar plates supplemented with ampicillin and incubate overnight at 37 °C.
4. Select individual clones and grow the selected transformants in 5 mL of LB medium supplemented with ampicillin.
5. Extract the DNA using an endotoxin free plasmid maxi kit according to the manufacturer's instructions and determine the DNA concentration and quality (*see* **Note 40**).
6. Restriction mapping of fusion genes. The correct cloning of the final gene constructs is analyzed by restriction mapping with *Hind* III and subsequent DNA sequencing (Table 7).
7. Analytical restriction mapping is performed by incubating the following reaction mixture at 37 °C for 2 h:
   - X μL (500 ng) of DNA.
   - 0.2 μL (1 U) of *Hind*III.
   - 0.5 μL of buffer.
   - ultrapure deionized water type I to a final volume of 5 μL.

Table 7
Size of fragments resulting from incubation of fusion genes with *Hind*III restriction enzyme

Gene construct	Size (bp)
wtGn	13; 1075; 4525
Gn-A80	13; 2275; 4525
Gn-I80	13; 2275; 4525
Gn-V84	13; 2335; 4525
Gn-E75	13; 2200; 4525
Gn-V168	13; 3595; 4525
Gn-V168-SILK	13; 3787; 4525

8. Load the samples on a 1% agarose gel and run the DNA electrophoresis at 10 V/cm for 90 min.
9. Among the colonies rendering the expected bands, verify the positive colonies by DNA sequencing.

3.2.3 Analysis Gn Glycoprotein Expression In Vitro

Expression of the chimeric polypeptides in the eukaryotic cells is evaluated by transfecting 293 T human embryonic kidney cells. 293 T cells are cultures in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

At the time of transfection, cells should have 70–80% confluence. Use black clear-bottomed 96-well plates to prevent signals from spreading between wells during the fluorescence measurements. The number of cells, quantities of DNA, and reagent volumes are optimized for 293 T cell cultures in 96-well-plates and 200 μL as final volume. Prepare fresh transfection reagent immediately before the transfection assay (*see* **Note 41**).

Lipofectamine® Transfection

1. Seed 293 T cells in 96-well plates (2×10^4 per well) and incubate at 37 °C and 5% CO_2 for 24 h.
2. Dilute 2 μL of Lipofectamine® LTX Reagent in 25 μL non-supplemented medium.
3. Dilute 2.5 μg of DNA in 125 μL of serum-free DMEM.
4. Add 2.5 μL of PLUS™ reagent in diluted DNA.
5. Add 25 μL of diluted DNA (with PLUS™ reagent) in 25 μL of diluted Lipofectamine® LTX Reagent.
6. Incubate at room temperature for 5 min.
7. Add 10 μL of the transfection mixture to each well.
8. Incubate at 37 °C for 24 h in a CO_2 incubator.
9. Replace cell medium with supplemented medium.
10. Analyze transgene expression after 24 h by immunocytochemistry and confocal microscopy.

Turbofect™ Transfection

1. Seed 293 T cells in 96-well plates (2×10^4 per well) and incubate at 37 °C and 5% CO_2 for 24 h.
2. Dilute 0.1 μg of DNA in 20 μL of serum-free DMEM.
3. Vortex the Turbofect™ reagent and add 0.4 μL to the diluted DNA.
4. Mix immediately by pipetting up and down.
5. Incubate at room temperature for 15 min.
6. Add 20 μL of the transfection reagent/DNA mixture to each well dropwise.
7. Gently rock the plate to achieve an even distribution of the complexes immediately after adding the transfection reagent.

8. Incubate at 37 °C for 24 h in a CO_2 incubator.
9. Replace the cell medium with supplemented medium.
10. Analyze transgene expression after 24 h by immunocytochemistry and confocal microscopy.

Immunocytochemistry Analysis

1. Seed 293 T cells in 96-well plates (2×10^4 per well) and incubate at 37 °C and 5% CO_2 for 24 h.
2. Transfect cells as described above.
3. After 24 h, wash with PBS 1× (200 μL per well), fix with PFA 4% (200 μL per well) for at least 30 min, and wash three times with PBS 1× (200 μL per well).
4. Permeabilize cells with Triton X-100 0.1% in PBS (200 μL per well) for 10 min and wash three times with PBS 1× (200 μL per well).
5. Block with FBS 2% for 1 h at room temperature.
6. Incubate cells with RVFV-immunized mouse serum for 1 h at 37 °C (*see* **Note 42**).
7. Wash three times with PBS 1× (200 μL per well).
8. Incubate with Alexa Fluor 488-labeled secondary antibody 5 μg/mL for 1 h at 37 °C (200 μL per well).
9. Measure the fluorescence intensity from the bottom using a microplate reader.

Confocal Microscopy

1. Seed 293 T cells in Milicell® EZ Slides (2×10^4 per well) and incubate at 37 °C and 5% CO_2 for 24 h.
2. Transfect the cells as described above.
3. After 24 h, wash with PBS 1× (500 μL per well), fix with PFA 4% (500 μL per well) for 30 min and wash three times with PBS 1× (500 μL per well).
4. Permeabilize cells with Triton X-100 0.1% in PBS (500 μL per well) for 10 min and wash three times with PBS 1× (500 μL per well).
5. Block with FBS 2% at room temperature for 1 h.
6. Incubate cells with RVFV-immunized mouse serum at 37 °C for 1 h.
7. Wash three times with PBS 1× (500 μL per well) and incubate with Alexa Fluor 488-labeled secondary antibody at 37 °C for 1 h (500 μL per well).
8. Wash cells with PBS 1× (500 μL per well).
9. Stain cell nuclei with DAPI for 5 min and wash with PBS 1× (500 μL per well) (*see* **Note 43**).

3.2.4 Analysis of Biodistribution in Mice In Vivo

Fluorescent Labeling of DNA Constructs

1. Adjust DNA to 100 μg/mL in TE buffer.
2. Dissolve Psoralen-PEG_3-Biotin in DMF at 20 mM.
3. Add the biotin solution to the DNA and mix well by pipetting up and down.
4. Irradiate the reaction tube from above with a 15 W UV lamp at 365 nm for 30 min on ice.
5. Precipitate the sample with 0.2 M potassium acetate and two volumes of 70% ethanol to remove unreacted biotin.
6. Centrifuge at 15,000 × *g* for 15 min.
7. Discard the supernatant, wash the pellet with 400 μL 70% ethanol and allow it to dry.
8. Dissolve the biotinylated sample in sterile PBS.
9. Dissolve the Qdot® 800 streptavidin conjugate in Secondary Incubation Buffer at 40 nM.
10. Add the Qdot® 800 streptavidin conjugate to biotinylated DNA and incubate at room temperature for 15 min. Use a 2:1 plasmid DNA:Qdot® 800 molar ratio to allow all the quantum dots to be complexed with the plasmid DNA.

Intravenous Administration of Fluorescently Labeled DNA in Mice

1. Inject the fluorescently labeled DNA into BALB/c mice (2.5 mg/kg mouse) intravenously via the tail vein. Use the diluted Qdot® 800 streptavidin conjugate immediately in the current experiment.
2. Anaesthetize the mice with isoflurane in oxygen (4%) in an induction chamber and transfer immediately to the IVIS (In Vivo Imaging System) with continuous anesthesia during measurement (1.5%). Use an untreated mouse (PBS) as control.
3. Scan the animals for fluorescence at excitation and emission wavelengths of 470 and 800 nm, respectively. Fluorescence of animals is plotted by subtracting the background from the untreated (PBS) mouse.

4 Notes

1. Different hydrophobic blocks have been reported in the literature, for example, tyrosine or alanine instead of isoleucine [13]. A higher hydrophobic content leads to better higher adjuvant properties [19].
2. Although the RDL method based on *Sap*I and *Ear*I is used in this chapter, there are possible variants, such as concatemerization, overlap elongation polymerase chain reaction (OEPCR), overlap-extension rolling circle amplification (OERCA), or

recursive directional ligation by plasmid reconstruction, in which two halves of a parent plasmid, each containing a copy of an oligomer, are ligated together, thereby dimerizing the oligomer and reconstituting a functional plasmid [10, 20]. RDL can also be performed with different enzymes provided they have compatible ends.

3. To optimize *E. coli* expression of recombinant protein, consider: (i) minimizing the GC content at the 5′-end to avoid the formation of secondary structures in the mRNA, which leads to interrupted translation; (ii) adding a transcriptional terminator or an additional one if one is already present; (iii) avoiding codons that have been associated with translation problems or use of *E. coli* strains that encode some of them; (iv) examining the second codon. Differences in expression of up to 15-fold have been found depending on the codon after the first methionine; (v) the addition of a fusion partner.
4. The percentage of agarose used depends on the size of the fragments to be separated.

Fragment size (bp)	Agarose (%)
1000–23,000	0.6
800–10,000	0.8
400–8000	1
300–7000	1.2
200–4000	1.5
100–200	2

Agaroses with different melting points are available on the market for selected range sizes of DNA fragments.

5. Some phosphatases require a metal cofactor such as Zn^{2+}, which makes them incompatible with common restriction enzyme buffers. In this case, we recommend performing dephosphorylation after DNA purification. If the buffers are compatible, dephosphorylation can be performed simultaneously with DNA digestion or before purifying the dephosphorylated DNA.
6. DNA loading buffer usually contains SDS and EDTA, which stop the dephosphorylation reaction, and is useful for phosphatases that do not heat-inactivate.
7. When performing an analytical electrophoresis, either TAE (tris-acetate-EDTA) or TBE (tris-borate-EDTA) buffer can be used. TBE provides higher resolution of the smallest bands in high agarose percentage gels. However, TBE is not recommended when the DNA fragment is required for further

purification and ligation steps because TBE gels afford poor gel recovery and borate ions are reported to inhibit DNA ligase. We recommend performing all the protocol in TAE buffer to avoid undesired mistakes.

8. The voltage applied depends on the cuvette dimensions and is usually set at 3–10 V/cm distance between the electrodes. For preparative electrophoresis we recommend the minimum voltage to obtain more defined bands. The run time depends on the size of the fragments, the percentage of agarose and the voltage applied. The electrophoresis is usually run until the band of interest has migrated 40–60% of the gel length.
9. Due to the low yield of the purification, we recommend measuring the plasmid concentration in a micro-volume UV–VIS Spectrophotometer to avoid loss of sample.
10. Most of the commercial strains of *Escherichia coli* can currently be used depending on the specific cloning requirements, such as high-quality plasmid preparations, blue/white screening, fast colony growth, methylation-free plasmid, etc. XL1-Blue, DH5alfa, and JM109 are some of the most widely used strains.
11. Although Star Activity should not be observed when *Ear*I is used according to the protocol, we have noticed problems of over-digestion when this reaction is run overnight and thus recommend not to exceed 5–6 h of digestion.
12. A 1:5 molar ratio of plasmid to insert is a good starting point, but this ratio can be changed depending on the size of the insert with respect to the plasmid. Shorter inserts generally need a higher ratio.
13. Transformation efficiencies will be approximately tenfold lower for reaction ligations than for a complete plasmid.
14. Expression plasmids contain features to maximize gene expression, such as a strong promoter, a strong termination codon, a transcription termination sequence, and a strong translation initiation sequence. Although different expression vectors are available, pET vectors, which work under the control of the T7 lac promoter and are induced by lactose, provide the highest level of protein expression.
15. It is important to consider the characteristics of both gene and protein before choosing the *E. coli* expression strain to use. The use of *E. coli* strains BL21(DE3) and BLR(DE3) allows the recombinant protein transcription to be closely regulated. Indeed, the expression process is under control of the bacteriophage T7 promoter, which is only recognized by the T7 RNA polymerase after induction. These strains reduce expression of potential toxic peptides by lowering the background expression. Moreover, BLR(DE3) strains lacking recombinase

systems (i.e., RecA-) stabilize target genes, which is an especially important characteristic for genes containing repetitive sequences. The deficiency of some proteases (lon cytoplasmic and OmpT periplasmic) increased expressed protein stability when required. Additionally, other commercial *E. coli* strains are available for various protein expression requirements, such as Origame BTM strains for expressions of proteins which require a folding with disulfide bonds, or Artic-Express™, which is used in protein expression processes at low temperatures.

16. Several commercial competent bacterial cells are available; in these cases, follow the protocol provided by the manufacturer. However, it is also possible to obtain competent cells in your own laboratory following processes like the TSS method. This method is based on two reagents (polyethylene glycol (PEG), and dimethyl sulfoxide or (DMSO)), which are able to permeabilize the bacterial wall and membrane, thus resulting in a suitable transformation efficiency. 1× TSS (Transformation and Storing Solution) comprises LB broth with 10% (w/v) PEG, 5% (v/v) DMSO, and 50 mM $MgCl_2$ at (pH 6.5). The mixture can be prepared without DMSO and autoclaved, subsequently adding the DMSO. Another option is to prepare the complete mixture and sterilize by filtration. Mix the cells with 2× TSS in a 1:1 volume ratio to achieve a 1× TSS final concentration and have your cells ready for use immediately. Then, transform the cells with the plasmid DNA of interest [21].
17. The auto-inducible modified TB medium (mTB) is Terrific Broth (TB) supplemented with lactose, glucose, glycerol, and salts. Glucose and glycerol can support bacterial culture growth during the first phase equally as effectively, thus doing away with the need for other carbon sources. In this phase, the biomass increases until it starts utilizing lactose as carbon source, which induces recombinant protein expression. This self-induction is due to lactose in the medium and its use allows higher final yields in the expression of recombinant proteins [22].
18. The most common antibiotics employed are used at the following final concentrations: Carbenicillin (disodium salt) 50 μg/mL, Ampicillin (sodium salt) 50 μg/mL, Chloramphenicol 34 μg/mL, Kanamycin (sulfate) 30 μg/mL, Tetracycline 12.5 μg/mL, and Streptomycin 25 μg/mL.
19. 1× Phosphate-buffered saline (1× PBS) is an isotonic solution that is used as wash buffer and contains 37 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4.

20. The washing steps for the bacterial culture prior to SDS-PAGE are short and are performed before cell rupture and denaturalization of the macromolecules by boiling in the presence of loading buffer. Washing can also be performed with deionized water without compromising the result. These washes need not preserve the osmotic balance with salt because cells will be ruptured in subsequent steps.
21. To prepare protein loading buffer with a 5× concentration. 2-Mercaptoethanol can be substituted by 500 mM DTT in the buffer composition. SDS interacts with positively charged amino acids in proteins, thereby disrupting the structural interactions of the proteins to separate them on the basis of their size. Moreover, a reducing agent such as 2-mercaptoethanol or DTT is present to reduce disulfide bonds.
22. To analyze high- and low-molecular weight bands on the same gel, develop a gradient gel. Acrylamide total percentage (%T) affects the protein migration, therefore it is especially important to select the gel type that offers optimum resolution of your sample. For example, use 10% T gel to separate proteins with a weight of 90–100 kDa, whereas 12% T gels are more appropriate for proteins of less than 60 kDa. Nevertheless, if you are interested in analyzing smaller peptides, a Tricine SDS-PAGE should be carried out (Bio-Rad Laboratories, I. A Guide to Polyacrylamide Gel Electrophoresis and Detection).
23. It is possible to preserve the clones of interest by making a long-term stock. One of the most widely used and practical methods is the production of a glycerol stock. Bacteria can be stored for years at -80C in a high percentage of glycerol. Thus, the selected colony is grown on LB with 0.5% (v/v) glucose at 37 °C with shaking (250 rpm). When the culture is in the exponential growth phase (optical density at 600 nm (OD_{600}) of 0.6–0.8), grown bacteria are transferred to a cryovial and 0.2 volumes of 80% (v/v) sterile glycerol are added. Due to the high viscosity of glycerol, it is recommended to use dilute solutions. The solution is obtained by diluting 100% glycerol in deionized water. A final concentration of 15–25% (v/v) glycerol is required.
24. Addition of a carbon source, such as glucose, to Luria-Bertani medium (LB medium) avoids the need to use amino acids as carbon source for cell metabolism when the cell volume decreases [23].
25. The production process can be scaled up using a bioreactor. This method allows a reduction in process time as well as greater control of the dissolved oxygen concentration (and IPTG, when needed), controlled bacterial growth and steady

pH, in addition to being a scalable and high-performance process. The use of bioreactors guarantees OD_{600} values greater than 10 [24].

26. The inoculated volume must not be higher than 1/30 of the final volume culture.
27. All flasks used must be sterilized previously. Furthermore, to boost the aeration of the culture and its level of dissolved oxygen, the final volume of the flask should be four times higher than the culture volume in it. When oxygen dissolved in the broth is limited, bacterial growth is slower and recombinant protein production is poor. Moreover, oxygen limitation induces the expression of anaerobic response genes and anaerobic metabolites, thus leading to spoilage of the culture medium and a lower yield of the protein of interest [25].
28. In our experience, the best way to determine the correct moment to stop production is to take samples at different times and measure their absorbance. The process should be stopped when the absorbance value has stabilized (stationary phase), before beginning to decline (death phase). The blank should be the supernatant after centrifuging one sample at 13,400 × *g* for 45 s. The optical density values obtained at 600 nm (OD_{600}) will rely on the production method. For example, if flasks are used, it is possible to obtain OD_{600} values of up to 10, whereas with a bioreactor OD_{600} value higher than 10 are routinely obtained.
29. TBS wash buffer must contain salt to maintain the osmotic balance and not damage bacteria. In addition, to obtain a higher yield, it is possible to harvest the cells by centrifuging all culture volume in the same containers over several steps.
30. TE sonication buffer comprises ethylenediaminetetraacetic acid (EDTA) a chemical chelating agent that removes contaminating divalent magnesium cations, a membrane-stabilizing ion, thereby reducing the activity of those proteases that need magnesium ions to function [26].
31. To avoid local overheating and consequent protein denaturation, maintain the sample at 0–4 °C. To keep samples cold, it is possible to place a chiller inside the disruptor or to place them in ice for 5 min after each pulse.
32. If purification is not to be started immediately, these phases can be stored at −20 °C for a long period. When ready for purification, allow the samples to thaw slowly at 0–4 °C the day before.
33. Depending on the ITC parameters and residue composition of the ELR, the supernatant salt concentration and possible pH changes must be evaluated. The salt usually used in this case is

NaCl. Indeed, it is possible to decrease the ELR transition temperature by increasing the salt concentration. Because a higher temperature could denature some proteins, this method is preferred [27].

34. Each specific polymer requires a different number of ITC cycles. Some proteins can be achieved in high purity after only a few cycles, whereas others require more than 5 cycles to be purified effectively. The potential loss of proteins in each cycle should be taken into account, especially with hydrophobic ones, which tend to be lost in the cell lysate [28].
35. It is possible to perform post-translational chemical modifications to increase the immunogenic activity of the protein. One example of these modifications is chemical glycosylation by carbohydrate chemical activation, PEGylation, or acylation [29, 30].
36. NMR and FT-IR spectroscopy can be used to verify the structure of the protein. Furthermore, amino acid analysis can be performed by HPLC, and the molecular weight can be obtained by MALDI-TOF. Turbidimetry and DSC allow the transition temperature of the protein to be calculated, whereas the nanoparticle size and zeta-potential can be studied by DLS. The loading of the different components can be evaluated by thermogravimetric analysis (TGA) or UV-vis absorbance, depend on their nature. The morphological characteristics of nanoparticles can also be determined using several microscopy techniques, such as TEM, SEM, or AFM.

    Once the ELR device that exposes the antigen is produced the more appropriate assay to quantify the immune response and its specificity should be selected (quantification of cytokines'level, ELISA tests, *etc.*).
37. Only plasmid DNA isolated from dam+ *E. coli* strains is suitable for this mutagenesis protocol as it is methylated.
38. To ensure successful amplification, the concentration of dsDNA template can be set at between 5 and 50 ng while keeping the primer concentration constant. In this case, 50 ng of dsDNA template is used.
39. High-quality DNA is critical for successful transfection. The A260 value should be within the instrument's linear range (0.1–1.0). 260/280 nm ratios >1.8 are recommended for high transfection efficiency. Lower and higher lower ratios indicate the presence of contaminants, such as RNA or proteins. Strong absorbance at around 230 nm indicates that organic compounds or chaotropic salts are present in the purified DNA. The A260/A230 ratio indicates the amount of salt in the purified DNA. The A260/A230 ratio should be greater than 1.5. Lower ratios indicate higher amounts of salt.

40. Endotoxin-free DNA improves transfection into eukaryotic cells, therefore endotoxin-free plastic tips and tubes must be used for elution.
41. Since either Lipofectamine® or Turbofect™ are some of the most standard transfection systems, both reagents were used in the cell transfection assays to discard any potential cytotoxicity due to the transfection process. Since cell viability was higher in Lipofectamine® treated cells compared with Turbofect™ ones [4], Lipofectamine® was used as a transfection reagent in the following experiments.
42. Do not wash between the blocking step with FBS 2% and incubation with the RVFV-immunized mouse serum that is used after 1:100 dilution in PBS-2% FBS. Incubation with RVFV-immunized mouse serum at 37 °C improves the fluorescence signal compared to incubation at room temperature.
43. Avoid prolonging the DAPI incubation time to prevent an excessively high signal from cell nuclei and merged images due to incorrect contrast between channels.

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Chapter 4

Display of Heterologous Proteins in *Bacillus Subtilis* Biofilms for Enteric Immunization

Claudio Aguilar, Ramona Wissmann, Cornel Fraefel, and Catherine Eichwald

Abstract

One of the foremost goals in vaccine development is the design of effective, heat-stable vaccines that simplify the distribution and delivery while conferring high levels of protective immunity. Here, we describe a method for developing a live, oral vaccine that relies on the biofilm-forming properties of the spore-former bacterium *Bacillus subtilis*. The amyloid protein TasA is an abundant component of the extracellular matrix of the biofilms formed by *B. subtilis* that can be genetically fused to an antigen of interest. Spores of the recombinant strain are then prepared and applied via the oral route in an animal model. Due to the intrinsic resistance of the spores, they can bypass the stomach barrier, germinate, and subsequently colonize the gut, where they develop into biofilms, expressing the antigen of interest. We describe here the steps necessary to produce spores, immunization, and downstream analysis of the vaccine efficacy.

Key words *Bacillus subtilis*, Endospores, Biofilm, Heterologous, Immunization

1 Introduction

Bacillus subtilis is a Gram-positive bacterium with several properties like the development into endospores and biofilms; it is safe, used as a probiotic [1–3] and has a well-described biology facilitating genetic manipulation [4]. For these reasons, *B. subtilis* is the microorganism of choice for diverse bio-applications such as vaccines [5–7] or bioremediation [8, 9].

Differing from laboratory-adapted strains, non-domesticated strains of *Bacillus subtilis* (as NCIB3610 strain) can form complex architectural structures denominated biofilms [10–12]. In general, biofilms are communities of surface-associated microorganisms encased in a self-produced extracellular matrix that commonly comprises lipids, amyloid-like proteins, eDNA, and exopolysaccharides [10, 13, 14]. Several proteins compose the *B. subtilis* biofilm matrix, and at least two of them, TasA and BslA, are

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essential in maintaining the biofilm structure [13]. TasA, in particular, shows to be a major component of the extracellular matrix [12] and forms amyloid-like fibers [15]. Additionally, *B. subtilis* form endospores that are highly resistant to harsh conditions as noxious chemicals, extreme temperature and pressure, being essential for preserving *B. subtilis* genetic material.

We demonstrated that TasA could be used to display heterologous proteins in the surface of biofilm [16], making a valuable tool for expressing an antigen of interest (AOI). Later, we showed in a dog [17] and mouse [18] models that oral application of recombinant *B. subtilis* spores harboring TasA fused to *Echinococcus granulosus* antigens, or even to the red fluorescent protein mCherry, are able to elicit a humoral intestinal immune response. This model is based on the ability of the recombinant *B. subtilis* spores to bypass the acidic stomach barrier and then germinate in the gut, forming a biofilm able to display TasA fused to an AO. Upon these conditions, the recombinant *B. subtilis* biofilm stimulates the gut-associated lymphoid tissue, resulting in the elicitation of a humoral immune response. Thus, the mouse model is a well-suited model for eliciting an immune response against antigens provided *via* the display in *B. subtilis* biofilm. Interestingly, the settlement of recombinant *B. subtilis* vegetative cells in the mouse gut environment require, as a preceding step, the eradication of the intestinal microbiota for an enhanced immunity which is achieved by pretreatment of the mice with an antibiotic mixture [18].

This chapter provides the essential tools for building a recombinant *B. subtilis* harboring TasA fused to an AOI and in vitro analysis of expression in recombinant biofilms. Next, we outline the methodology for preparation and application by oral gavage feeding tube of recombinant *B. subtilis* spores. Lastly, we provide the tool for analyzing local humoral immune responses.

2 Materials

2.1 Transformation of B. subtilis

1. *Bacillus subtilis* subsp. *subtilis* 168 (ATCC 23857).
2. LB broth (Miller) autoclaved.
3. Glycerol, sterilized by autoclaving.
4. Liquid nitrogen (1 L).
5. 1.5 mL test tubes, sterile.
6. Water bath, set at 37 °C.
7. Static incubator, set at 30 °C.
8. Static incubator, set at 37 °C.
9. Sterile tubes for culture, 15 mL.

10. T-Base media: $(NH_4)_2SO_4$, 2 g/L; $K_2HPO_4 \cdot 3H_2O$, 18.3 g/L; KH_2PO_4, 6 g/L; trisodium citrate$\cdot 2H_2O$, 1 g/L. Sterilize by autoclaving.
11. SpC media: T-Base, 50% glucose, 1.2% $MgSO_4$, 10% Bacto yeast extract, 1% casamino acids, 10 mg/mL Phenylalanine, 10 mg/mL Tryptophan.
12. SpII media: T-Base, 50% glucose, 1.2% $MgSO_4$, 10% Bacto yeast extract, 1% casamino acids, 10 mg/mL Phenylalanine, 10 mg/mL Tryptophan, 0.1 M $CaCl_2$.
13. 0.1 M EGTA, pH 8.
14. SpII + EGTA media: SpII without $CaCl_2$ containing 20 mM EGTA pH 8.
15. 100 mg/mL Spectinomycin, in dH_2O. Sterilize using a 0.22 μm filter.
16. 10 mg/mL Kanamycin, in dH_2O. Sterilize using a 0.22 μm filter.
17. Spectrophotometer.
18. Rotary shaker.
19. 200 mL Erlenmeyer flask.
20. 2 L Erlenmeyer flask.
21. Selective LB: LB media fortified with 1.5% Bacto agar. Antibiotics for selection are added when the media reaches 50 °C (Spectinomycin 100 μg/mL, Kanamycin 10 μg/mL).
22. Glass beads of 3 mm diameter. Sterilize by autoclaving.

2.2 Transduction of B. subtilis *NCIB3610*

1. TY media: Tryptone, 10 g/L; yeast extract, 5 g/L, NaCl, 5 g/L; $MgSO_4$, 2.46 g/L, $MnSO_4$, 0.017 g/L. Adjust to pH 7.2.
2. *Bacillus subtilis* bacteriophage SPP1.
3. *Bacillus subtilis* subsp. *subtilis* NCIB 3610 (ATCC 6051, DSM 10).
4. 15 mL test tubes, sterile.
5. Cell scraper.
6. 1 M $MgSO_4$. Sterilize by autoclaving.
7. DNase I recombinant (Roche).
8. Syringe filter, 0.45 μm.
9. 1.5 mL cryotubes.
10. Chloroform ($CHCl_3$).
11. TY top-agar media: TY media fortified with 0.5% Bacto agar.
12. TY agar media: TY media fortified with 1.5% Bacto agar.
23. Selective LB: LB media fortified with 1.5% Bacto agar. Antibiotics for selection are added when the media reaches 50 °C

(Spectinomycin 100 μg/mL, Kanamycin 10 μg/mL). When needed, add Sodium citrate to final concentration of 10 mM.

13. Centrifuge.
14. Glass beads, ø 3 mm. Sterilize by autoclaving.

2.3 *In Vitro* B. Subtilis *Biofilm*

1. LB broth (Miller).
2. MSgg broth [12]: 50 mM potassium phosphate buffer, 0.1 M MOPS pH 7.0, 0.05 mM $FeCl_3$, 2 mM $MgCl_2$, 0.0001 mM $ZnCl_2$, 0.002 mM thiamine, 50 μg/mL tryptophan, 50 μg/mL phenylalanine, 50 μg/mL threonine, 0.5% glycerol, 0.5% glutamate, 0.7 mM $CaCl_2$ in distillated Milli-Q water. All the components are filtered at exception of $CaCl_2$ that it is autoclaved. The MSgg broth can be stored in dark at 4 °C for up to 1 week.
3. MSgg semi-solid media: To prepare 500 mL of MSgg semi-solid media, all the above components for MSgg broth are mixed to a volume of 200 mL and pre-warmed in a water bath at 37 °C for 20 min. Meanwhile, an autoclaved solution containing 7.5 g of Bacto agar in 300 mL of distillate is pre-warmed in a water bath at 50 °C. The two components are mixed in orbital motion and immediately used to prepare Petri dishes. The final agar concentration is 1.5% (w/v). Thus, exactly 25 mL of MSgg semi-solid media per 10 cm diameter Petri dish is added by pipetting using a 25 mL disposable pipet. The MSgg plates can be stored in the dark for 1 week at 4 °C.
4. Petri dishes 10 cm diameter.
5. 25 mL pipets.
6. 12-well multi-well tissue culture plates.
7. 37 °C shaker.
8. 30 °C incubator without a ventilator.
9. Stereo microscope equipped with a 1.25 × S objective.

2.4 Preparation of Biofilm Samples for Tas-AOI Fusion

1. Sample buffer 4×: 8% SDS, 40% glycerol, 200 mM Tris pH 6.8, 4% 2-mercaptoethanol, 0.4% bromophenol blue).
2. Sonicator.
3. Spectrophotometer.
4. Cuvettes.

2.5 Preparation of Spores

2.5.1 Growing Culture

1. LB broth (Miller).
2. LB agar plates (semi-solid media): LB broth (Miller), 1.5% Bacto agar.
3. 1 L Erlenmeyer flask.
4. Incubator at 37 °C (e.g., Heraeus).

5. 4 L of Difco sporulation medium (DSM) is prepared as previously described [4] (*see* **Note 1**). DSM: 0.8% Bacto Nutrient Broth, 0.1% KCl, 0.012% $MgSO_4$, 0.5 mM NaOH. The solution is autoclaved and immediately cooled in a water bath at 50 °C. The following reagents are added once the temperature is equilibrated at 50 °C: 1 mM $Ca(NO_3)_2$, 0.01 mM $MnCl_2$, 1 μM $FeSO_4$. Semi-solid DSM plates are obtained by the addition of 1.5% Bacto agar to the solution before autoclaving.
6. Eight Erlenmeyer flasks of 2 L.
7. Serological pipets, 25 mL.
8. Shaker at 37 °C and 180 rpm (Kuhner, Climo-Shaker ISF4-X) with a Tray for 2 L Erlenmeyer flasks (Kuhner).

2.5.2 Spore Purification

1. Centrifuge (e.g., Sorvall RC6+ Centrifuge ThermoFisher).
2. Rotors for the centrifuge (Fiberlite F12-6x500 LEX by Thermo Scientific for the 500 mL centrifuge tubes, Fiberlite F14-6x250y by Thermo Scientific for the 250 mL centrifuge tubes and SS-34 by Sorvall® for the 33 mL centrifuge tubes).
3. Centrifuge bottles (500 mL, 250 mL, and 33 mL).
4. Serological pipet, 10 mL.
5. 1 L of KCl/NaCl solution: 1 M KCl and 0.5 M NaCl.
6. 1 L of 50 mM Tris pH 7.5 with lysozyme 50 μg/mL (Fluka Analytical) (*see* **Note 2**).
7. 1 L of 1 M NaCl solution.
8. 4 L of autoclaved deionized water.
9. PBS.
10. 15 mL test tubes.
11. 1.5 mL test tubes.

2.5.3 Spore Quantification

1. LB agar plates (semi-solid media).
2. PBS.
3. 96-well multi-well tissue culture plate.

2.5.4 Electron Microscopy of the Spores

1. 4% trypticase soy agar.
2. Cigarette paper.
3. Copper block.
4. Liquid nitrogen.
5. Freezing device (KF 80, Reichert-Jung, Austria).
6. Freeze substitution unit (FS 7500, Boeckler Instruments).
7. Acetone.
8. 0.25% glutaraldehyde.

9. 0.5% osmium tetroxide.
10. Epon.
11. Ultrathin Section cutting device.
12. Uranyl-acetate and lead-citrate.
13. Transmission electron microscope with a CCD camera.

3 Methods

3.1 Transformation of B. subtilis

3.1.1 Competent Cells Preparation

1. Using LB media fortified with 1.5% agar, grow *B. subtilis* 168 in an incubator overnight at 30 °C.
2. Use the freshly grown bacteria to inoculate 20 mL of pre-warmed SpC medium. The inoculated media should give an $OD_{600\ nm}$ reading of approximately 0.5.
3. Incubate at 37 °C with vigorous shaking and take samples periodically to follow cell growth by reading at $OD_{600\ nm}$.
4. When the culture reaches the stationary phase (no significant increase in $OD_{600\ nm}$ over 20 min), use 2 mL of the culture to inoculate 200 mL of pre-warmed SpII media and continue incubation at 37 °C with agitation.
5. After 90 min of incubation, harvest the cells by centrifugation at 8000 × *g* for 5 min, at room temperature.
6. Carefully decant the supernatant into a sterile container and save (*see* **Note 3**).
7. The cells are resuspended in 18 mL of the saved supernatant and added 2 mL of sterile glycerol. Mix gently, avoiding the formation of bubbles or foam.
8. Distribute the mix of competent cells in sterile centrifuge tubes, labeled, making aliquots of 0.5 mL. Snap-freeze in liquid nitrogen and store at −80 °C.
9. Prepare 20 mL of SpII + EGTA and distribute in sterile centrifuge tubes labeled, making aliquots of 0.5 mL. Snap-freeze in liquid nitrogen and store at −80 °C.

3.1.2 Competent Cells Transformation

1. Thaw a tube containing SpII + EGTA by immersing frozen tubes in a 37 °C water bath. After thawing, place the tube in a rack at room temperature.
2. Thaw a tube containing competent cells by immersing frozen tubes in a 37 °C water bath. Immediately after thawing, add one volume of SpII + EGTA and mix gently.

3. In a sterile 15 mL test tube, add 200 μL of the mix of competent cells and SpII + EGTA to the DNA solution (<0.1 mL) and incubate for 30 min in a rotary shaker at 37 °C. Make sure to have one control with no addition of DNA.
4. Plate cells in selective LB media and incubate at 37 °C.

3.1.3 SPP1 Transduction of B. subtilis NCIB3610

The method of generalized transduction of *B. subtilis* is performed over two consecutive days. On day 1, the *B. subtilis* 168 donor strain (harboring the genetic elements transferred to *B. subtilis* NCIB3610) will be used to prepare an SPP1 phage lysate stock. On day 2, the newly made SPP1 stock will be used for the generalized transduction of *B. subtilis* NCIB 3610.

Day 1

1. Use a fresh culture of the donor strain of *B. subtilis* 168, grown on a plate of LB media fortified with 1.5% agar.
2. Inoculate 3 mL of TY media in a 15 mL test sterile tube to an $OD_{600\ nm}$ of about 0.1.
3. Incubate in an orbital shaker at 37 °C until the culture reaches the late-log/early-stationary phase (usually $OD_{600\ nm}$ of 1.0–1.5).
4. In a 15 mL sterile tube, mix 200 μL of the culture with 100 μL of diluted phage stock (*see* **Note 4**) and incubate without agitation at 37 °C for 15 min.
5. Prepare TY top agar and equilibrate at 50 °C in a water bath. Add 3 mL of top agar into the 15 mL tube containing the cells, mix gently and thoroughly, and pour on top of a fresh TY plate, making sure to cover all the surface of the plate (*see* **Note 5**).
6. Incubate at 37 °C for 16 h.
7. Grow the recipient strain (i.e., *B. subtilis* NCIB3610) on a plate of LB media fortified with 1.5% agar at 37 °C.

Day 2

1. Choose a plate that is covered with plaques but that still has clearplaque outlines. Then, using a 10 mL pipet, add 3 mL TY, carefully break up the top agar with a cell scraper, and transfer the agar suspension to a sterile 15 mL test tube. Next, wash the plate with another 3 mL TY and add this volume to the same 15 mL test tube.
2. Gently vortex to help break up the agar, then centrifuge at 2000 × *g* for 10 min.
3. The supernatant is transferred to a new 15 mL test tube and add $MgSO_4$ to 10 mM. Next, add 100 U of DNAse I and incubate at room temperature for 5 min.

4. The solution is then filtered with a 0.45 μm syringe filter into a labeled cryotube. Next, add a drop (30–50 μL) of $CHCl_3$. The SPP1 phage lysate stock can be stored at 4 °C until use.
5. Using the fresh plate of the recipient strain, inoculate 3 mL of TY media in a 15 mL sterile tube to an $OD_{600\ nm}$ of 0.1.
6. Incubate the culture at 37 °C until it reaches the late-log/early-stationary phase (usually OD_{600nm} of 1.0–1.5).
7. Add 1 mL of the culture to a sterile 15 mL test tube containing 9 mL of the diluted lysate (*see* **Note 6**). Incubate at 37 °C for 30 min without agitation.
8. Centrifuge at 2500 × *g* for 10 min at room temperature. Using a 10 mL pipet, carefully remove the supernatant, leaving approximately 200–300 μL. Use this remaining supernatant to resuspend the pellet. Spread the cells on a plate containing selective antibiotics plus 10 mM sodium citrate; incubate at 37 °C for 12–36 h (*see* **Note 7**).

3.1.4 Genetic Background for the Expression of TasA-AOI Fusion Proteins

The TasA-AOI fusion proteins need to be expressed in a genetic background lacking both a functional SinR, repressor of the *tasA* operon, and the native TasA to obtain the maximum expression [16]. This bacterial strain is prepared by allele replacement using long-flanking homology PCR (LFH–PCR) [19]. To create the mutant *tasA/sinR*::km^R, genomic DNA of *B. subtilis* NCIB3610 was used as a template in a PCR containing the following primers (Table 1): (i) FtasAUP and RtasADO; (ii) FsinRUP and RsinRDO. The km^R gene was obtained by amplification of genomic DNA from *B. subtilis tasA* (CA017) strain [20] using the primers FKmrUP and RkmrDo (Table 1). The products were joined by PCR, and the amplicon was then gel-purified. The purified amplicon was transformed into *B. subtilis strain* 168 and the positive clones were then used for allele replacement into *B. subtilis* strain NCIB3610 employing SPP1-mediated generalized transduction (see Subheading 3.1.3).

3.1.5 Plasmids Construction

The plasmid pBS-TapAop is obtained by PCR amplification of the *tapA-sipW-tasA* operon from genomic DNA of *B. subtilis* NCIB3610, using primers TapAopF_BamHI and TapAopF_XbaI. The fragment is digested with *Xba*I and *BamH*I and cloned in the corresponding sites of pBluescript II KS(+) (Stratagene).

The plasmid pBS-TapAop-mCherry is obtained by PCR amplification of *mCherry* from pRSET-mCherry [21] using primers mCherryF_XbaI and mCherryR_SB. The PCR product is digested with *Xba*I and *Sac*I and subcloned into the respective sites of pBS-TapAop [16].

The plasmid pDG-TasA-mCherry is obtained by digestion of pBS-TapAop-mCherry with *BamH*I and ligated in the corresponding sites of pDG1662 (*Bacillus* genetic stock center,

Table 1
Primers used recombinant *B. subtilis*

Primer name	Oligonucleotide sequence
FtasAUP	5-ACAATAAGTCATGGCCGGA-3′
RtasADO	5-CCTATCACCTCAAATGGTTCGCTGGTTCGCTGGTTTAATACGCTGGCCAA-3′
FsinRUP	5-CGAGCGCCTACGAGGAATTTGTATCGGCTCCCCTTTTATTGAATG-3
RsinRDO	5-TATGCCGGCTATATGCTT-3
FKmrUP	5-CAGCGAACCATTTGAGGTGATAGG-3
RKmrDo	5-CGATACAAATTCCTCGTAGGCGCTCGG-3
TapAopF_BamHI	5-ATGCGGATCCTCAGAGTTAAATGGTATTGCT-3
TapAopF_XbaI	5-GCATTCTAGAATTTTTATCCTCGCTATGCGC-3
mCherryF_XbaI	5-GATCTCTAGAATGGTGAGCAAGGGCGAGGAG-3
mCherryR_SB	5-GATCGAGCTCGGATCC**TTA**CTTGTACAGCTCGTCCAT-3’

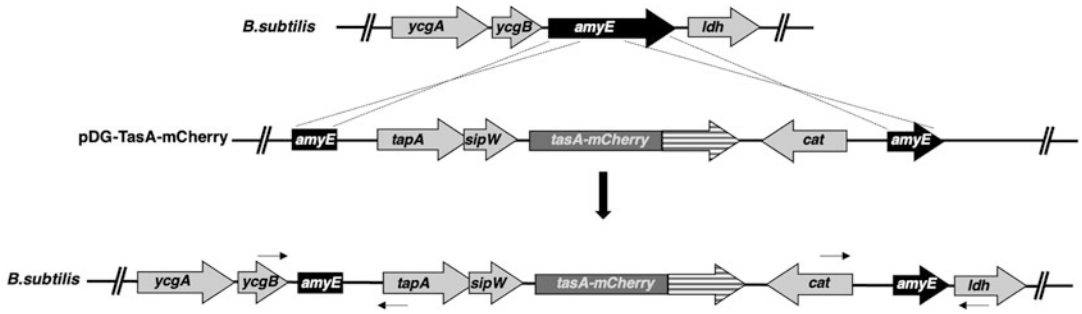

Fig. 1 Allelic replacement at the *amyE* locus. The plasmid pDG-TasA-mCherry was linearized and transformed into *B. subtilis*. The allelic replacement occurs via a homologous recombination event at the *amyE* locus, and selection is conferred by chloramphenicol resistance located inside the recombination zone. After selection, clones are analyzed by PCR for the correct insertion, with oligos that anneal outside the zone of recombination (represented by small arrows on the bottom of the image)

http://www.bgsc.org/). Next, the plasmid pDG-TasA-mCherry is linearized by digestion with *Xho*I and then transformed into *B. subtilis* strain 168. The positive clones are then used for allele replacement into *B. subtilis* strain NCIB3610 employing SPP1-mediated generalized transduction (see Subheading 3.1.3).

Clones of *B. subtilis* strain NCIB3610 are subsequently screened for the correct insertion of the recombinant locus at the native *amyE* locus. This is done by PCR, with one oligonucleotide annealing outside the recombination zone and another one annealing in the recombinant locus. Typically, two PCR reactions with oligos annealing upstream or downstream of *amyE* are performed as evidence for the correct recombination event (Fig. 1).

3.2 Characterization of Biofilms

3.2.1 Growing Biofilms of B. subtilis in MSgg Semi-Solid Media

1. One isolated colony of undomesticated *B. subtilis* is inoculated in 3 mL of LB broth and incubated for 6 h in a shaker at 200 rpm and 37 °C.
2. Samples are normalized to $OD_{600\ nm}$ of 1. For this, each culture sample was diluted 1:10 in LB broth (100 μL bacteria culture plus 900 μL LB). Blank is LB broth. Samples were measured in a spectrophotometer. The formula used to reach $OD_{600\ nm}$ of 1 is: vol. bacteria (μL) = 1 $OD_{600\ nm}$ × 1000 μL/ measured $OD_{600\ nm}$.

   Then, the volume of bacteria obtained from the equation is completed up to 1000 μL with LB broth.
3. Upon sterile conditions, a drop of 2 μL of the normalized culture is added to the surface of MSgg semi-solid media and let to dry (*see* **Note 8**).
4. The plates are incubated at 30 °C for 72 h post-inoculation with bacteria facing up. This time allows featuring well-defined biofilm architectonic phenotype.

3.2.2 Growing Biofilms of B. subtilis in MSgg Liquid Medium

1. One isolated colony of undomesticated *B. subtilis* is inoculated in 3 mL of LB broth and incubated for 6 h in a shaker at 200 rpm and 37 °C.
2. Samples were normalized to $OD_{600\ nm}$ of 1. For this, each culture sample was diluted 1:10 in LB broth (100 μL bacteria culture plus 900 μL LB). Blank is LB broth. Samples were measured in a spectrophotometer. The formula used to reach $OD_{600\ nm}$ of 1 is: vol. bacteria (μL) = 1 $OD_{600\ nm}$ × 1000 μL/ measured $OD_{600\ nm}$. Then, the volume of bacteria obtained from the equation is completed up to 1000 μL with LB broth.
3. Upon sterile conditions, 10 μL of the normalized culture is inoculated in 8 mL per well of MSgg media of a 6-well muti-well plate. The MSgg media volume has been standardized to generate an optimal architectonical phenotype.
4. The multi-well plates are incubated at room temperature in the dark for 96 h post-inoculation. This time allows featuring well-defined biofilm architectonic phenotype.
5. Alternatively, 3 mL per well of MSgg in a 12-well plate can be inoculated with 10 μL of normalized culture. In this condition, mature biofilms pellicles will be observed at 48–72 h post-inoculation.

1. After 72 h post-inoculation in semi-solid or 48 h post-inoculation in liquid media, the whole biofilm is collected in a 2 mL test tube containing 1 mL distilled water with protease inhibitor (cOmplete™ EDTA-free protease inhibitor cocktail,

3.3 Characterization of TasA-Heterologous Protein Fusion by Western Blotting

3.3.1 Sample Preparation

Roche, Switzerland). One tablet of protease inhibitor dissolved in 5 mL water.

2. The biofilm cells are disaggregated by sonication with five pulses of 10 s at 14 Hz.
3. The biofilm samples are then normalized to OD_{600nm} of 1.
4. 25 μL of each normalized sample are mixed with 8 μL of sample buffer 4× and heated in a thermoblock for 5 min at 95 °C.
5. The heated samples are spin-down, followed by 10-s sonication at 14 Hz. This step is required for shearing of the DNA present in the sample and to facilitate loading in SDS-polyacrylamide gel.

3.3.2 Gel Migration and Immunoblotting

1. It is necessary to prepare a 12% SDS-polyacrylamide gel using 1.5 mm spacers adapted for Bio-Rad Mini-PROTEAN vertical electrophoresis cell. A pre-stained molecular weight marker is used, such as Precision Plus Protein Dual Color Standard (Bio-Rad).
2. The loaded samples are resolved at 30 mA per gel until the bromophenol blue migration front reaches the bottom end of the gel.
3. The proteins are transferred to 0.45 μm nitrocellulose (Protan, Amersham) in a wet chamber using transfer buffer for 2 h at 200 mA or overnight at 50 mA.
4. To evaluate that all the proteins are transferred correctly and in similar amounts, the membrane is stained with Ponceau S solution (Sigma) for 1 min in a shaker at room temperature. A picture acquisition is advised to be kept in the laboratory registry. Finally, the membrane is rinsed with PBS to remove Ponceau S staining.
5. The membrane is blocked with gentle agitation in a shaker with 5% non-fat milk in PBS for 30 min at room temperature or overnight at 4 °C.
6. The TasA-AOI fusion is identified either by incubating the membrane with rabbit anti-TasA [18] diluted (1:5000) or with an antibody specifically recognizing the AOI. In both cases, the antibodies are diluted in 5% non-fat milk in PBS and incubated for 1 h at room temperature.
7. The membrane is washed three times in an orbital shaker for 2 min using 5 mL of 5% non-fat milk in PBS each time.
8. The secondary antibodies are directed against the host species used to incubate the membrane with the primary antibody. Thus, IRDye® 800CW goat anti-Rabbit IgG (LI-COR) secondary antibody is used to bind anti-TasA. The secondary

antibody is diluted in 5% non-fat milk PBS and incubated in the dark with agitation for 1 h at room temperature.

9. The membrane is washed three consecutive times for 5 min at room temperature with 5% non-fat milk in PBS. Finally, the membrane is rinsed with PBS until no traces of milk are observed.
10. The membrane signal is acquired using an Odyssey Fc (LI-COR) imaging system.
11. Alternatively, the membrane can be incubated with a secondary antibody conjugated with horseradish peroxidase. The membrane is developed using a commercial kit for chemiluminescence, as ECL™ western blotting detection reagents (Pierce), and the image is also acquired at the Odyssey Fc imaging system.

3.3.3 Data Analysis

Since the molecular weight of TasA corresponds to 26 kDa, it is expected that TasA fused to the AOI (TasA-AOI) has a molecular weight of 26 kDa plus the molecular weight of the AOI peptide (Table 2). For example, as indicated in Fig. 2, the immunoblot anti-TasA showed a band at 52 kD corresponding to the expected MW for TasA-mCherry fusion protein. This band is confirmed by incubating the membrane with an anti-mCherry antibody that colocalized with the 52 kDa band and ensuring that this band is TasA-mCherry. Furthermore, as expression control of our biofilm, we included a sample of *B. subtilis* NCIB3610 biofilm showing a unique band at 26 kDa that corresponds to TasA. Similarly, the deletion mutant for TasA, *B. subtilis* strain *tasA/sinR*, did not show TasA after incubation of the membrane with an anti-TasA antibody. Thus, this methodology allows fast identification of the TasA fused to AOI expressed within the biofilm.

3.4 Preparation of Spores

The whole process needs to be done in a sterile environment. Each preparation of *B. subtilis* spores requires a total volume of 4 L DSM.

3.4.1 Growing Culture

This method has been adapted from Nicholson and Setlow [4].

1. Grow *B. subtilis* strains on LB semi-solid media for 16 h at 37 °C.
2. Inoculate a colony in 250 mL LB broth media in a 1 L Erlenmeyer flask and incubate at 37 °C for overnight in an orbital shaker at 180 rpm.
3. Dilute 28 mL of the culture in 500 mL DSM contained in a 2 L Erlenmeyer flask and incubate for 72 h at 37 °C in an orbital shaker at 180 rpm. Therefore, the total amount of Erlenmeyer flasks is 8.

Table 2
Characterization by immunoblotting of TasA fused to heterologous proteins in recombinant *B. subtilis* biofilms

Bacterial strain	Protein	Expected MW (kDa)	Reference/source
NCIB3610 (wt)	TasA	26	Kolter Lab., Harvard Medical School
tasA/TasA-mCherry	TasA-mCherry	52	Vogt et al. [16]
tasA/TasA-AOI	TasA-AOI	26 + MW of AOI	
tasA/sinR	–	–	Vogt et al. [16]

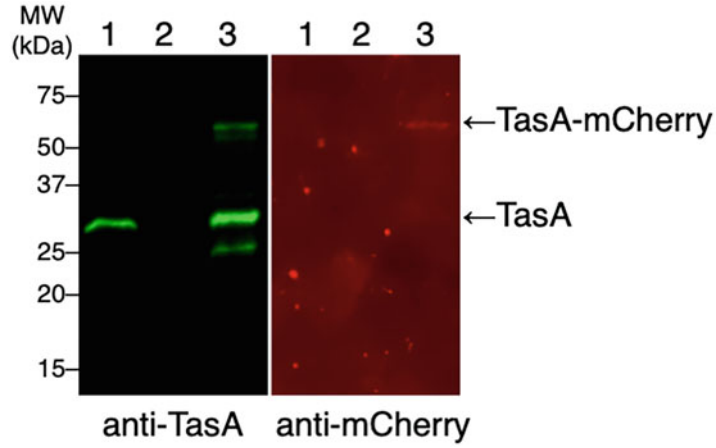

Fig. 2 Immunoblotting of *B. subtilis tasA/sinR*/TasA-mCherry biofilm extracts at 72 h post-inoculation. Biofilm extracts at 72 h post-inoculation were prepared from *B. subtilis* strains NCIB3610 (wt, lane 1), *tasA/sinR* (lane 2), and *tasA/sinR*/TasA-mCherry (lane 3) grown in MSgg semi-solid media. The membrane (left panel) was incubated with rabbit anti-TasA (diluted 1:5000) followed by an anti-rabbit conjugated to IRDye® 800CW (diluted 1:5000). Next, the membrane (right panel) was incubated with mouse monoclonal anti DsRed2 (sc-1,015,126, diluted 1:1000) followed by secondary goat anti-mouse conjugate to IRDye® 680RD (1:5000). The arrows point to the localization of TasA-mCherry and TasA. The molecular weight standard is indicated at the left

4. Pour the culture into 500 mL centrifuge tubes (*see* **Note 1**) and spin-down at 17,000 × *g* for 20 min at 4 °C.
5. Remove the supernatant (*see* **Note 2**) and store the pellet at −80 °C overnight.

3.4.2 Spore Purification

1. Incubate the pellets in an 80 °C hot water bath for 30 min.
2. Keep working close to the flame: resuspend the pellets in ten volumes of a solution containing 1 M KCl and 0.5 M NaCl.

Transfer the resuspended pellets from a 500 mL to a 250 mL centrifuge tube. Centrifuge at 15,000 × *g* for 20 min at 4 °C.

3. Discard the supernatant.
4. Resuspend the pellets in ten volumes of a solution containing 50 mM Tris pH 7.5 and 50 μg/mL lysozyme. Then, incubate for 60 min at 37 °C without agitation. This procedure will permit the proteolysis of the bacteria cell wall.
5. Centrifuge at 15,000 × *g* for 20 min at 4 °C.
6. Discard the supernatant.
7. The pellets are washed with ten volumes 1 M NaCl and centrifuged at 15,000 × *g* for 20 min at 4 °C.
8. Discard the supernatant.
9. Wash pellets with ten volumes with sterile deionized water and centrifuge at 15,000 × *g* for 20 min at 4 °C.
10. Discard the supernatant and repeat from **step 9** twice.
11. Resuspend the pellet in ca. 5–10 mL PBS, transfer the resuspended pellet to a 33 mL centrifuge bottle. For this, pool the pellets of the 250 mL centrifuge bottles in one bottle and then transfer to a new 33 mL centrifuge bottle. Centrifuge for 20 min at 12,000 × *g* and 4 °C.
12. Remove the supernatant, resuspend the pellet in 20 mL PBS, and centrifuge for 20 min at 12,000 × *g* and 4 °C.
13. Remove the supernatant, resuspend the pellet in 10 mL PBS, and transfer everything into a 15 mL test tube. To maximize the spore yield, rinse the 33 mL centrifuge tubes again and transfer the supernatant to another 15 mL test tube.
14. Reserve an aliquot of 50 μL of purified spores for further quantification.

3.4.3 Spores Quantification

1. Prepare ten-fold serial dilutions of the aliquot of spore preparation, as follows:
   (a) Pipet 180 μL of PBS in each well of a 96-well multi-well plate (tissue culture plate with flat bottom).
   (b) Add 20 μL of the spore preparation in the first well of each row (dilution 10^{-1}).
   (c) Mix each dilution volume by pipetting up and down at least three times.
   (d) Transfer 20 μL of the diluted volume to the next well. It is relevant to change the pipet tips at this point.
   (e) Repeat from **step** (**c**) until you reach the dilution of 10^{-12}.

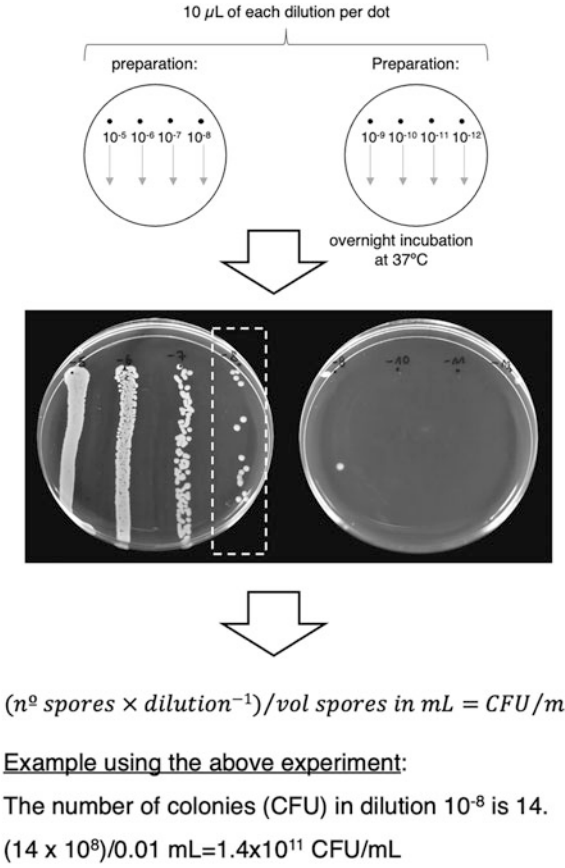

Fig. 3 Quantification of *B. subtilis* spores. Ten-fold serial dilutions of *B. subtilis* are prepared, and 10 μL are inoculated over LB agar plates as indicated at the top of the diagram. The plates are vertically inclined to allow the spread of each drop until the next end. After overnight incubation at 37 °C, the spores germinate and form colonies that can be easily counted. The number of colonies at the highest dilution is considered to determine the number of colony-forming units (CFU). Thus, one spore generates one colony. The data are processed as indicated in the example

2. Label the LB agar plates as in Fig. 3.
3. Pipet aliquots of 10 μL of each of the dilutions from 10^{-5} to 10^{-12} on the plate, as indicated in Fig. 3.
4. Hold plates vertically so the drops flow to the opposite end of the bottom of the dish.
5. Incubate at 37 °C overnight.
6. The concentration of spores (CFU/mL) is determined as described in Fig. 3.

3.4.4 Electron Microscopy of the Spores

This experiment allowed to determine if the recombinant *B. subtilis* spores have the same morphology as wild type spores.

1. Plate a 3 μL aliquot of freshly prepared spores on small pieces (2 × 3 mm) of 4% trypticase soy agar mounted on cigarette paper and slammed on a highly polished copper block cooled by liquid nitrogen in a freezing device.
2. Transfer the samples to a freeze substitution unit precooled at −88 °C for substitution with acetone.
3. Fix the samples with 0.25% glutaraldehyde and 0.5% osmium tetroxide, raising the temperatures gradually to +2 °C.
4. The samples are then kept at a temperature of −88 °C for up to 2 days.
5. Embed the samples in epon and let polymerize at 60 °C for 2.5 days.
6. Cut ultrathin sections (70–80 nm) and stain with uranyl-acetate and lead-citrate before analysis in a transmission electron microscope equipped with a CCD camera at an acceleration voltage of 100 kV.
7. Data are processed and analyzed using ImageJ software (version 2.1.0/1.53c via a Creative Commons license).

3.4.5 Spores Storage

1. The spore preparation is stored in a 15 mL sterile conical test tube (polypropylene centrifuge tube) at −80 °C until further usage.
2. An 50 μL aliquot can be stored at −20 °C only when used for immediate spore quantification.

3.5 Animal immunization

Each animal experiment requires approval of national legislation and the international agreement based on harm-benefit analysis of the conducted experiment.

The animal model of choice corresponds to BALB/c or C57BL/6 mice because they are susceptible to elicit a favorable cellular and humoral immune response to AOI.

3.5.1 Pretreatment with an Antibiotic Mixture

Mice are treated with an antibiotic mixture to eliminate the gut microbiota, as described by Shan *et al.* [22]. This procedure is necessary to reduce the intestinal microflora and allows the settlement of the recombinant *B. subtilis* in the mice gut.

1. For this purpose, a mixture containing 0.5 mg/mL ampicillin (PanReac AppliChem, Spain), 0.5 mg/mL gentamycin (PanReac AppliChem, Spain), 0.25 mg/mL vancomycin (Alfa Aesar, Germany), metronidazole (Alfa Aesar, Germany), and 20 mg/mL sucrose (Sigma) dissolved in sterile drinking water was provided as a source of water to the mice for 5 days.

Sucrose is required to increase the palatability of the antibiotic mixture.

2. The mice are grouped randomly in a maximum of five animals per cage and provided with *ad-libitum* food. The drinking water is replaced for 5 days with the antibiotic cocktail.
3. Every day, up to 1 g of feces is collected randomly from each cage. This step is necessary to monitor the reduction of the intestinal microflora by growing bacteria from feces in enriched non-selective media. The samples need to be treated during the same day and therefore cannot be stored.
4. Each sample of feces is resuspended in five volumes of PBS and vortexed to homogeneity. Then bacteria are ten-fold serially diluted from 10^{-1} to 10^{-12} using 96-well multi-well plates. For this, 20 μL of resuspended feces are pipetted in 180 μL PBS to reach dilution 10^{-1} and then followed by serial dilutions up to 10^{-12}. It is crucial to change the pipet tips between dilutions.
5. Each feces dilution (10 μL) is seeded, similarly as described in Fig. 3, in three types of non-selective agar media: LB, heart-brain infusion, and nutrient broth.
6. The plates are incubated overnight at 37 °C.
7. The reduction of the intestinal microbiota was monitored by counting the number of bacterial colonies isolated from feces in non-selective media. The data collected for each selective media are plotted using the *x*-axis on the day of treatment, and in the *y*-axis the number of CFU/g of feces. It is expected that on days 4 and 5, no bacterial colonies are growing in the plates.
8. Please *see* **Note 9**.

3.5.2 Oral Gavage

1. The oral immunization is performed using an oral gavage feeding tube containing a maximum volume of 200 μL of recombinant *B. subtilis* spores in PBS. Each dose contain 5×10^{10} CFU of recombinant *B. subtilis* spores.
2. When indicated, the immunization will be performed a maximum of three times on days 1, 21, and 42 of the experimental schedules (Fig. 4).
3. For this purpose, we will use round head oral gavage feeding tubes of 0.8 × 40 mm from Delvo (cat #191181).
4. The animal will be double-handed restrained as follows: Slide the thumb and index fingers of the hand up to the animal body and grasp the scruff of the neck to restrain the head. Extra restraint is achieved by holding the tail with the fourth and fifth fingers.

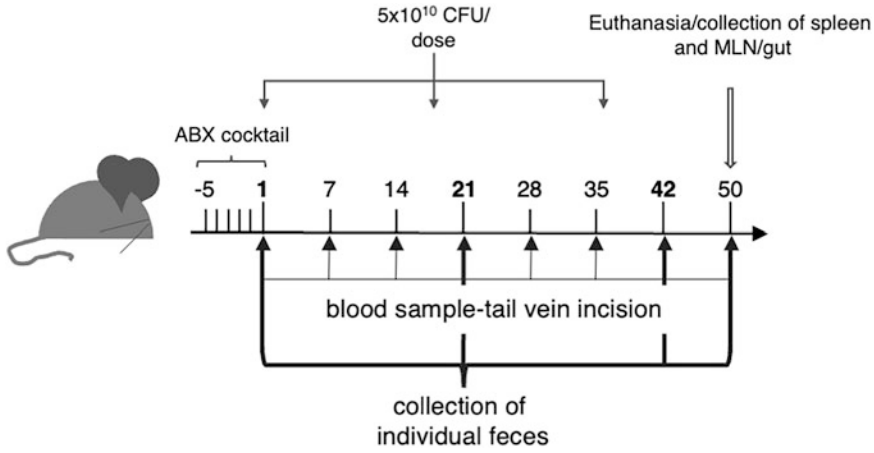

Fig. 4 Schematic schedule for a mouse model of immunization with recombinant *B. subtilis* spores. Mice are treated with an antibiotic cocktail for 5 days to eradicate the intestinal microbiota. Then, recombinant *B. subtilis* spores are orally applied using gavage feeding tubes with $5x10^{10}$ CFU per dose in a volume of 200 μL on days 1, 21, and 42 of the experimental schedule. It is necessary to considerer experimental control such as placebo (saline solution) and background genotype spores as *B. subtilis* strain *tasA/sinR*. Blood samples are collected weekly, on days 1, 7, 14, 21, 28, 35, 42, 50, to monitor elicited humoral response. Feces collection permits the monitoring perseverance of shedding of recombinant spores and characterization of local intestinal immunity against display antigens in biofilm. The animals are sacrificed on day 50 to recover: (i) spleen and MLN to evaluate the cellular immune response and (ii) intestine sections (duodenum, jejunum, ileum, cecum, and colon) to characterize the localization of recombinant bacteria and local humoral immunity

5. Then for oral gavage, the animal is held vertically at chest height.
6. The water moisten gavage feeding tube is inserted 1/5 of its length above the tongue into the animal mouth.
7. It is essential to stimulate the throat and evoke swallowing reflex, and at the same time turn up the animal head with the gavage feeding tube until the top of the head and the body form a straight line.
8. Slide the probe along the esophagus until completely inserted (tip at the entrance of the stomach).
9. Apply solution (not too fast).
10. Then, the tube is carefully removed from the esophagus.
11. Finally, it is required to check the proper placement of the solution (entire volume applied, no reflux to mouth).

3.5.3 Collection of Blood and Feces Samples

IMPORTANT: The feces and blood samples need to be collected just before the animals are orally provided with recombinant *B. subtilis* spores.

Blood Sample Collection

1. Blood sample collection is performed by tail vein incision. This methodology allows the collection of 100 μL of blood once a week.
2. Anesthesia is unnecessary, which makes this route suited for repeated blood sampling.
3. Pressure is applied after blood collection to prevent hematomas. Blood samples are collected eight times, specifically on days 1, 7, 14, 21, 28, 35, 42, and 50 of the experimentation schedules (Fig. 4).
4. For this purpose, blood extraction is performed by employing a syringe/needle (26 gauge) or directly by a stab puncture of the lateral tail vein.
5. Vasodilation is necessary to promote bleeding. For this purpose, the animal is exposed at 37 °C for 5–8 min or by local warming of the tail.
6. The blood sample is collected in a 1.5 mL test tube and immediately processed for serum extraction.
7. For serum extraction: the blood samples are incubated for 30 min at 37 °C and then centrifuged at 1,500 × *g* for 15 min at 4°C. This procedure will permit the separation of PBMC, red blood cells, and coagulation factor from the serum that are found in the pellet and supernatant, respectively.
8. The recovered sera can be stored at −20 °C or used immediately to test the humoral immune response.

Feces Sample Collection

Feces collection is required to determine intestinal secretory antibodies and evaluate the persistence and germination of recombinant *B. subtilis* in the gut of the animals.

1. For this purpose, the mice need to be isolated into individual cages (16 cm × 21.5 cm SPF cages) during a maximum period of 24 h on days 1, 21, 42, and 50 of the experimentation schedules (Fig. 4).
2. All feces found in a cage need to be collected in a 10 mL test tube. Instead of regular litter, the animals are provided with abundant paper towel which facilitates the feces collection.
3. The animals are regrouped immediately in their original experimental groups (maximum number of mice per cage: 3; size SPF cage 19 cm × 37 cm). Please, *see* **Note 10**.
4. The feces are stored at −20 °C before processing. These samples are required for both to determine spore germination and elicitation of local humoral response.

3.6 Data Analysis

3.6.1 Determination of Recombinant B. subtilis in Mouse Feces

1. Feces are weighted and resuspended in 10 volumes of their weight with PBS.
2. Samples are vigorously vortexed until homogeneity.
3. 1 mL of the homogenized solution is collected in a new 1.5 mL test tube and heated for 30 min at 80 °C. This step is required to kill the vegetative cells and to allow the germination of recombinant *B. subtilis* spores.
4. Each sample is then ten-fold serially diluted as described in Subheading 3.4.3.
5. 10 μL of each serial dilution is then inoculated in media containing a selective media for the recombinant *B. subtilis* (*see* Subheading 3.1.4) and the drops are inclined vertically until reaching the opposite end of the plate.
6. The plates are incubated at 37 °C for overnight.
7. The number of colonies for each dilution is counted to determine the concentration of spores as CFU/mL (*see* Fig. 3).
8. The obtained concentration is normalized according to the weight of feces to obtain CFU/g of feces. For this, the following equation is applied:

$$\begin{aligned}&\text{spore conc.(CFU/mL)}\\&\quad\times \text{Vol.resuspension (mL)/weight of feces (g)}\\&\quad= \text{normalized spores value (CFU/g)}\end{aligned}$$

3.6.2 ELISA for Detection of the Humoral Response

1. One gram of feces is resuspended in 5 mL PBS.
2. The sample is vortex homogenized for 30 s and centrifuged at 800 × *g* for 10 min.
3. 360 μL of recovered supernatant is mixed with 40 μL of feces buffer (1% BSA, 0.01% Triton X-100, 0.1% 2-mercaptoethanol, and protease inhibitor (cOmplete™ EDTA-free protease inhibitor cocktail, Roche, Switzerland)) and kept in ice.
4. Recombinant purified protein (500 ng/well) or 72 h biofilm extract (OD_{600nm} of 0.0002/well) in 0.2 M bicarbonate buffer pH 9.4 is coated for 16 h at 4 °C in 96 well multi-well plates (Nunc-Immuno Maxisorp, Thermo Scientific). The biofilm extract is prepared, resuspended, and normalized as described in Subheading 3.2.1.
5. Plates are incubated for 2 h at room temperature with blocking buffer (1% BSA in PBS).
6. A 100 μL aliquot of feces mixture is added to each well and incubated for 2 h at 37 °C in a humid chamber.

7. The plates are washed three times with 200 μL per well of 0.05% Tween-20 in PBS.
8. The plate is incubated with 100 μL of secondary antibody diluted in 1% BSA-PBS and incubated for 1 h at 37 °C in a humid chamber. The following secondary antibodies are used: goat anti-mouse IgA conjugated to peroxidase (diluted 1:600, Sigma) or rabbit anti-mouse IgG (whole molecule) conjugated to peroxidase (diluted 1:2000, Sigma).
9. The plates are washed three times with 200 μL per well of 0.05% Tween-20 in PBS.
10. The plates are incubated with 100 μL per well of 3,3′,5,5-′-tetramethylbenzidine (TMB) substrate in the dark for 30 min at room temperature.
11. The reaction is stopped by the addition of 100 μL 1 M H_2SO_4.
12. The plates are read at an $OD_{450\ nm}$ using a Tecan i-control microplate reader (Tecan US Inc.).
13. The data are analyzed and processed using Microsoft®Excel® for Mac version 16.55.

    For data analysis:

    (a) The cut-off is determined as the average of three negative controls.

    (b) The negative control value is obtained by incubating the antigen, followed by the secondary antibody conjugated to HRP.

    (c) The cut-off is subtracted from all the sample values. In addition, each value has been subtracted from its corresponding pre-immune (PI) value.

4 Notes

1. Using fewer and smaller centrifuge bottles increases the number of spores that can be harvested. Just ensure that the washing is enough.
2. For minimal spore loss: When discarding the supernatant, hold the centrifuge bottle with the pellet showing to the bottom. Only toss as long as the supernatant is clear.
3. The supernatant contains the competence factors necessary for transformation. Make sure to save the supernatant since no transformant will be obtained using fresh SpII media.
4. Depending on the SPP1 stock preparation, different dilutions can be tested, typically from 10^{-3} to 10^{-5}, using TY media as diluent.

5. To prevent undesired gelification of the top agar before the surface of the plate is completely covered, TY plates need to be at room temperature. Ideally, they should be fresh, poured earlier that day, or taken out of the fridge a few hours earlier.
6. Several dilutions of lysate can be tested using TY as the diluent. Usually, 30 μL lysate in 9 mL TY works well. Include a control with no addition of SPP1 phage.
7. Use a stock solution of 1 M sodium citrate, adjusted to pH 7 (the low pH of the solution may inhibit growth). The sodium citrate prevents superinfection and lysis of the recipient.
8. It is essential to dry excess humidity in the plate before inoculation by leaving the plates open in a laminar hood for 10 min before inoculation.
9. The bottles will be weighed before and after treating with antibiotics to determine if animals have drunk enough water. Thus, according to Wolfensohn and Lloyd [23] a mouse can drink up to 15 mL/100 g of body weight. Therefore, if a mouse weights 25 g, it will drink 3.75 mL per day, then, five mice will drink 18.75 mL of water per cage. Also, we need to consider the possibility that some water drops fall from the bottle (approx. 3 mL), which means that five animals can drink close to 22 mL per cage per day. In addition, the animals are weighed daily to ensure their well-being.
10. Metabolic cages are not required for these types of experiments.

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Chapter 5

Production of Influenza H5 Vaccine Oligomers in Plants

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Abstract

The transient expression of veterinary vaccines in plants is a promising tool because of its low cost connected with a practically unlimited scale-up. To achieve these goals, two major challenges, high immunogenicity of vaccines and minimal of down-stream processing cost, have to be overcome. Here we present and discuss protocols enabling to generate highly immunogenic H5 influenza candidate vaccines as H5 oligomers, by transient expression in *Nicotiana benthamiana* plants and to perform analytical experiments as Western blot, ELISA, and hemagglutination and hemagglutination inhibition assays.

Key words Influenza vaccine, H5 oligomers, Plant-derived hemagglutinin

1 Introduction

Transient or stable expression of therapeutic proteins in plants is promising tools to achieve unlimited scale-up potential and relatively low production cost [1, 2]. Nevertheless, the down-stream 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]. The high down-stream cost is a major bottleneck that limits the commercial production of plant-based pharmaceuticals [4]. It is essentially true for veterinary vaccines, where cost has to be low to fit into economical parameters of animal-based production. In order to develop plant-made recombinant vaccines for veterinary application, two major challenges have to be addressed: high immunogenicity of veterinary vaccines and minimal down-stream efforts. Here, the design and use of subunit influenza H5 vaccines as H5 oligomer derivatives could help to address these limitations. In general, H5 oligomers were produced from H5 trimers stabilized by GCN4pII trimeric motif [5]. H5 trimers were linked via connectors that are introduced after the trimeric motif to form H5 oligomers, outlined in Fig. 1a, b [6]. The connectors are disulfide bonds formed by

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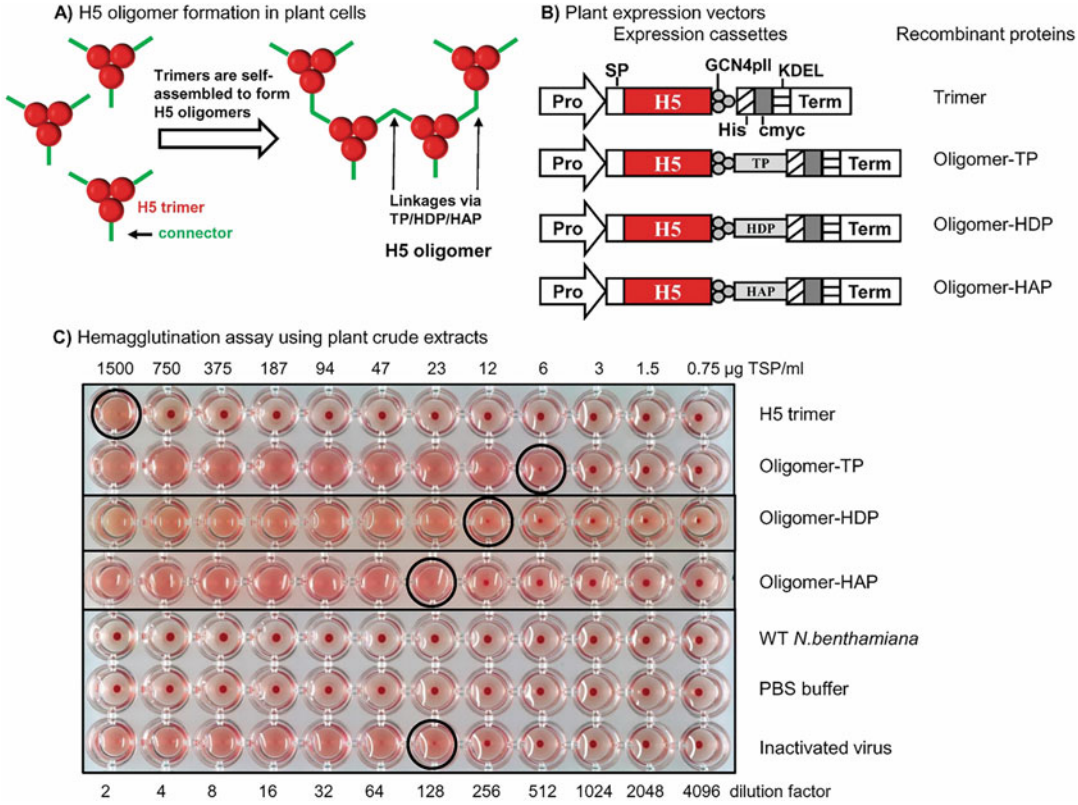

Fig. 1 Production and characterization of H5 trimers and H5 oligomers from plants. (**a**) Model of H5 oligomer formation by H5 trimers self-assembled. H5 trimers are connected by cysteine residues (via disulfide bonds) in the tail piece sequence (TP) from the C-terminal sequence of the mouse IgM [6, 7], homoantiparallel peptides (HAP) [6, 8–10], and homodimer proteins (HDP) [6, 11, 12]. (**b**) Expression cassettes for H5 oligomers. Starting with the construct mentioned above, the tail piece (TP) sequence of the mouse IgM antibody [7], the homoantiparallel peptide (HAP) sequence [6, 8–10], or the homodimer proteins (HDPs) sequence [6, 11, 12] were introduced directly after the GCN4pII trimer motif. The legumin B4 signal peptide (SP) and the KDEL motif caused ER retention. *Pro* cauliflower mosaic virus 35S ubiquitous promoter, *term* cauliflower mosaic virus 35S terminator. (**c**) Screening for H5 oligomers by the hemagglutination assay. Twofold serial dilutions of plant extracts that contain H5 trimers or H5 oligomers, WT, PBS buffer and inactivated virus are mixed with the given amounts of chicken red blood cells, and hemagglutination is recorded. Circles indicate hemagglutination titers. Inactivated rg A/swan/Germany/R65/2006 (H5N1) virus, *WT* wild-type *N. benthamiana*

cysteine residues in the tail piece sequence (TP) from the C-terminal sequence of IgM [6, 7] (Fig. 1a, b), homoantiparallel peptides (HAP) [6, 8–10] (Fig. 1a, b), and homodimer proteins (HDP) [6, 11, 12] (Fig. 1a, b).

Oligomers have been shown to enhance the immunogenicity of hemagglutinins in mice and chicken [5, 6, 13, 14]. Their high immunogenicity enables to minimize down-stream processing procedure by using plant crude extracts containing H5 oligomers as vaccine sources for veterinary immunization. As a result, high neutralizing antibody titers were observed in mice and chickens

vaccinated with plant crude extracts containing H5 oligomers. Importantly, more than 90% of those vaccinated chickens were protected from wild type highly pathogenic avian flu viruses [6]. This achievement is 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 cheap vaccination methods. The oligomerization of the major flu antigen hemagglutinin (HA) seems to be an essential tool to achieve sufficient antigenicity [6].

In this chapter we provide and describe methods to produce H5 oligomers in plants by transient expression and test their immunogenicity.

2 Materials

2.1 Plant Transient Expression

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 a 50 mg/ml stock solution. Antibiotic solutions are sterilized by passing through 0.2 μm cellulose acetate membrane.
3. 0.1 M MES buffer: Dissolve 19.5 g of MES (2-(N-morpholino) ethanesulfonic acid) in 1 l water.
4. 1 M $MgSO_4$ solution: Dissolve 246.5 g $MgSO_4 \times 7$ hydrate in 1 l water.
5. Infiltration buffer (10 mM MES, 10 mM $MgCl_2$, pH 5.6): 100 ml of 01. M MES buffer (in **step 3**), 10 ml of 1 M $MgSO_4$ solution (in **step 4**), and 890 ml of water were freshly mixed. pH is adjusted to 5.6.
6. *Nicotiana benthamiana* plants: Plants are cultivated in the greenhouse at 21 °C, 16 h light per day. After 6–8 weeks, they are ready for agro-infiltration.
7. Agrobacterial C58C1 strains harboring the shuttle vectors. The original pCB 301-Kan vector backbone [15] was used to design plant expression shuttle vectors. These vectors are used for expression of H5 trimer, H5 oligomer-TP, oligomer-HDP, and oligomer-HAP (Fig. 1a, b).

8. Agrobacterial strain harboring the shuttle vector for expression of HcPro [13] which is a suppressor of gene silencing as an enhancer. This has been found to enhance the expression levels of recombinant proteins in plant cells.

2.2 SDS-PAGE and Immunoblotting

1. Hand casted SDS polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGEs are prepared by using the protocol described by Laemmli [16].
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. Prestained molecular weight protein ladder (i.e PageRuler™ or similar from other suppliers).
5. Nitrocellulose membranes (i.e., Whatman or similar).
6. Transfer buffer: 10% methanol (v/v), 24 mM Tris, 194 mM Glycine.
7. Tris buffered saline pH 7.8 (TBS buffer): 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. Western blotting detection reagents (i.e., ECL™ or similar).
11. Antibodies: anti-c-Myc monoclonal antibody 9E10 [17], sheep anti-mouse IgG horseradish peroxidase linked whole antibody (GE healthcare).
12. Homogeneizer (i.e., Mixer Mill MM 300 Retsch, Haan, Germany).

2.3 ELISA

1. P-nitrophenyl phosphate (pNPP) solution: 1 mg/ml in 0.1 M diethanolamine-HCl (pH 9.8).
2. Microtiter plates (i.e., ImmunoPlate Maxisorp).
3. Mouse sera from mice vaccinated with H5 trimer, oligomer-TP, oligomer-HDP or oligomer-HAP crude extracts, and WT crude extracts are prepared as described (*see* Subheading 3.4).
4. Secondary antibody: Rabbit anti-mouse IgG (whole molecule), alkaline phosphatase conjugate.
5. Purified antigens: Plant-derived H5 trimer. The protein was enriched by immobilized metal affinity chromatography and was further purified by size exclusion chromatography. A detailed protocol for the purification methods was described by Phan and co-workers [13].
6. Wash buffer: 0.05% Tween 20 in PBS (PBST).

7. Blocking solution: 3% BSA in PBST Keep at 4 °C.
8. ELISA plate reader.

2.4 Hemagglutination Assay

1. Inactivated virus: rg A/swan/Germany/R65/2006(H5N1).
2. Plant crude extracts from wild-type *N. benthamiana* leaves, infiltrated *N. benthamiana* leaves expressing H5 trimer or H5 oligomers: H5 oligomer-TP, oligomer-HDP, oligomer-HAP.
3. 1% chicken erythrocytes: 2 ml of 3% sodium citrate solution (pH 5.1–5.3) are taken up in a 10-ml syringe. 8 ml of fresh blood from a rooster vein is added and immediately carefully mixed and put into 1.5 ml tubes. The mixture is centrifuged at 900 × *g* for 5 min, 4 °C. The supernatant is removed. 1 ml PBS is added into pellets, mixed and centrifuged again. This step is repeated, 1 ml PBS is added and the cells are centrifuged down again for 5 min. The supernatant is carefully removed and the red blood cells are suspended and stored at 4 °C overnight. 1 ml of packed red blood cells (RBC) are diluted 1:100 in PBS to result 1% chicken erythrocytes.
4. PBS buffer, pH 7.4 (1.424 g Na_2HPO_4 ($2H_2O$), 0.272 g KH_2PO_4, 8.7 g NaCl and 1 l H_2O).
5. Plastic V-bottom microtiter plates.

2.5 Hemagglutination Inhibition Assay

1. Inactivated virus: Four hemagglutination unit (HAU) of rg A/swan/Germany/R65/2006 (H5N1).
2. Mouse sera from mice vaccinated with H5 trimer, oligomer-TP, oligomer-HDP or oligomer-HAP crude extracts and WT crude extracts are prepared as described (*see* Subheading 3.4).
3. 1% chicken erythrocyte.
4. PBS buffer, pH 7.4.
5. Plastic V-bottom microtiter plates.

2.6 Mouse Immunization

1. H5 trimer, oligomer-TP, oligomer-HDP, or oligomer-HAP crude extracts and WT crude extracts.
2. 6- to 8-weeks-old male BL6 (C57/Black6J) mice (12 per group).
3. Emulsigen®-D adjuvant (MVP Technologies, USA). We have exclusively used this adjuvant in the mouse experiments.

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. 1b) 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 (300 ml + 300 ml, each) and concentrated by centrifugation at 4000 × *g*, 30 min, 4 °C.
4. Bacterial pellets are suspended in the fresh infiltration buffer. Agrobacterium suspensions are adjusted by dilution with 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 at 1.0 bar for 90 s 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.

3.2 Western Blot Analyses

1. Frozen leaf discs are homogenized and the resulting powder is suspended in SDS sample buffer.
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.
4. The concentration of total soluble protein (TSP) is determined using the Bradford assay (*see* **Note 3**).
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 blocking solution for 2 h.
8. The membrane is incubated for 2 h at room temperature with a monoclonal anti-c-Myc antibody in blocking solution (*see* **Note 4**).
9. The membrane is washed 5× with blocking solution containing 0.5% milk.
10. The membrane is incubated for 2 h at room temperature with a HRP-conjugated sheep anti-mouse IgG in blocking solution (*see* **Note 5**).
11. The membrane is washed three times with blocking solution containing 0.5% milk. TBS and PBS are used for the penultimate and final washes.

12. Peroxidase activity is detected by applying equal volume of ECL Western Blotting Detection Reagents
13. The membranes are then exposed to X-ray film (*see* **Note 6**).

3.3 Hemagglutination Assay

3.3.1 Total Soluble Protein Extraction

1. Infiltrated leaves are ground in liquid nitrogen and homogenized in PBS buffer (1:3 ratios; mass: volume) with a commercial blender. The extracts are centrifuged at 16,200 × *g*, 30 min, at 4 °C. Protein concentrations of clarified plant extracts were determined by Bradford assay.
2. Protein concentrations of all plant crude extracts are diluted to 3 μg TSP/μl, as used for hemagglutination assay.

3.3.2 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.
3. Twofold serial dilution is made across the entire row.
4. 25 μl of 1% red blood cells (RBC) are 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) (Figs. 1c and 2).

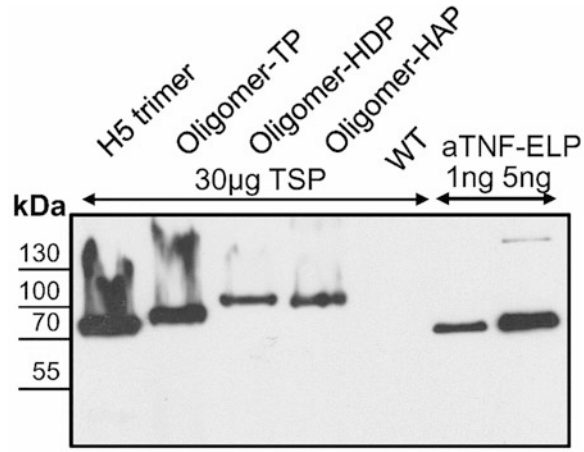

Fig. 2 Expression of recombinant influenza H5 variants in plants, as demonstrated by Western blot. Extracted proteins from leaf materials expressing H5 trimer, oligomer-TP, oligomer-HDP, oligomer-HAP and *N. benthamiana* (a negative control) are separated in a reduced SDS-PAGE gel. Known amounts of purified anti TNFα-nanobody-ELP [18] are included as positive controls and for semi-quantification. Recombinant proteins are visualized by Western blotting with an anti-c-Myc monoclonal antibody

3.4 Mouse Vaccination

3.4.1 Total Protein Extraction

1. Infiltrated leaves are ground in liquid nitrogen and homogenized in PBS buffer (1:3 ratios; mass: volume) with a commercial blender. The extracts are centrifuged at 16,200 × *g*, 30 min, at 4 °C. Protein concentrations of clarified plant extracts were determined by Bradford assay.
2. H5 contents in crude plant extracts are semi-quantified by Western blotting. Known concentrations of the anti-TNF-α-nanobody-ELP standard protein [17] for the c-Myc-tag are used to compare blot signal intensities. A total of 100 ng of H5 antigens are given to one mouse in each vaccination.

3.4.2 Mouse Vaccination

1. H5 trimer, oligomer-TP, oligomer-HDP or oligomer-HAP crude extracts are formulated with Emulsigen®-D adjuvant to the 20% final adjuvant concentration referring to extract volume.
2. Mice were immunized subcutaneously with the formulated candidate vaccines on day 0, 14, and 28 (Fig. 3a).
3. In control groups, mice received WT crude extracts plus adjuvant.
4. Mice are then retro-orbitally bled 1 week after the second and third immunization.
5. Blood samples are centrifuged twice at 900 × *g*, room temperature, 15 min.
6. Mouse sera are collected individually for ELISA tests.

3.4.3 Hemagglutination Inhibition Assay

1. 25 μl of PBS are added into all wells of a plastic V-bottom microtiter plate.
2. 25 μl of mouse serum are added into the first well of each now of the plate.
3. Twofold serial dilution is made across the entire row.
4. 25 μl containing 4 HAU were added into every well (*see* **Note 7**).
5. Plates are incubated in 25 °C for 30 min with shaking.
6. 25 μl of 1% red blood cells (RBCs) are added.
7. Results are read after incubation of plates at 25 °C for 30 min. The endpoint dilution that inhibits hemagglutination was defined as one hemagglutination inhibition unit (Fig. 3b, c).

3.5 ELISA

1. For testing, mouse sera are coated with 100 μl of 1 μg/ml recombinant H5 antigen in PBS and incubated overnight at room temperature (*see* **Note 8**).
2. After blocking with blocking solution for 2 h at 25 °C, 100 μl of the specific serum dilution (2×10^{-4}) are applied and incubated at 25 °C for 1.5 h.

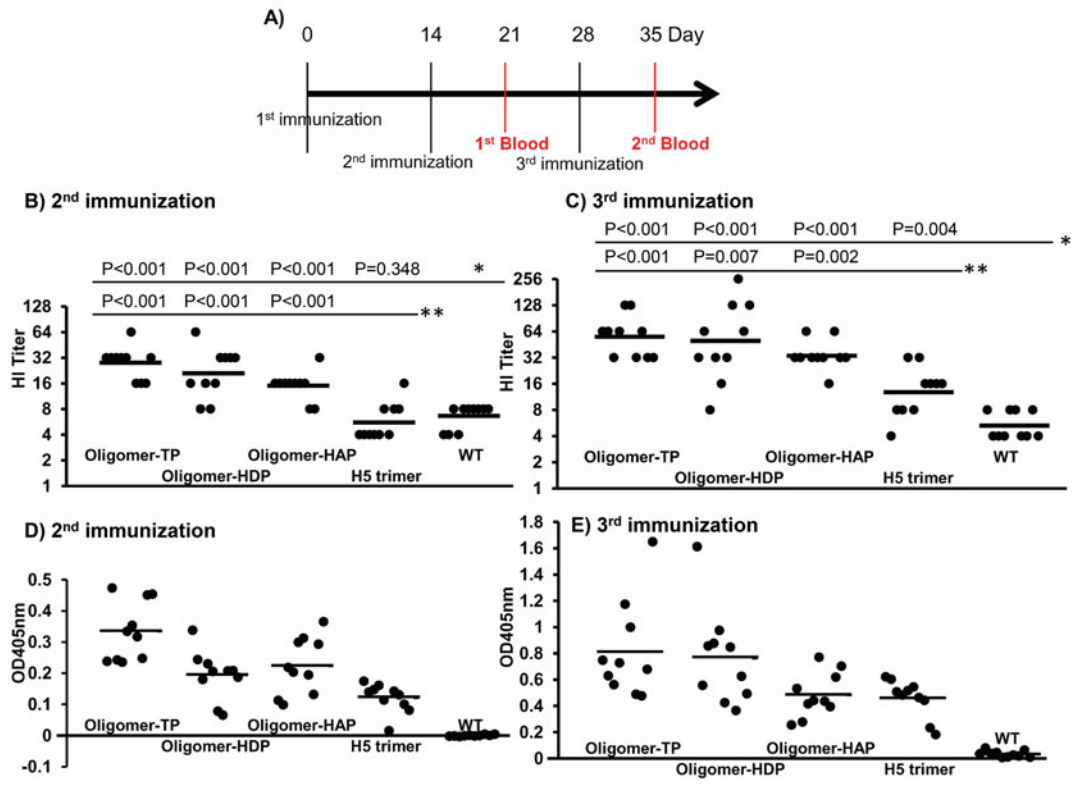

Fig. 3 Immune responses of mice immunized with plant crude extracts containing H5 trimers and H5 oligomers. (**a**) Mouse immunization and bleeding schedule. Each mouse is immunized either with crude extracts containing 0.1 μg (oligomer-TP, oligomer-HDP, oligomer-HAP or H5 trimer, respectively) formulated with the Emulsigen®-D adjuvant at 20% final concentration. In the negative control group, mice are injected with *N. benthamiana* wild-type leaf extracts formulated with the Emulsigen®-D adjuvant at 20% final concentration. Mice are vaccinated with the formulated vaccines at days 0 and are boosted at days 14 and 28. Sera are collected 1 week after the second and third immunizations. (**b**, **c**) Antibody responses are estimated by hemagglutination inhibition assay after the second and third immunization. Measurement of neutralizing antibodies raised by injection of different extracts into mice after two (**b**) and three (**c**) immunizations are done by hemagglutination inhibition assay. Single dots represent the HI titers of single serum samples and bars are the geometric mean titers of each test group. Statistical analyses of HI data were performed using the Mann–Whitney rank-sum test (Sigma Plot software). *p* values less than 0.05 were defined as significant. *: *P* values for HI GMTs comparison from mice vaccinated with H5 oligomer crude extracts and mice vaccinated with WT crude extract. **: *P* values for HI GMTs comparison from mice vaccinated with H5 oligomer crude extracts and mice vaccinated with H5 trimer crude extract. (**d**, **e**) Antibody responses are estimated by indirect ELISA after the second and third immunization. Measurement of antibodies raised by injection of different extracts into mice after two (**d**) and three (**e**) immunizations are done by indirect ELISAs. Single dots represent the OD405nm value of single serum samples and bars are the average OD405nm values of each test group

3. Plates are washed 5 times with PBST, 100 μl of a rabbit anti-mouse IgG alkaline phosphatase conjugate diluted (1/2000) in 1% (w/v) BSA in PBST are then added.
4. The enzymatic substrate, p-nitrophenyl phosphate (pNPP) is added and the absorbance signal is measured at 405 nm after 1 h incubation at 37 °C (Fig. 3d, e) (*see* **Note 9**).

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. 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.
4. The dilution factors should be conducted following the specific manufacturer's recommendations. In this chapter, a 1:50 dilution is applied for the anti-c-Myc monoclonal antibody (9E10) from hybridoma cell culture supernatant (Fig. 2).
5. The dilution factors should be conducted following the specific manufacturer's recommendations. In this chapter, a 1:2000 dilution is applied for the sheep anti-mouse IgG, HRP linked whole antibody from GE healthcare.
6. 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.
7. The dilution causing a complete hemagglutination is defined as 1 hemagglutination unit (HAU).
8. To minimize cross-reactive signals, the plant-derived H5 enriched by immobilized metal affinity chromatography was further purified by size exclusion chromatography.
9. To obtain absolute values, blank controls are included in every plate. Serum at given dilution, which results in ELISA OD values of approximately 1, is included in every ELISA plate as an internal control and used to normalize the different ELISA values.

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Chapter 6

DNA Vaccines in Pigs: From Immunization to Antigen Identification

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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 very basic protocols describing the generation and in vivo application of a prototypic DNA vaccine. The future will say the last word regarding the definitive implementation of DNA vaccination in the field.

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

1 Introduction

Immunization by means of nucleic acid administration was described in the early 1990s [1], opening a new and promising way in the vaccination field. The most common nucleic acid vaccines are based on DNA plasmids, and they are based on a simple albeit smart principle: the capability of cells to in vivo uptake DNA and intracellularly express the encoded protein, which will serve as antigen (Ag). In turn, the immune system can recognize the aforementioned foreign Ag and mount a specific immune response against it. To obtain an optimal expression of the encoded proteins, the gene construct is placed under the control of a mammalian promoter, usually the human cytomegalovirus (CMV) promoter that drives high gene expression. DNA vaccines may be administered in many ways, being intramuscular and intradermal inoculation the two most common procedures. In the first case, the plasmid is primarily taken up by muscle cells, whereas in the second

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case the cells that receive the plasmid are dermis cells, among them Langerhans cells (tissue-resident antigen-presenting cells found in the skin). Independently of the immunization route, the success of DNA immunization relies on the final uptake of DNA and/or the plasmid-encoded antigens by professional Ag-presenting cells (APCs) [2].

The advantages of DNA vaccines are many, but chiefly, their safety. We do not have to worry at all about residual virulence, which is the main concern of attenuated vaccines. In addition, DNA vaccines have proven to be able to induce both antibody and cellular responses. Cellular responses are key for fighting intracellular pathogens, and the potential of DNA vaccines to induce them is one of the main advantages in comparison to inactivated vaccines. Moreover, DNA plasmids have intrinsic adjuvant properties due to the presence of unmethylated CpG motifs, which have been described to rapidly trigger an innate immune response [3]. Last but not least, DNA vaccines can be easily *à la carte* designed. Hence, on the one hand, we have the possibility of modulating the immune response elicited depending on the DNA construct used, forcing the induction of either antibody or cellular responses, or both. On the other hand, as with all other next-generation vaccines, immunization with only a few antigens instead of using the whole pathogen facilitates the development of DIVA vaccines (vaccines that allow Differentiating Infected from Vaccinated Animals), an essential concept in veterinary medicine. Anyhow, an advantage of DNA vaccines in comparison to protein-based subunit vaccines is that the production costs are lower, and they render enhanced stability for storage and transportation. 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 [4], which work extremely well, and for horses [5]. Nevertheless, one of the major scepticisms generated by DNA vaccination in large animals lies in its low immunogenicity, on 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 [6]. Other methods to increase DNA transfection efficacy, such as biolistic or nanoparticles, have also been proposed. Besides the methodologies used to enhance DNA uptake, research has provided many other strategies to improve the immune response induced and the protection afforded. Still, it must be kept in mind that the first step for designing rational DNA vaccines is the identification of promising antigens, which is not that obvious when working with complex pathogens. Far from providing a single universal strategy of vaccination, our experience has shown that vaccines should be tailored to the target animal species and the pathogen to be fought.

The present chapter will review: (1) methods used to enhance the efficacy of the DNA delivery in the animal, mainly focusing on in vivo electroporation; (2) strategies to enhance the immunogenicity of DNA vaccines, mainly focused on those successfully used in our laboratory in the swine model; (3) the use of plasmid cocktails and expression library immunization (ELI), ideal protocols to search for protective antigens within complex pathogens; and (4) a simple protocol describing the steps involving the construction of a DNA vaccine as well as swine immunization and a brief consideration about prime-boost strategies.

2 Design and Deliver a DNA Vaccine

As above mentioned, the first step prior to beginning with the design of a DNA vaccine is knowing what is relevant and what is not in protection against the pathogen we are dealing with. Which kind of immune response are we looking for? Do we need the production of antibodies or the induction of a cellular cytotoxic response? Maybe we should need both. Obviously, the answer to such questions lies in the immunological mechanisms underlying protection against a given pathogen as well as the nature of its antigens. For some viruses it may be simple, e.g., a DNA vaccine encoding the E2 Ag from Classical swine fever virus induced both humoral and cellular responses and conferred sterilizing immunity [7]. However, it is not that simple for much more complex pathogens, such as the African swine fever virus (ASFV), harboring more than 150 ORFs and many of them coding for unknown proteins or proteins with unknown functions. In addition, research from our group based on DNA vaccination found out that the induction of antibodies unable to neutralize the virus or to inhibit the infection may even have deleterious effects, thus leading to exacerbation of the disease [8].

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.

2.1 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 or fusing with the lipid bilayer of the cell membrane. Liposome-entrapped DNA has shown to enhance both humoral and cell-mediated immune response more effectively 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 [9]. 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 to the plasmid DNA against DNases present in the digestive tract. In the mice model, the efficacy of a liposome-droved oral DNA vaccine has been successfully assayed showing protection against influenza challenge [10].

2.2 The Gene Gun

The biolistics approach involves 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. Consequently, 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 [11]. This particle-mediated DNA vaccination approach has shown to be effective in swine, inducing comparable $CD8^+$ T-cell responses and superior antibody production using 100- to 1000-fold less DNA compared to naked DNA injection [12]. When epidermis cells are renewed, the transformed cells expressing the DNA will disappear, therefore stopping the production of Ag. Quite a different panorama of what happens with intramuscular DNA injection, where cells can produce the proteins for a longer period. Some authors [13] claim that, due to the limitations of the approach, 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.

2.3 Electroporation

The principle behind electroporation is quite simple: to induce 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, the membranes recover their 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). Logically, electroporation conditions must be optimized for each animal species [6]. 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 increased release of the antigen proteins from injured cells, thereby improving antigen presentation [14]. Some authors pointed out the possibility of performing the delivery of a DNA vaccine into subcutaneous adipose tissue cells using non-invasive electroporation [15].

Various devices developed to perform in vivo electroporation are available 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 anaesthetized to receive the treatment, together with the bulky appearance of most devices, makes in vivo electroporation currently unfeasible for swine veterinary practice, therefore remaining to be employed in research or small animals and human medicine. It is expected that a not-too-distant future will bring us more portable devices, suitable to be used in massive vaccination as it happens in a regular swine farm.

2.4 Microneedle Array Patch

Microneedle array patch (MAP) consists of minimally invasive micron-sized needles that can penetrate the skin's primary barrier and deliver a vaccine or other kinds of drugs. Due to their slow sustained release of the inoculated substance, MAPs are a strong vaccine delivery system. There are several types of MAPs: solid (S-MAP), hollow (H-MAP), coated (C-MAP), and dissolving (D-MAP). All of them can be used to deliver DNA vaccines. S-MAP are inserted into the skin and then removed to form micron-sized pores on the skin surface. Therapeutics solutions can then be applied to the surface, which contains the micropores. Quite differently, H-MAPs are miniature versions of the conventional hypodermic needles and in contrast to the other MAP-types, are more challenging to produce due to their structure and fragility. Another possible choice is C-MAP, which consists of solid microneedles that have been coated with the solution that we intend to deliver. Finally, D-MAP is prepared using biodegradable materials, such as polymers or sugars, loaded with the drug. After the D-MAP is applied to the skin, its needles dissolve to release, in our case, the DNA vaccine into the skin [16]. Several preclinical studies of vaccines delivered by means of MAP have been conducted in a wide range of animal models, from rodents to primates, including swine. Most of such studies are being carried out with the C-MAP or the D-MAP systems and several microneedle manufacturers with mass-production capabilities are developing vaccine C-MAP and D-MAP in cooperation with vaccine companies, which makes us optimistic about the future of this technology [17].

3 Enhancing the Immunogenicity of DNA Vaccines

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 suits your interests considering 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.

3.1 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 on the fact that after in vivo administration, cytokines will act concomitantly and locally in the zone of Ag expression, therefore avoiding undesirable effects observed when they are systemically administered and providing a more robust and long-term stimulation. The selection of the cytokine to be included (IFN-γ, IL-18, IL-2, and IL-12) will depend on the type of response we want to elicit. Most of the reports of cytokines as DNA-adjuvant are carried out in mice models and, although there are not many studies in veterinary vaccination [18, 19], this strategy looks promising for the near future [13].

3.2 Targeting the Encoded Ag

From the many potential strategies to be used, this review is focused on those successfully used for swine in our laboratory: (1) The employment of strategies aiming to drive the vaccine antigens to APCs; (2) to direct the vaccine-encoded antigens to the MHC-I pathway, in occasions avoiding Ab production; and (3) the use of plasmid cocktails including expression library immunization (ELI), consisting of inoculation of plasmids cocktails encoding random or directed gene fragments of the pathogen's genome.

One of our favorite options is to target the viral Ag to sites of the immune induction, a strategy first described in a mouse model with the use of CTLA-4 as a genetic adjuvant [20]. Following a similar approach, we used the APCH1 molecule as a carrier. APCH1 is a single-chain antibody that recognizes an epitope of the class-II swine leukocyte antigen (SLA-II) molecule, highly expressed in swine APCs [21]. By fusing our DNA construct to APCH1, the encoded fusion products were efficiently directed in vitro to SLA-II positive cells and enhanced the in vivo induction of both specific antibodies and T-cell responses [8, 22]. Interestingly enough, the protection afforded by the vaccines varied depending on the antigens and pathogen used, ranging from sterilizing protection in some pigs against foot-and-mouth disease virus (FMDV) challenge [22] to viremia exacerbation in the case of the ASFV challenge [8]. These results demonstrate once more that adjuvants

are not universal, and also that to design rational vaccines against a given disease it is required to have a deep knowledge of its pathogenesis.

The second choice we used in our laboratory was based on the so-called sHA; the extracellular domain of the ASFV hemagglutinin, a molecule with important similarities to the CD2 leukocyte molecule [23]. As described for the APCH1, the fusion of antigens to the sHA allowed in vitro binding to APCs, most probably due to the expression on their surface of CD2 receptors. Mirroring the in vitro findings and as described for APCH1, this fusion exponentially enhanced both antibodies and T-cell responses induced in pigs but, again, did not result in any protection against ASFV lethal challenge [24].

Of course, there are other strategies to target antigens to APCs, but not many have been successfully used in swine [25]. In this way, the use of CD169 or CD163, two endocytic receptors mainly expressed by 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 humoral immunity [26]. The use of TLR-2, a member of the Toll-like receptor family, looks also promising in swine although the enhancement of antibody production was not as outstanding as the obtained with CD163 or CD169 [25].

The lack of success of our ASFV vaccines could be explained by either a failure in the induction of protective CTL responses, in view that specific CD8-T cell responses have been described as key players in ASFV protection [27], or simply due to the bad selection of the vaccine antigens (just 3 from the more than 150 antigens encoded by ASFV). To solve this "dilemma," we decided to obtain a vaccine prototype encoding our favorite antigens as a fusion with ubiquitin; a strategy successfully used in mice to force class I antigen presentation of the encoded antigens, thus enhancing the CTL responses induced in vivo. Briefly, after transcription of our DNA construct, the antigen is 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 transporter associated with antigen processing (TAP transporters) to the endoplasmic reticulum. There, such peptides are bound to MHC class I molecules which will be finally trafficked to the cell surface to interact with 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 CTL induction but also abrogated antibody induction in vivo, as it was also described before in mice [28] and most importantly, allowed conferring partial protection against ASFV lethal challenge for the first-time using subunit vaccines [24].

Once more, DNA vaccines provided new lessons: one same antigen can induce from exacerbation to protection, depending on the immunological outcome that it provokes. Ubiquitin, like any other genetic adjuvant, can fail to exhort its effects, as it happened in the case of Aujeszky glycoproteins. Ubiquitination of such glycoproteins did not lead to an enhancement of the protection afforded, most probably due to the lack of efficient degradation in the proteasome as demonstrated in vitro [29]. This result demonstrates once more the impossibility of designing universal vaccine strategies, and that it will always depend on the nature of the antigens to be used and likewise on the mechanisms involved in protection against the given pathogen.

4 Searching for Potential Antigens in Complex Pathogens: 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 the MHC class-II pathway, 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 a blend of strategies but repeated many times for different Ag. In addition, we must keep in mind that plasmids used in DNA vaccines present less size limitation in comparison with other vectors used in the vaccination field [30]. Nevertheless, we must consider that the immune response is so complex and delicate that we should be cautious when manipulating such a response. Everything fits on paper, but we must always keep in mind that it may not be what will happen in vivo: the immune system depends 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 [31] and later extended to many other target species, is a method for the systematic 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 thereafter 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 through ELI immunization [32].

5 A prototypic Approach to Obtain and Test a DNA Vaccine Specifically Designed for Swine

5.1 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, CMV); and (4) a polyadenylation signal sequence (that provides stability and effective translation) [33]. Moreover, some authors point out the fact that the plasmid itself has immunogenic properties due to its repetitive CpG motifs, which can induce strong B-cell and T-cell responses [34]. 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 a 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-grown 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). 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) commented are: (1) APCH1: the single chain of an antibody that recognizes the DR allele of the class-II SLA-II molecule; (2) sHA: the extracellular domain of the ASFV hemagglutinin (sHA), with homology to the CD2 leukocyte antigen; and (3) Ub: a monomer of the mutated Ubiquitin (A76).

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 cuts commonly every 300–500 bp.

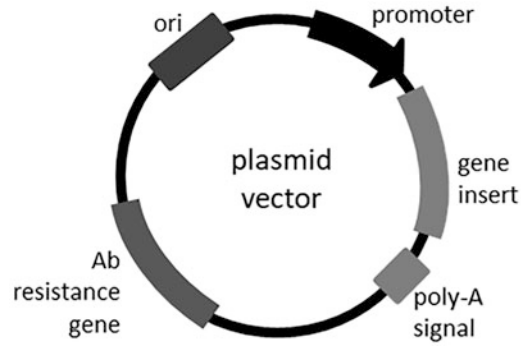

Fig. 1 Schematic representation showing essential components of a plasmid DNA vaccine construct

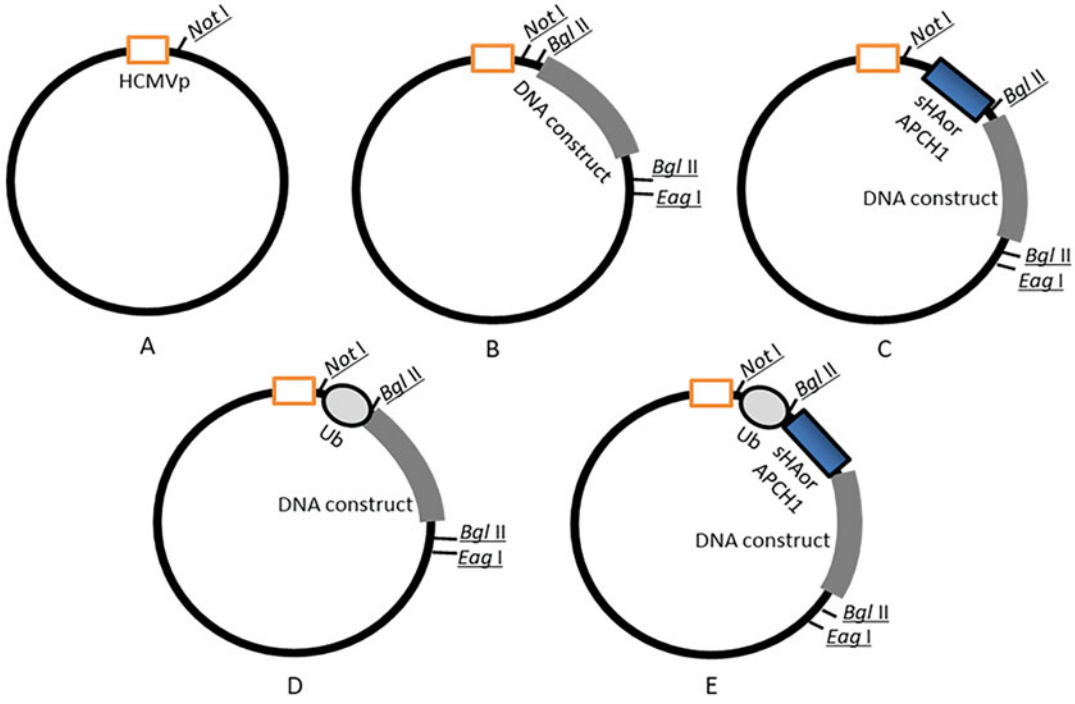

Fig. 2 Schematic representation of plasmids used for DNA immunization. (**a**) Control plasmid, with no insert gene; (**b**) plasmid with a DNA insert; (**c**) plasmid containing the DNA construct fused to the ORF encoding for APCH1 or sHA; (**d**) plasmid containing the DNA construct fused to the ORF 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

3. Clone fragments into the selected eukaryotic expression vector. There are many options available in the market, having all in common the presence of a promoter capable of being recognized by the target species. In our case we used the pCMV plasmid from Clontech (Palo Alto, California), to express the encoded product under the control of the immediate early promoter of human cytomegalovirus (CMV). We also recommend the use of pVAX™200-DES (Invitrogen, California) which meets U.S. Food and Drug Administration (FDA) guidelines for the design of DNA vaccines. To clone the DNA insert inside the plasmid backbone we normally use the Quick Ligase Kit (New England BioLab). To purify DNA products, we commonly use Qiagen Minelute Reaction Cleanup Kit (Qiagen, The Netherlands).
4. Transform plasmids in selected bacteria (we usually use electrocompetent *Escherichia coli* ElectroMAX™ DH10B™ T1 Phage-Resistant Competent Cells, from Invitrogen) and afterwards plate in the suitable medium to select the properly transformed bacteria.
5. Pick up a precise number of individual colonies for each restriction fragment and plasmid frame, to be individually inoculated into a 96-well culture plate. The number of colonies to be picked up to ensure the representation of all *Sau3A*I fragments in the three possible frames was calculated following a formula that considers the length of each original viral DNA restriction fragment and the number of fragments generated by the complete Sau3AI digestion.

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

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.
6. Perform and store replicas of all plates at −70 °C with 15% (v/v) of glycerol.
7. To obtain DNA material for vaccination purposes, a mixture of the generated plasmids must be done (we prepare a pool by taking 0.5 ml from each culture. This pool is used as a starter culture to inoculate 1 l of the proper broth culture, we normally use LB medium, supplemented with the appropriate antibiotic) and, therefore, the DNA plasmid pool must be produced at large scale. Finally, the obtained DNA must be purified to be free from bacterial toxins (we usually use Endofree Plasmid kits, from Qiagen, The Netherlands).

5.2 Assessing Ag Expression In Vitro

To prove that the obtained plasmids correctly express the inserted genes, it is recommendable to analyze their expression by Western blotting using specific polyclonal or monoclonal antibodies. Briefly, suitable cell lines shall be transfected with the vector, while 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, and calcium phosphate-based transfection). Cells will be incubated for 24–72 h, and then harvested to evaluate its optimal in vitro expression kinetics. If a specific antibody against the selected antigens is not available, a peptide tag, e.g. His-tag or FLAG-tag, can be added in frame with the protein. In this way, the use of an anti-tag antibody will allow confirming protein expression. If everything is correct, the plasmid DNA is ready to be injected into the animal to elicit the desired immune response.

Taking a step forward, the antigenicity of the proteins encoded by our DNA plasmids could be ex vivo evaluated before going to in vivo administration. For this purpose, in our laboratory we have developed an ELISpot assay in which PBMC from ASF-surviving pigs are co-cultured with ear-derived autologous fibroblasts transfected with the plasmids of interest [35]. In this case, the plasmids encoded individual ASFV proteins in frame with ubiquitin, aiming to force MHC-I presentation and the induction of $CD8^+$ T-cell responses. After in vitro plasmid electroporation, fibroblasts serve as APCs: they express the encoded proteins and present epitopes at the cell surface by SLA-I molecules. If SLA-I-peptide complexes are recognized by $CD8^+$ T-cells within PBMC from ASF-surviving animals, these cells will secrete IFNγ and the number of IFN-γ-secreting cells will be assessed by ELISpot. This assay can be used not only to confirm that the selected proteins are antigenic but also to follow the specific responses induced by the encoded antigens after DNA administration, as well as to identify novel antigens with protective potential.

5.3 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 [24]. 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 into animals it is compulsory to purify it from bacterial toxins.

1. Prepare a stock solution of 400 μg DNA/ml in a commercial sterile saline solution.
2. In an aseptic environment, put a sterile needle into 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. It is preferable to prepare the vaccine doses on the same day than the inoculation. If this is not possible, syringes should be kept under refrigeration (4 °C) until their use. If different DNA constructs are going to be used, it is highly recommendable to mark the syringes with different color tapes to avoid confusions during the immunization.

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. Please allow the DNA doses to reach room temperature: If the injection is too cold, it may cause undesired additional pain to the animals.
4. Immobilize the animal according to the animal welfare policies of your institution and clean and disinfect the injection points with absorbent paper imbibed with ethanol 70% before 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, repeat **step 5** but perform the injections on the left side of the animal.

6 Concluding Remarks

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. The maximal revolution seemed to occur with the arrival of prime-boosting protocols. Even for the most sceptical, DNA electroporation demonstrated to be the ideal protocol for immune priming, followed by boosting with recombinant viruses encoding the same antigens or with recombinant proteins. Prime-boost strategies have been proven to improve humoral immunity and also enhance the DNA-primed CTL responses [36]. The most used viral vector platforms are, among others, the modified Vaccinia virus Ankara (MVA) and, especially, the adenoviral vectors [36]. 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 [37]. The efficacy of heterologous prime-boost regimes has led to several human and non-human primates trials for important diseases such as HIV [38, 39], albeit in occasions homologous

prime-boost strategies have demonstrated to give optimal results [40]; 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 a Rift Valley fever virus attenuated vaccine in sheep [41]. A 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 [42]. This concept could perfectly be extended to other diseases.

A prime-boost immunization regime has also been tested in our laboratory administering as prime DNA plasmids encoding preselected ASFV proteins in frame with the ubiquitin gene (to enhance $CD8^+$ T-cell responses), followed by a suboptimal dose of a recombinant live attenuated vaccine prototype. DNA priming improved protection against ASFV lethal challenge, in comparison to a control group receiving only the suboptimal dose of the live attenuated virus [43]. Far from aiming to provide an optimal formulation for vaccination in the field, in this case the prime-boost strategy proved a valuable tool to identify novel ASFV antigens with protective potential.

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Chapter 7

Use of Foot-and-Mouth Disease Virus Non-coding Synthetic RNAs as Vaccine Adjuvants

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Abstract

The ncRNAs are short RNA transcripts with sequence and structure resembling that of specific domains in the non-coding regions of the foot-and-mouth disease (FMD) virus (FMDV) genome. These synthetic molecules induce a robust antiviral response and have been shown to enhance the immune response and protection induced by an FMD inactivated vaccine in pigs. Here, we describe the method for ncRNAs synthesis, formulation, and delivery into mice and pigs for studies focused on testing the adjuvant effect of RNA-based strategies in combination with veterinarian vaccines.

Key words Non-coding RNA, Foot-and-mouth disease virus, Foot-and-mouth disease vaccines, Vaccine adjuvants, Immunomodulatory RNA, Non-coding RNA, RNA delivery

1 Introduction

Foot-and-mouth disease (FMD) is an acute, highly contagious, and economically devastating vesicular disease affecting pigs, cattle, and other wild and domestic animals worldwide [1–3]. FMD is considered a major concern in animal health due to the rapid spread of the virus and the subsequent restrictions in international trade of animals and animal products in case of an outbreak. The causative agent, foot-and-mouth disease virus (FMDV) is a member of the *Picornaviridae* family and its genome consists of a single-stranded RNA molecule containing an open reading frame encoding all viral proteins and flanked by two highly structured 5′ and 3′ non-coding regions (NCRs) (Fig. 1) [4, 5]. In the 5′NCR, the S fragment is a 360-nt region predicted to fold into a stable hairpin in the 5′-terminus of the viral RNA. The 450-nt long internal ribosome entry site (IRES) is a multi-domain region which mediates the cap-independent translation of the FMDV genome. On the

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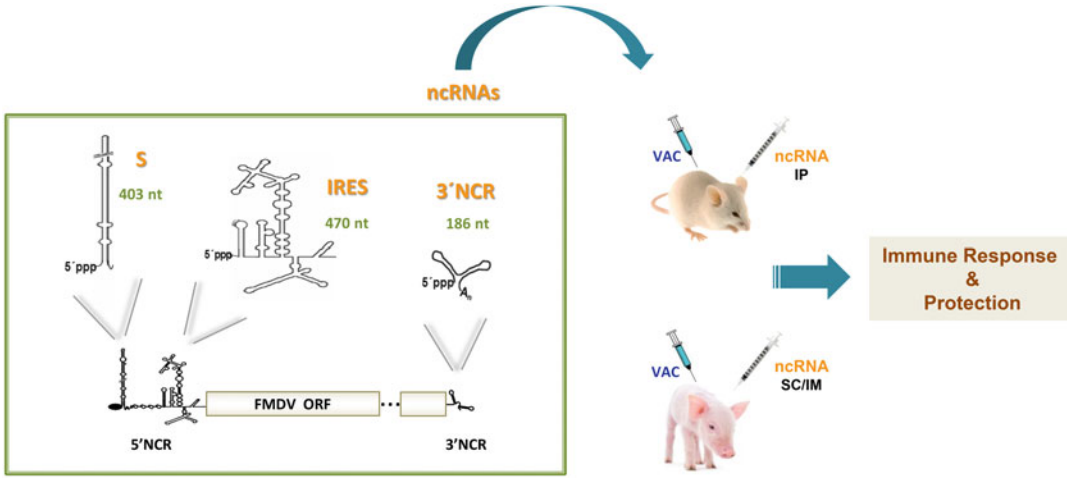

Fig. 1 The structure, length, and specific location in the FMDV genome of the three ncRNAs are indicated. A schematic representation of their use as vaccine adjuvants in pigs and mice is also shown. SC, IP, and IM indicate subcutaneous, intraperitoneal, and intramuscular inoculation, respectively

opposite site of the viral RNA the 3′NCR is a region of 90 nt enclosing two stem-loop structures and a 3′-terminal poly A tail [6].

By in vitro transcription using plasmids which contain their cDNA as templates we are able to generate RNA molecules corresponding in sequence to the FMDV IRES, S fragment and 3′NCR (Fig. 2). When these transcripts, generally referred to as ncRNAs, are delivered into mammalian cells, they are recognized as pathogen-associated molecular patterns (PAMPs) by viral sensors of different families and an antiviral state is activated. These molecules are known to elicit a robust antiviral effect based on type I interferon (IFN) induction through both Toll-like and retinoic acid-inducible gene-I (RIG-I)-like receptors (TLR and RLR, respectively) signaling pathways [7]. The ncRNAs have been successfully assayed both in cultured cells and in a mice model, against a variety of viral pathogens from different viral families, including FMDV (*Picornaviridae*) and zoonotic viruses causing severe disease in humans like West Nile virus (*Flaviviridae*) and Rift Valley fever virus (*Phenuiviridae*) [8–10]. The biological activity of the ncRNAs has been extensively described in swine and bovine cells and we have recently shown also their antiviral activity in an FMDV wild animal host species, the wild boar, against a variety of swine viruses including African swine fever (ASFV, *Asfarviridae*), a complex DNA virus [11]. With the aim to explore the immunomodulatory properties of the FMDV ncRNAs and address whether their ability to induce a robust type I IFN response could be exploited for their use as immune adjuvants, the IRES transcripts were initially tested in combination with an inactivated FMD vaccine in mice

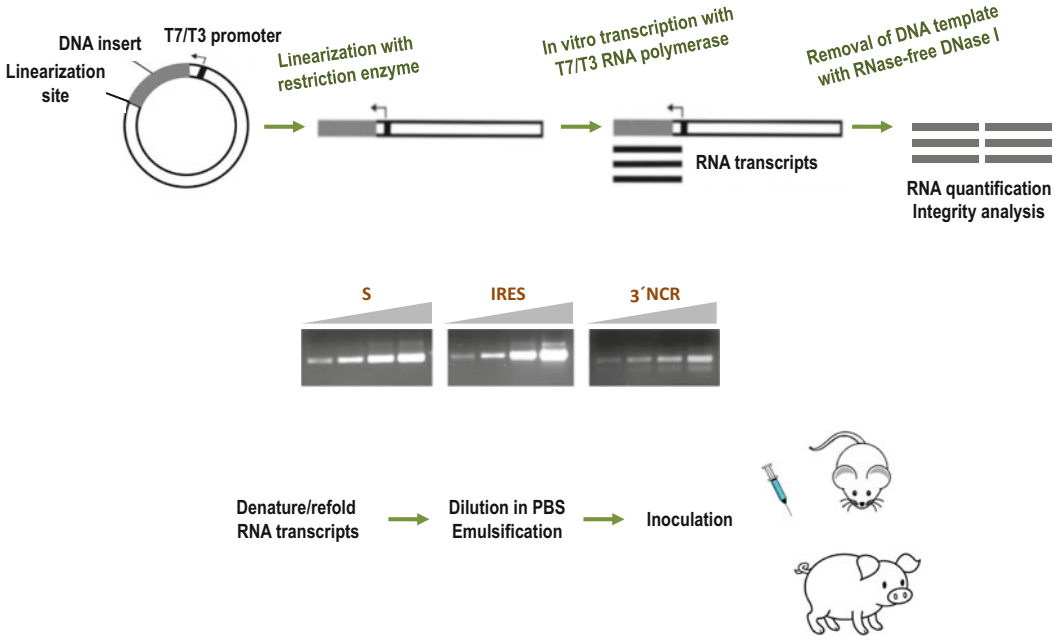

Fig. 2 The different steps and reactions followed for ncRNA synthesis, formulation, and delivery into mice and pigs are indicated. Images show a typical result of the electrophoretic analysis of 1, 2, 3, or 5 μL of the in vitro transcription reaction onto 2% agarose gels. The RNA amounts range from 100 ng (1 μL of 3′NCR transcripts) to 1100 ng (5 μL of IRES transcripts) according to quantification by spectrophotometry

(Fig. 1). The results of this study showed that RNA inoculation can enhance the immune response elicited after vaccination in terms of level and duration of the specific antibodies and promote long-term protection after a single vaccination [12]. We had previously observed that transfection of swine peripheral blood mononuclear cells with the ncRNAs induced robust antiviral and cytokine responses with IFN-α production linked to pDC-enriched subpopulations [7]. Based on those observations and the encouraging results in mice, we addressed the effect of ncRNA delivery on the immune response and protection induced by an FMD vaccine in swine (Fig. 1). Our results showed that direct inoculation of the IRES transcripts in pigs in combination with an FMD vaccine had an enhancing effect on the specific B- and T-cell mediated immune responses elicited, also increasing the rate of protection against FMDV challenge [13]. Here, we describe the protocols of ncRNA synthesis, proper folding and formulation, as well as procedures for RNA delivery in vivo into mice and swine that can be useful for further studies on RNA-based therapeutics and vaccine adjuvants in livestock.

2 Materials

2.1 RNA Synthesis

1. RNase-free water: We use diethylpyrocarbonate (DEPC)-treated water (*see* **Note 1**).
2. DNA template: Generated by linearization of the corresponding plasmid using a unique restriction site located at the 3′-terminus of the sequence (Fig. 2).
3. 5× Transcription buffer: 200 mM Tris-HCl, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl, pH 7.9. Usually provided by the supplier with the RNA polymerase.
4. 100 mM dithiothreitol (DTT). Usually provided by the supplier with the RNA polymerase.
5. Magnesium chloride ($MgCl_2$): 1 M solution in DEPC-treated water (*see* **Note 2**).
6. 25 mM rNTP mix: 25 mM rATP, 25 mM rCTP, 25 mM rGTP, 25 mM rUTP. Prepared by mixing equal volumes of the four 100 mM rNTPs individual solutions.
7. RNase Inhibitor (20–40 U/μL). We use recombinant RNasin® Ribonuclease Inhibitor (40 U/μL) from Promega.
8. T3 or T7 RNA polymerase (20 U/μL).
9. Thermostatic water bath or thermoblock for incubation at 37 °C.

2.2 Removal of DNA Template

1. DEPC-treated water.
2. 5× Transcription buffer: 200 mM Tris-HCl, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl, pH 7.9. Usually provided by the supplier with the RNA polymerase.
3. 100 mM dithiothreitol (DTT) solution. Usually provided by the supplier with the RNA polymerase.
4. RNase-free deoxyribonuclease (DNase) I (1–2 U/μL). We use RQ1 RNase-free DNase (Promega).
5. Thermostatic water bath or thermoblock for incubation at 37 °C.
6. Refrigerated microcentrifuge.

2.3 Purification of RNA Transcripts

1. Phenol/chloroform/isoamyl alcohol (25:24:1 mixture). We use a solution saturated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA from Sigma-Aldrich.
2. Sodium acetate: a 3 M solution, pH 5.2.
3. Absolute ethanol (ethyl alcohol stored and used at −20 °C).
4. 70% ethanol in DEPC-treated water (stored and used at −20 °C).
5. DEPC-treated water for RNA resuspension.

6. A −20 °C, −80 °C freezer or dry ice for RNA precipitation.
7. Refrigerated microcentrifuge.
8. A −80 °C freezer for RNA storage.

2.4 RNA Formulation

1. RNA transcripts (purified and quantitated).
2. RNase-free water (we use DEPC-treated water).
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
4. PBS containing 5% glucose.
5. Cationic lipid-based transfection reagent (i.e., Lipofectin®).
6. Vortex mixer.
7. Thermoblock.
8. Spectrophotometer (i.e., Nanodrop ND1000).

2.5 Vaccine Formulation

1. Antigen: BEI-inactivated FMDV.
2. Adjuvant: Montanide ISA 50 V2 or ISA 206 (Seppic) for mice or pigs, respectively.

3 Methods

The following protocols describe the procedures for RNA synthesis and inoculation of cross-bred Landrace female pigs (a weight of about 20 kg). The modifications for inoculation of adult mice (Swiss ICR-CD1or BALB/c) are also described.

3.1 RNA Synthesis

1. Prepare a 1 μg/μL solution of each DNA template in RNase-free water. The DNA was previously linearized, purified from the restriction enzyme digestion, resuspended in RNase-free water, quantified by spectrophotometry and kept at −20 °C until use (*see* **Note 3**).
2. Set up the in vitro transcription reaction. A standard 120 μL-reaction for 1.5 μg of template DNA is prepared in a 1.5 mL tube as follows: add 69.3 μL of DEPC-treated water, 24 μL of 5× transcription buffer, 6 μL of 100 mM DTT, 1.2 μL of 1 M $MgCl_2$, 2 μL of RNase inhibitor (80 U), 12 μL of a 25 mM rNTPs mix solution, 1.5 μL of a 10 μg /μL dilution of the DNA template (1.5 μg), and 4 μL of the corresponding RNA polymerase (80 U). We use T3 or T7 RNA polymerase depending on the specific ncRNA to be synthesized (*see* **Note 4**). The use of each RNA polymerase is determined by the promoter under which the sequence to be transcribed has been cloned into the DNA plasmid in each case (Fig. 2).
3. Incubate the transcription reaction at 37 °C for 2.5–3 h in a water bath or thermoblock.

3.2 Removal of DNA Template

1. Following in vitro transcription, template DNA is digested with RNase-free DNase I. For that, centrifuge the reaction samples at 3000 × *g* for 30 s to bring down the condensate. Next, add the following reagents: 80 μL of DEPC-treated water, 24 μL of 5× transcription buffer, 6 μL of 100 mM DTT, and 10 μL of RQ1 RNase-free DNase (10 U). This step can be performed at room temperature but always keeping the DNase at −20 °C or briefly on ice (just before adding the enzyme to the mixture).
2. Mix well the 240 μL mixture pipetting up and down and incubate at 37 °C for 1–1.5 h in a water bath or thermoblock. At this point, the integrity and approximate concentration of the RNA transcripts can be checked by electrophoresis on agarose gels (*see* **Note 5**).

3.3 Purification of RNA Transcripts

1. Add 240 μL of phenol:choroform:isoamyl alcohol (25:24:1) to the sample and vortex virgorously. Use this reagent according to the safety information provided by the supplier avoiding inhalation and contact with eyes or skin.
2. Centrifuge the sample at 16,000 × *g* at room temperature for 5 min. Centrifugation contributes to the formation of two phases. Carefully collect the aqueous, polar phase at the top of the solution containing the RNA and water and transfer it to a clean tube. Discard the organic phase at the bottom containing denatured proteins.
3. Repeat **steps 1** and **2**.
4. Precipitate the RNA adding 24 μL of 3 M sodium acetate (pH 5.2) (0.1 volume) and 600 μL (2.5 volumes) of cold absolute ethanol (kept at −20 °C). Depending on how efficient the recovery of the aqueous phase was, the volume will be around 200–240 μL. The volumes of the sodium acetate solution and ethanol required for precipitation can be precisely recalculated in each case depending on the final volume collected but it is not strictly necessary.
5. Incubate at −80 °C (in a freezer or on dry ice) for 10–15 min. Low temperature accelerates RNA precipitation. The samples can be also incubated for a few hours or overnight at −20 °C. As the amount of RNA in the sample will be large, even incubation on ice for about 30 min would work.
6. Centrifuge at 16,000 × *g* at 4 °C for 30 min. The RNA pellet should be clearly visible. Remove and discard the supernatant.
7. Wash the RNA pellet with 200 μL of cold 70% ethanol prepared in DEPC-treated water (kept at −20 °C) to remove the excess salt.
8. Centrifuge at 16,000 × *g* at 4 °C for 5 min. Remove and discard the supernatant.

9. Air dry the RNA pellet at room temperature for 10 min. After removal of salts and drying, an RNA translucent pellet will be obtained.
10. Resuspend the RNA pellet in 40 μL of DEPC-treated water. This step might take some time (*see* **Note 6**). Save 1 μL of the sample for quantification and store the samples at −80 °C (*see* **Note 7**).
11. Prepare a 1/10 dilution of RNA with 1 μL of the sample adding 9 μL of DEPC-treated water. Quantify RNA concentration by spectrometry. The final RNA concentration should be around 10 μg/μL (1 μg/μL in the 1/10 dilution).

3.4 RNA Folding and Emulsification

1. Prepare the appropriate volume of a 10 μg/μL dilution of RNA considering the ratio 500 μg RNA/pig and keep it on ice (*see* **Note 8**).
2. Prepare the total volume of the transfection reagent needed (40 μL/pig) and keep it at 4 °C (*see* **Note 8**).
3. Heat the 10 μg/μL dilution of the RNA at 92 °C for 5 min.
4. Add 5% glucose-PBS (510 μL/pig) to the RNA tube, mix pipetting up and down, and cool it down to room temperature for 10 min.
5. Add the transfection reagent to the RNA in PBS, mix briefly but vigorously with a vortex and incubate at room temperature for 15 min.
6. Transfer the RNA mixture to ice and keep it refrigerated until the time of inoculation. Then, let it warm up at room temperature for 5–10 min. The mixture is now ready to be injected (*see* **Note 9**).
7. For inoculation of mice, use an RNA dose of 200 μg in a volume of 200 μL per mouse, prepared as explained above.

3.5 Vaccine Formulation

1. The FMD inactivated vaccine is prepared by emulsification of the inactivated virus in a 1:1 water/oil emulsion in Montanide ISA50 V2 or ISA 206 (for mice or pigs, respectively) by vigorously mixing until the emulsion is stable, according to the manufacturer's instructions. For pigs a suboptimal antigen payload of 3 μg was used per animal and the equivalent of 2×10^5 plaque forming units per mice.
2. For a different vaccine, prepare the formulation to be tested following the specific procedures in each case.

3.6 RNA Delivery and Vaccination in Pigs

1. The RNA dose per pig (500 μg) in a volume of 600 μL of the RNA mixture consists of: 50 μL of the 10 μg/μL RNA stock +40 μL transfection reagent (Lipofectin) + 510 μL 5% glucose-PBS (*see* **Notes 10** and **11**).

2. Split in two each individual dose for inoculation in two shots.
3. Inject 300 μL (250 μg RNA) intramuscularly in the tabloid neck, behind and below the ear, but in front of the shoulder.
4. Inject 300 μL (250 μg RNA) subcutaneously in the loose skin behind the ear.
5. Inoculate the vaccine formulation intramuscularly in the neck in a single dose at the opposite site of RNA injection (*see* **Note 12**).

3.7 RNA Delivery and Vaccination in Mice

1. The RNA dose per mice consists of 200 μg RNA prepared as above in a volume of 200 μL (*see* **Notes 10** and **11**).
2. Inject the 200 μL of RNA mixture intraperitoneally (*see* **Note 13**).
3. Inject the vaccine intraperitoneally at a different site of the abdomen about 2 cm apart from the RNA inoculation site (*see* **Note 14**).

4 Notes

1. All solutions and buffers should be RNase-free to prevent RNA degradation. An alternative to DEPC-treated water is to purchase Nuclease-free water (DNase/RNase-free distilled water) from suppliers of molecular biology reagents (*see* Promega, Sigma, Qiagen). Make sure that pipettes, bottles, and any other supplies are handled with gloves.
2. When trying to scale up the RNA synthesis reaction to make large amounts of ncRNAs for inoculation of mice and pigs, we tested several different protocols and found that the presence of 10 mM $MgCl_2$ in the in vitro transcription reaction increased the RNA yield.
3. RNA in vitro transcription is a very efficient reaction. Following the protocols described here, a minimum yield of 300× the amount of template DNA should be achieved. Our average is around 300–400×. That means that we make 300–400 μg of RNA from 1 μg of template DNA. This should be taken into account when preparing the required amount of linearized plasmid (Fig. 2).
4. For enzymatic synthesis of RNA, DNA-dependent RNA polymerases from bacteriophages T3, T7, or SP6 are usually used. Among their advantages, they do not require additional protein factors for initiation, elongation, or termination; they are very active and initiate the transcription very stringently from their own promoters. Such properties make these polymerases a very convenient tool for in vitro transcription.

5. We recommend checking the integrity, size, and concentration of the newly synthesized RNA transcripts by electrophoresis in native agarose gels. Load 1–5 μL of the 240 μL reaction during treatment with DNase and make sure that a clear band and not a smear (indicative of RNA degradation) is detected. The migration in native gels will not allow the accurate determination of RNA bands size but will be a good reference if compared with the migration of known DNA or RNA molecular weight markers. This check point is particularly useful when setting up the technique. Also, DNA removal is a quite inefficient step. If a DNA template band is still clearly detectable at the end of the incubation, more DNase can be added (about 3 U). Alternatively (or both), continue incubation for a longer period of time (up to 3 h).
6. If the pellet is overdried, resuspension may be difficult. In that case, leaving the pellet to rehydrate for a few min at room temperature will help.
7. RNA transcripts are stable for months at −80 °C. Avoid freezing-thawing of the RNA, make small volume aliquots.
8. Prepare some extra doses of the RNA mixture (and vaccine formulation also) to make sure that the last animal in each group can be inoculated after serial injection, despite of the dead space syringe volume and pipetting error. We usually prepare RNA mixture for 12 animals for a group of $n = 10$.
9. In order to prevent RNA degradation during the synthesis and handling of the RNA transcripts, RNase inhibitors are added to the reactions and RNase-free reagents should be used in all steps avoiding contact with dirty surfaces or potentially contaminated with human or animal bodily fluids. Despite the fact that RNA is prone to degradation by ubiquitous RNases, we have learned that if made and handled with basic precautions, no degradation problems should be encountered. This includes the preparation of the RNA and vaccine formulations in a farm environment.
10. When scaling up the RNA amount needed for a different animal species do not extrapolate directly in terms of weight from mice data. Notice that the weight ratio of pig (20 kg) to mouse (20 g) is 1000 but the RNA amount is only increased in 2.5-fold while being biologically active. An RNA dose about 0.5–1 mg might be sufficient and adequate for large animals. Though we have observed that protection against viral infection is RNA dose-dependent, inoculation of suckling mice with as little as a few micrograms of IRES transcripts (1–10 μg) still showed remarkable antiviral effect with a 10–40% decrease in survival compared with the lowest dose conferring 100% (100 μg) [10].

11. We have limited evidence from in vivo antiviral activity assays in suckling mice showing that the ncRNAs are biologically active even in the absence of a transfection reagent (in our case Lipofectamin), though with a 30% decrease in their antiviral capacity [10]. Titration of this transfection reagent (or any other used) in order to reduce it to the minimum amount ensuring full activity of the ncRNAs may be useful in reducing costs when setting up a new vaccination experiment in an animal model.
12. We inject RNA and vaccine in separate areas of the neck to avoid any physical or inflammatory interference between both shots but ideally a formulation combining all components would be desirable for future developments. For RNA and vaccine injections use the proper size and length needle to ensure the efficient delivery of the samples in the target tissues. Depending on the age of the pigs, different sizes and lengths may be needed.
13. In mice, the innate immune stimulation exerted by ncRNA inoculation can be easily measured by ELISA of IFN-α in serum at 6–8 h after RNA delivery [8]. Also, an IFN bioassay based on protection against vesicular stomatitis virus (VSV) infection of L929 murine cells pretreated with RNA-inoculated mice sera may be informative [14].
14. Though simultaneous inoculation of RNA and vaccine seems to be the most convenient schedule of delivery, we have evidence of both RNA immunostimulatory and its subsequent antiviral effect when administered either 24 h before or 24 h after viral infection in mice [8, 10]. It seems reasonable to assume that in case that co-administration of RNA and vaccine is problematic for logistic reasons, there would be a ±24 h time window for vaccination with respect to RNA inoculation.

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Chapter 8

Evaluation of Innate Lymphoid Cells and Dendritic Cells Following Viral Vector Vaccination

Zheyi Li, Sreeja Roy, and Charani Ranasinghe

Abstract

Recently, we have shown that fate of a vaccine is determined by the cytokine milieu in the innate immune compartment at the early stage of vaccination. Specifically, 24 h post-delivery, level of innate lymphoid cell type 2 (ILC2)-derived IL-13/IL-13Rα2 are the master regulators of DC and also different ILC subsets responsible for modulating the downstream immune outcomes. Here, we provide step-by-step details how to assess different ILC and DC subsets in lung and muscle following intranasal and intramuscular viral vector vaccination, respectively, using multi-color flow cytometry and confocal microscopy.

Key words Innate lymphoid cells (ILC), Dendritic cells (DC), Viral vector vaccination, Recombinant fowl poxvirus (rFPV), IL-4/IL-13 regulation, Intranasal (i.n.)/intramuscular (i.m.) vaccination, Lung and muscle single cell preparation, Flow cytometry, Confocal microscopy

1 Introduction

Vaccine studies in our laboratory have shown that route of delivery, viral vector combination, and cytokine milieu can significantly alter the quality, avidity/poly-functionality of $CD4^+$/$CD8^+$ T cells, and protective efficacy [1–5]. Specifically, (1) vaccines delivered mucosally (intranasal) can induce high avidity poly-functional T cells with better protective efficacy compared to systemic (intramuscular) delivery and (2) this was associated with mucosal vaccination inducing reduced IL-4/IL-13 expression by T cells compared to systemic delivery [3]. We have also shown that IL-13Rα2 inhibitor and IL-4R antagonist adjuvanted vaccines that transiently block IL-13 and/or IL-4 signaling at the vaccination site can induce higher avidity/poly-functional vaccine-specific effector/memory $CD4^+$/$CD8^+$ T cells with improved $CD8^+$ T cell mediated protective efficacy in mice and macaques [6–9]. But interestingly, using these vaccines we have also shown that presence of IL-13 was

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necessary for effective antibody differentiation, where unlike IL-13Rα2 inhibitor adjuvanted vaccine, IL-4R antagonist adjuvant vaccine can also induce effective B cell immunity [7–10].

Recently, trying to unravel the mechanisms by which these vaccines modulate immune outcomes, we have found that the cytokine milieu at the vaccination site 24 h post-delivery, significantly modulate the fate of a vaccine (e.g., quality or avidity of T and/or B cells). Specifically, ILC2s are the main source of IL-13 at the vaccination site that regulates the different ILC and DC subsets giving rise to different adaptive immune outcomes [11–16].

Although ILC have been well studied in allergy and asthma [17, 18], not much is known about their function following infection or vaccination. ILC are derive from common lymphoid progenitor cells, but are lineage negative cytokine-producing cells which do not express classical lymphoid differentiation lineage markers present on T or B cells [19]. ILC were classified as innate cells as they do not require RAG1 or RAG2 expression (recombination activating genes) for their development. Three main groups of ILC (ILC1, ILC2, and ILC3) have been identified according to the transcription factors and cytokines they express. ILC2 have shown to express different cytokine receptors, according to the tissue or anatomical location (for example, ST2/IL-33R$^+$ lung ILC2, IL-25R$^+$ muscle, ILC2 thymic stromal lymphopoietin receptor—TSLPR$^+$ skin ILC2), mainly cytokines IL-4, IL-5, and/or IL-13, and transcription factor GATA3 [11, 16, 20, 21]. ILC1 and ILC3 have been defined by the expression of NKp46, and their capacity to produce IFN-γ, IL-22, IL-17A and linked to expression of transcription factors T-bet and RORγt [21]. However, several studies have shown that the ILC populations can be highly plastic and change according to different tissue milieu or environment factors [11, 16, 22, 23].

Similarly distinct DC subsets carry out differential functions based on the anatomical location and/or the invading pathogen [24]. Recent, studies have shown that the activity of a particular DC subset is determined by the specific infection and the cytokine milieu they induce. For example, although control of acute influenza virus infection has been associated with CD11b$^-$ CD103$^+$ cross presenting DCs [25], CD11b$^-$ CD8$^+$ DCs have been associated with activation of cytotoxic CD8 T cells against non-respiratory pathogens such as West Nile Virus [26]. Interestingly, different viral vector-based vaccines have also shown to activate uniquely different LC/DC subsets. Recombinant fowl pox virus (rFPV) vector-based vaccines which induce very low ILC2-derived IL-13 at the vaccination site have shown to promote CD11b$^+$ CD103$^-$ conventional DC (cDC) recruitment and high avidity T cell induction. In contrast, recombinant vaccinia virus (rVV) based vaccines which induce elevated ILC2-derived IL-13 have shown to promote cross presenting DC and induction of low

avidity T cells [12, 13]. Moreover, adenoviral vector-based vaccines, which induce moderate ILC2-derived IL-13 have shown greater plasmacytoid DCs (pDC) activity compared to cDC [12].

Given our findings, profiling the ILC, their cytokine expression and DCs at the vaccination site 24–72 h post-delivery (1) provide the ability to predict the downstream adaptive immune outcomes as well as (2) understand the different molecular mechanisms governing these processes. In this chapter we described multi-color flow cytometry-based techniques to assess different ILC and DC profiles following viral vector-based vaccination. Methods described can also be adapted to profile innate immune responses to any vaccine candidate and/or adjuvant.

2 Materials

2.1 Immunization

1. Female Balb/c mice, 6–7 weeks of age (*see* **Note 1**).
2. Viral vector-based vaccine(s) or any other vaccine or adjuvant to be tested.
3. For intramuscular immunization, 1 ml syringes with needle (needle 30 gauge).
4. For intranasal immunization, P20–40 pipette and filter tips.
5. Sterile 1× Dulbecco's phosphate buffered saline (DPBS).
6. Ice bucket.
7. Sonicator (only for pox-viral vector-based vaccines).
8. Anesthetic machine and isoflurane or similar set-up.
9. PCII biosafety cabinet.

2.2 Sample Collection and Preparation

1. Autoclaved individually wrapped scissors and forceps.
2. Sterile 5 ml tubes.
3. Sterile 50 ml Falcon tubes.
4. Sterile 70 μm Falcon cell strainers.
5. Autoclaved sterile syringe plunger without rubber part.
6. Sterile gauze.
7. Assorted set of Gilson pipettes (or similar) and tips.
8. Transfer pipettes (Sigma).
9. Sterile U-bottom 96 well plates with lids.
10. Complete RPMI: 500 ml RPMI-1640 media, 50 ml heat inactivated fetal calf serum (FCS), 10 ml 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 ml 100 mM sodium pyruvate, 500 μl stabilized penicillin, streptolysin and neomyiosin solution (PSN), 4 μl mercaptoethanol (2-ME).

11. Red blood cell lysis buffer: 0.16 M NH_4Cl, 0.017 M Tris HCl (pH 7.65) (*see* **Note 2**).
12. Lung tissue digestion buffer: 1 mg/ml Collagenase, 1.2 mg/ml Dispase, 5 units/ml DNase, Complete RPMI media (*see* **Note 3**).
13. Muscle tissue digestion buffer: 0.5 mg/ml Collagenase, 2.4 mg/ml Dispase, 5 units/ml DNase, Complete RPMI media (*see* **Note 3**).
14. 37 °C Incubator with 5% CO_2.
15. Hemocytometer, 0.4% trypan blue and microscope for cell counting.
16. Centrifuge that can spin 50 ml tubes.
17. 37 °C water bath with floating rack.
18. PCII biosafety cabinet.
19. 70% ethanol.

2.3 Flow Cytometry Analysis (FACS)

1. Brefeldin A (BFA) solution (*see* **Note 4**).
2. FACS buffer: DPBS with 1% FCS.
3. IC Fix buffer (BioLegend).
4. 1× IC-Perm buffer (BioLegend) (*see* **Note 5**).
5. 0.5% Paraformaldehyde (w/v) in DPBS (*see* **Note 6**).
6. FACS antibodies (*see* Tables 1, 2, and 3).
7. FACS cluster tubes.
8. Assorted set of Gilson pipettes (or similar) and tips.
9. Ice bucket.
10. 37 °C Incubator with 5% CO_2.
11. Bench centrifuge that can spin both plates and 50 ml tubes (for example, Beckman Coulter Allegra X-12R).
12. BD LSR Fortessa or equivalent cytometer.
13. Latest version of FlowJo software or similar FACS data analysis software.

2.4 Immunofluorescence

1. Materials mentioned in FACS Subheading 2.3.
2. Slides and glass cover slips.
3. Poly-L-Lysin.
4. Antifade Vectashield mounting medium *with* 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, #H-1200).
5. Antifade Vectashield mounting medium *without* 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, #H-1000).
6. Confocal microscope (Recommended - Leica TCS SP5 confocal microscope).

Table 1
Recommended ILC2 antibodies and dilutions (*see* Note 7)

Antibody	Fluorochrome	Clone	Source	Working dilution
Lineage cocktail with isotype control	FITC	include CD3e, Ly-6G/Ly-6C, CD11b, CD45R/B220, TER-119/erythroid cells	BioLegend	1:50
CD45	APC/Cy7	30-F11	BioLegend	1:200
ST2	PerCP/Cy5.5	DIH9	BioLegend	1:100
CD127	Brilliant Violet 510	A7R34	BioLegend	1:100
GATA3	Brilliant Violet 421	16E10A23	BioLegend	1:50
IL-13	PE	EBio13A	eBioscience	1:100
IL-4	PE/Cy7	11B11	BioLegend	1:100
FC block	N/A	2.4G2	PharMingen	1:200

Table 2
Recommended ILC1 and ILC3 antibodies and dilutions

ILC1/3 antibody	Fluorochrome	Clone	Source	Working dilution
Lineage cocktail with isotype control	FITC		BioLegend	1:50
CD45	APC/Cy7	30-F11	BioLegend	1:200
ST2	PerCP/Cy5.5	DIH9	BioLegend	1:100
NKp46	Brilliant Violet 421	29A1.4	BioLegend	1:100
RORγt	PE	AFKJS-9	BioLegend	1:50
T-bet	PE/Cy7	4B10	BioLegend	1:50
IL-17A	Alexa Flour 700	TC11-18H10.2	BioLegend	1:100
IFN-γ	APC	XMG1.2	BioLegend	1:100
FC block	N/A	2.4G2	PharMingen	1:200

Table 3
Recommended DC antibodies and dilutions

Anti-mouse antibody	Fluorochrome	Clone	Source	Working dilution
Fc block	N/A	2.4G2	BD Biosciences	1:200
MHC-II I-A	APC	M5/114.15.2	eBioscience	1:1600
CD11c	Biotin	N418	BioLegend	1:200
Streptavidin	Brilliant Violet 421	N/A	BioLegend	1:400
CD8	APC-eFluor780	53-6.7	eBiosceince	1:300
B220	PerCPCy5.5	RA3-6B2	eBioscience	1:300
CD11b	Alexa Fluor 700	M1170	BioLegend	1:300
CD103	FITC	2E7	eBioscience	1:200
7-amino-actinomycin D viability staining solution (7AAD)	N/A	N/A	BioLegend	1:100
IL-4Rα	PE	I015F8	BioLegend	1:100
IL-13Rα1	PE	13MOKA	eBioscience	1:100
IL-13Rα2	Biotin	110815	R&D	1:100
Streptavidin	PE	N/A	BioLegend	1:100
IFN-γRα	Biotin	2E2	BioLegend	1:400
Streptavidin	APC	N/A	BioLegend	1:100
γc	PE	TUGm2	BioLegend	1:100

3 Methods

3.1 Immunization

1. Thaw the viral vaccine stocks on ice and dilute to appropriate concentration using sterile DPBS.
2. For intranasal vaccination, if using pox-viral vector-based vaccines (example recombinant Fowl pox virus or Modified Vaccinia Ankara) dilute vaccine stock to 1×10^7 PFU in 20–30 μl per mouse. For intramuscular vaccination, dilute vaccine stock to 1×10^7 PFU in 100 μl per mouse (dilute vaccine as bulk with extra 2–3 doses).
3. When using pox-viral vector-based vaccines sonicate diluted vaccines in an ice bath 3 × 15 s to break up clumps (if using cell-based laboratory preparations). Keep on ice and immunize animals as soon as possible. This procedure is not needed when using other viral vector-based or protein-based vaccines.

4. Place animals under mild isoflurane anesthesia in a PCII biosafety cabinet according to manufactures instructions or approved animal ethics guidelines.
5. Intranasal immunization: gently grab each mouse by their neck and turn over (nostril facing up), then using a P20–40 pipette with filter tips slowly release 10–15 μl of vaccine into each nostril.
6. Intramuscular immunization: after anesthetizing mice as in **step 4**, place the mouse belly facing down in the PCII biosafety cabinet legs spread apart. It is recommended to use a nosecone anesthetic set-up to keep the animals under minimum discomfort/ duress during vaccination.
7. Place index or middle finger under the leg and slightly push up to reveal the muscle, and then slowly deliver 50 μl of diluted vaccine per leg muscle using a 1 ml syringe.

3.2 Sample Collection and Preparation

3.2.1 Lung Tissue Collection and Preparation Following Intranasal Immunization

1. 24 h post immunization (*see* **Note 8**), euthanize the mice using cervical dislocation (according to approved institutional animal experimentation ethics committee guidelines), in a PCII biosafety cabinet.
2. After placing mice belly up, using sterile scissors & forceps, gently harvest lung tissues and keep in a 5 ml tube containing complete RPMI medium on ice until use.
3. First, remove the complete RPMI medium, then using scissors cut lung into small pieces. All tissue preparation should be conducted in a PCII biosafety cabinet.
4. Add 1 ml of freshly prepared lung digestion buffer per lung to enzymatically digest the tissues to release single cells.
5. During digestion, gently vortex every 10 min and incubate in a 37 °C water bath for 45 min (here water bath is crucial not an incubator).
6. Then pass the digested lung tissues through a 70 μm Falcon cell strainer into a 50 ml Falcon tube and gently mash the digested preparation through by washing constantly with complete RPMI.
7. Next, centrifuge the lung suspension for 15 min at 524 × *g* at 4 °C.
8. Remove the supernatant by tipping out or using a transfer pipette and gently resuspend cells in 3 ml red blood cell lysis buffer, leave at room temperature for 3 min (*see* **Note 9**).
9. Immediately add 30 ml complete RPMI to dilute out the lysis buffer to minimize cell death and centrifuge at 524 × *g* for 5 min at 4 °C.

10. Remove/pour out the resulting supernatant carefully, then wash pellet once using complete RPMI to remove any remaining lysis buffer.
11. Centrifuged the lung suspension for 15 min at 524 × *g* at 4 °C.
12. Pass the cells through sterile gauze (four layers) into another 50 ml Falcon tube to remove any remaining debris.
13. Wash the cells twice using complete RPMI medium by centrifugation 15 min at 524 × *g* at 4 °C.
14. Remove supernatant and resuspend final pellet in 1 ml complete RPMI medium.
15. Count cells using a hemocytometer, using trypan blue to distinguish deal cells.
16. Plate 2×10^6 cells in U-bottomed 96-well plate.
17. Rest cells overnight (16 h) in an incubator at 37 °C with 5% CO_2 (*see* **Note 10**) and proceed to Subheading 3.3.1 or Subheading 3.3.2.

3.2.2 Muscle Tissue Collection and Preparation Following Intramuscular Immunization

1. Euthanize animals using the same methods as per above.
2. After placing mice belly down, using sterile scissors and forceps, gently remove quadriceps muscle from both rear legs and keep in complete RPMI medium on ice until use.
3. First, remove the complete RPMI medium, then using scissors cut muscle into small pieces. All tissue preparation should be conducted in a PCII biosafety cabinet.
4. Add 1 ml muscle digestion buffer (prepared fresh) to each tube to enzymatically digest the tissues to release single cells.
5. During digestion, vortex every 10 min and incubate in a 37 °C water bath for 45 min (as per for lung).
6. Gently pass the digested muscle tissues through a 70 μm Falcon cell strainer into a 50 ml Falcon tube and gently pass the digested preparation through using complete RPMI (*see* **Note 11**).
7. Next, centrifuge muscle cell suspension for 15 min at 524 × *g* at 4 °C and remove supernatant.
8. Resuspend the muscle cells in complete RPMI and pass through sterile gauze similar to lung preparation to further remove debris.
9. Wash the cells twice using complete RPMI medium by centrifugation 15 min at 524 × *g* at 4 °C.
10. Remove supernatant and resuspend final pellet in 0.5 ml complete RPMI medium.
11. Count cells using a hemocytometer, using trypan blue to distinguish dead cells.

12. Plate 5×10^5 cells per sample into U-bottomed 96-well plate (*see* **Note 12**).
13. Rest cells overnight (16 h) at 37 °C with 5% CO_2 in an incubator as per lung.

3.3 Flow Cytometry Analysis

3.3.1 ILC Staining

1. Prior to staining, add 1× BFA (*see* **Note 4**) to each well and incubate at 37 °C with 5% CO_2 for 5 h to prevent cytokine release.
2. Centrifuge the plate at 524 × *g* on a bench top centrifuge for 2 min at 4 °C and discard the supernatant by flicking the plate in a single motion.
3. Add 100 μl FACS buffer into each well, centrifuge for 2 min at 524 × *g* at 4 °C, discard supernatant.
4. Repeat **step 3**.
5. Add Fc block antibodies to each well in a total volume of 40 μl and incubate on ice in the dark for 20 min, to prevent non-specific binding of antibodies.
6. Add 100 μl FACS buffer into each well, centrifuge for 2 min at 524 × *g* at 4 °C, discard supernatant.
7. Add surface staining antibodies to each well in a total volume of 40 μl and incubate on ice in the dark for 40 min.
8. Add 100 μl FACS buffer into each well, centrifuge plate for 2 min at 524 × *g* at 4 °C, discard supernatant.
9. Resuspend in 100 μl IC Fix buffer and incubate on ice in the dark for 10 min.
10. Add 100 μl FACS buffer into each well, centrifuge for 2 min at 524 × *g* at 4 °C, discard supernatant.
11. Add 100 μl IC-Perm buffer to permeabilize cells at room temperature in the dark for 10 min.
12. Centrifuge for 2 min at 524 × *g* at 4 °C, discard supernatant.
13. Add 25 μl of intracellular antibodies to each well diluted in IC-Perm buffer, and incubate on ice in the dark for 40 min.
14. Add 100 μl FACS buffer into each well, centrifuge for 2 min at 524 × *g* at 4 °C, discard supernatant.
15. Repeat **step 13**.
16. Resuspend the resulting cell pellets in 100 μl 0.5% PFA to fix cells and then transfer to cluster tubes (the extra PFA fixation helps preserve the cells better).
17. Acquire minimum 1×10^6 events per lung sample or 3×10^5 events per muscle sample on a BD LSR Fortessa or equivalent cytometer.

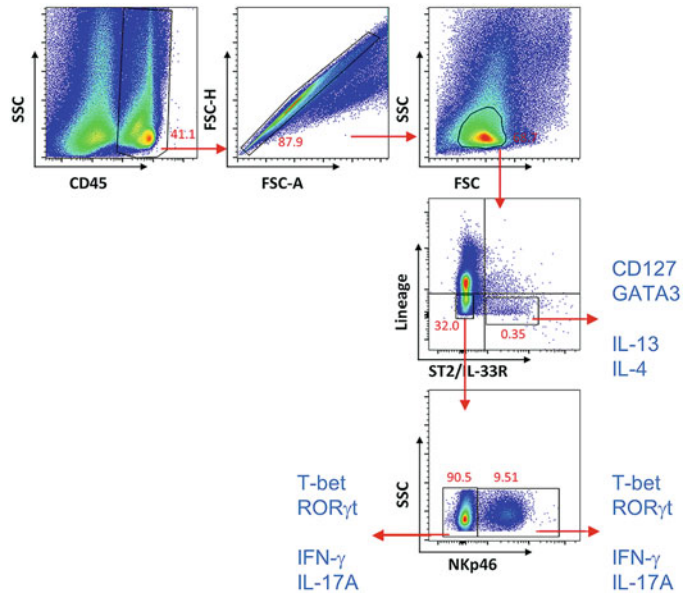

Fig. 1 Lung ILC gating strategy

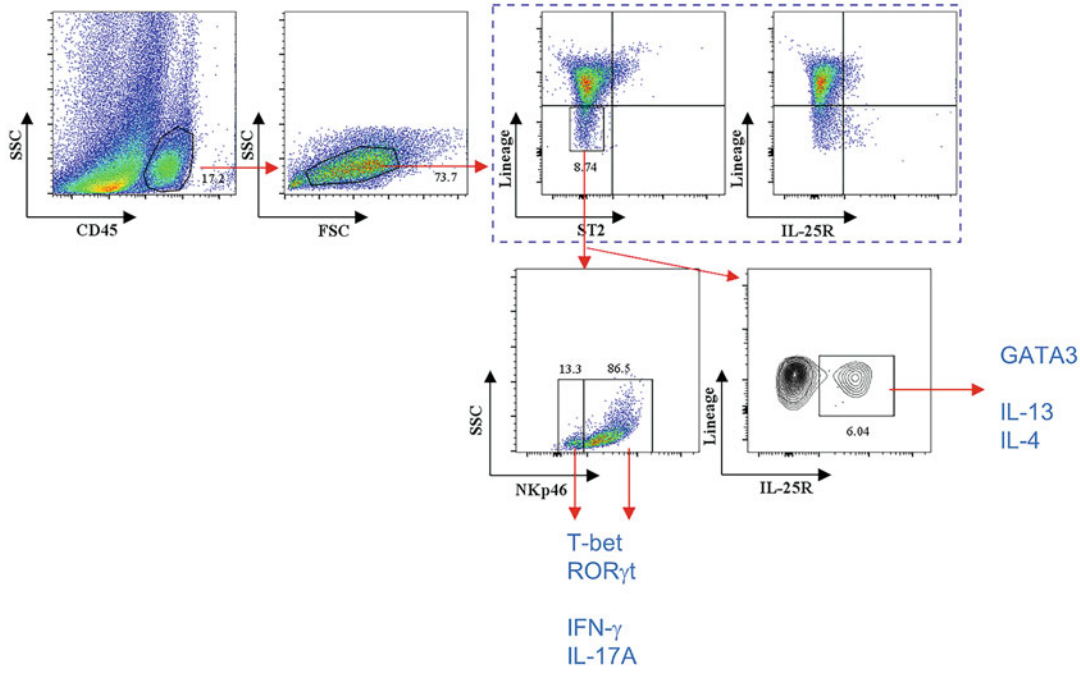

Fig. 2 Muscle ILC gating strategy

18. Analyze data using FlowJo or similar software (*see* **Note 13**), using gating strategy indicated in Figs. 1 and 2. All gates should be placed using appropriate positive and negative controls (*see* **Note 16**).

3.3.2 DC Surface Staining

1. Centrifuge cell suspension for 2 min at 400 × *g* at 4 °C, discard supernatant.
2. Wash cells with 200 µl FACS buffer and centrifuge for 2 min at 400 × *g* at 4 °C.
3. Add 50 µl of CD16/CD32 Fc block per well and incubate for 20 min at 4 °C in the dark.
4. Top up each well with 100 µl FACS buffer, centrifuge for 2 min at 400 × *g* at 4 °C, discard supernatant.
5. Add 50 µl of CD11c biotin antibody solution and incubate for 20 min at 4 °C in the dark.
6. Top up each well with 100 µl FACS buffer, centrifuge for 2 min at 400 × *g* at 4 °C in the dark.
7. Add 50 µl of BV421 conjugated streptavidin and incubate for 20 min at 4 °C in the dark.
8. Top up each well with 100 µl FACS buffer, centrifuge for 2 min at 400 × *g* at 4 °C.
9. Add 50 µl of primary surface antibody staining solution (containing MHC-II, CD8, B220, CD11b, and CD103) per well and incubate for 20 min at 4 °C in the dark.
10. Top up each well with 100 µl FACS buffer, centrifuge for 2 min at 400 × *g* at 4 °C, discard supernatant, proceed to next step or continue to intracellular staining.
11. Resuspend cells in 100 µl FACS buffer (If viability dye is used) or 100 µl 0.5% PFA to fix cells and transfer to FACS cluster tubes.
12. If viability dye is used, add 7-AAD (live/dead marker) to unfixed cells (*see* **Note 14**).
13. Acquire minimum 1×10^6 events per sample data on a BD LSR Fortessa or equivalent cytometer.
14. Analyze data using FlowJo or similar software using gating strategy indicated in Fig. 3. All gates should be placed using appropriate positive and negative controls (*see* **Note 16**).

3.3.3 DC Intracellular Staining

1. Centrifuge the surface-stained cell suspension for 2 min at 400 × *g* at 4 °C, gently remove the supernatant.
2. Add 100 µl of Fixation buffer per well.
3. Incubate for 10 min at 4 °C in the dark.
4. Twice: Top up with 100 µl FACS buffer per well followed by centrifugation for 2 min at 400 × *g* at 4 °C.
5. Discard supernatant and add 100 µl 1× IC-Perm per well.
6. Incubate for 10 min at room temperature in the dark.

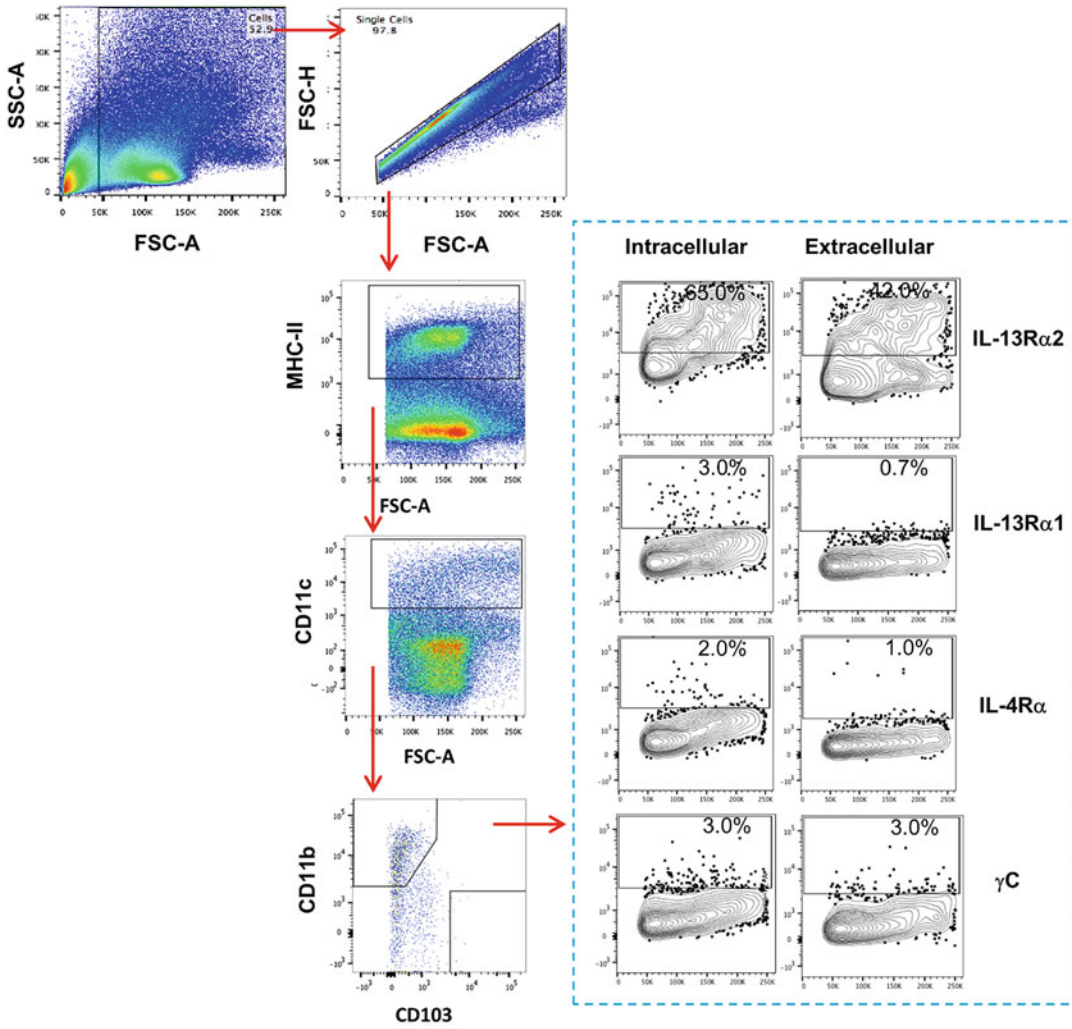

Fig. 3 DC gating strategy

7. Twice: Top up with 100 μl FACS buffer per well followed by centrifugation for 2 min at 400 × *g* at 4 °C.
8. Discard supernatant and add 25 μl of primary antibody solution per well, incubate for 30 min at 4 °C in the dark.
9. Twice: Top up with 100 μl FACS buffer per well followed by centrifugation for 2 min at 400 × *g* at 4 °C.
10. Discard supernatant and add 25 μl of PE (for anti-IL-13Rα2 stained wells) or APC (for anti-IFNγRα stained wells) conjugated streptavidin solution per well.
11. Incubate for 20 min at 4 °C in the dark.
12. Twice: Top up with 100 μl FACS buffer per well followed by centrifugation for 2 min at 400 × *g* at 4 °C.

13. Discard supernatant and fix cells by adding 100 μl 1.5% PFA per well.
14. Incubate cells for 10 min at 4 °C in the dark.
15. Twice: Top up with 100 μl FACS buffer per well followed by centrifugation for 2 min at 400 × *g* at 4 °C.
16. Discard supernatant and resuspend cells in 100 μl FACS buffer per well and transfer to cluster tubes.
17. Acquire minimum 1×10^6 events per sample on a BD LSR Fortessa or equivalent cytometer.
18. Analyze data using FlowJo or similar software using gating strategy indicated in Fig. 3. All gates should be placed using appropriate positive and negative controls (*see* **Note 16**).

3.4 Immunofluorescence to Detect Intracellular and Extracellular Receptor Expression (IL-13 and IFN-γ) Using Confocal Microscopy

1. Coat glass cover slips with Poly-L-Lysin and leave it to dry overnight.
2. Centrifuge lung suspension cells (stained for both surface and intracellular antigens) at 400 × *g* for 2 min to immobilize cells onto coated cover slips (*see* **Note 15**).
3. Add 10 μl of Antifade Vectashield mounting medium with or without 4′,6-diamidino-2-phenylindole (DAPI) onto cells.
4. Mount cover slips onto clean glass slide.
5. Border the coverslip corners with nail varnish to seal slips onto slides.
6. Perform imaging and analysis of slides at 60× using Leica TCS SP5 confocal microscope.

3.5 Flow Cytometry Gating Strategy for ILC and DC Analysis

3.5.1 ILC Gating Strategy (Figs. 1 and 2)

To gate lung ILC, firstly, gate $CD45^+$ cells from the total lung population and following doublet discrimination, place FSC^{low} SSC^{low} gate to identify the lymphocyte subset. From this subset, gate lung ILC2 as $lineage^-$ $ST2/IL\text{-}33R^+$ cells and place subsequent gates to evaluate intracellular expression of IL-13 and IL-4. Next place gate on $lineage^-$ $ST2/IL\text{-}33R^-$ population to identify the ILC1 and ILC3 subsets, as $NKp46^+$ or $NKp46^-$ ILCs, followed by subsequent gating to evaluate expression of IFN-γ and IL-17A (*see* **Note 16**).

Note that, CD127 and transcription factor GATA3 can also be used to identify ILC2 subset. Similarly, transcription factors T-bet and RORγt can be used to identify ILC1 and ILC3 subsets. It is recommended to choose appropriate fluorescent markers (non-overlapping colors) to identify different ILC subsets and their cytokine and transcription factor expression profiles based on the actual need and focus of the experiment.

To gate muscle ILC, firstly, gate $CD45^+$ cells from the total muscle population and following doublet discrimination, place FSC^{low} SSC^{low} gate to identify the lymphocyte subset. From this

subset, gate muscle ILC2 as lineage$^-$ ST2/IL-33R$^-$ IL-25R$^+$ cells and place subsequent gates to evaluate intracellular expression of IL-13 and IL-4. Also perform lineage marker vs. IL-25R to doubly confirm that muscle cells are IL-25R$^+$ (top extreme right plot). Next as per lung place gate on lineage$^-$ ST2/IL-33R$^-$ population to identify the ILC1 and ILC3 subsets, as NKp46$^+$ or NKp46$^-$ ILCs, followed by subsequent gating to evaluate expression of IFN-γ and IL-17A (*see* **Note 16**).

3.5.2 DC Gating Strategy (Fig. 3)

First, gate all live cells (Fig. 3 top left plot). Then performe doublet discrimination and place gate to identify the MHC-II$^+$ cell subset. From the MHC-II$^+$ total DC subset place respective gates to identify the CD11c$^+$ CD11b$^+$ CD103$^-$ population (using appropriate positive and negative controls), including extracellular and intracellular IL-4/IL-13 cytokine receptor expression (*see* **Note 16**). Similar sequential gating can be placed to identify the other DC subsets and their receptor expression. Note that these cytokine receptors can also be evaluated on ILC subsets using the appropriate antibody panel to avoid spectral overlap.

4 Notes

1. Different ILC profiles are observed in female and male mice; therefore, it is important to use the gender appropriate for the experiment.
2. When making the RBCL, first, prepare 1 l of 0.16 M NH_4Cl (8.6 g NH_4Cl in 1 l ddH_2O), next also prepare 500 ml of 0.17 M Tris HCl at pH 7.65 (10.3 g Tris HCl in 500 ml ddH_2O). Then mix 900 ml NH_4Cl solution with 100 ml Tris HCl solution, filter-sterilize, and store in fridge at 4 °C.
3. The digestion enzymes used normally come as a powder and should be reconstructed in sterile DPBS to an appropriate stock concentration (recommended 20× collagenase, 20× Dispase and 100× DNase) and stored at −20 °C until use. On the day of the experiment, the stock solutions should be thawed on ice and 1× working digestion buffer mixture should be freshly prepared in complete media. The recommended suppliers for the enzymes are Sigma-Aldrich (Collagenase), Gibco (Dispase), Calbiochem (DNase).
4. Stock BFA comes as a 1000× solution. When ready to use, freshly dilute to 10× BFA in complete media, and then further dilute to assay concentration (1×) when adding into the 96 well plate containing the cells. For example, assuming cells were seeded in a 100 μl volume per well, 10 μl of 10× BFA per well should be added to each well. When dispensing make sure to add to the side of the well.

5. IC-perm (used in this protocol) usually comes as a 10× concentration. Dilute to 1× using ddH_2O or miliQ water (not media or DPBS).
6. Make sure to wear appropriate PPEs (gloves, safety glasses, and face mask) when handling PFA as it is mutagenic. Weigh appropriate amount of PFA powder and add into 1× PBS in a sterile glass bottle. Cover with aluminum foil, place on a hot plate, and gently stir until powder is fully dissolved. Store solution in the fridge at 4 °C, completely cover in aluminum foil away from light. PFA solution will degrade after a while and/or when exposed to light. Therefore, it is recommended to prepare in small quantities and store no longer than 1–2 months.
7. Fluorochrome of antibodies can be changed accordingly. However, each new antibody batch should be titred before use, as there can be batch to batch variation. The dilutions mentioned are only a guide.
8. We recommended to assess ILC and DC profiles 24 h postviral-vector vaccination. For other vaccines, a timeline (24–72 h) could be performed to determine the optimal evaluation timepoint [11].
9. To ensure maximum RBC lysis efficiency, it is recommended to aliquot and equilibrate the RBC lysis buffer to room temperature before use. Cell should not be left in lysis buffer for too long, (should be less than 5 min), as over lysis result in significant cell death. Once the lysis is completed, cell suspensions should be immediately diluted out with complete RPMI to minimize cell death.
10. Resting the cells overnight after digestion is important to allow the recovery of cell surface markers before performing the staining (for optimal surface antibody staining).
11. In the context of muscle cell preparation (unlike the lung cell preparation) to avoid creating small cell debris, muscle should not be mashed/processed too hard through the strainer.
12. Cell yields from muscle tissue are significantly lower than lung tissue, therefore, less cells are plated into each well.
13. Proper controls should be used in flow cytometry, including single color controls, unstained controls, positive controls (if possible), and FMO (fluorochrome minus one) controls. These are important when setting gates during analysis.
14. When using viability dye such as 7-AAD, cells should not be fixed with PFA to achieve optimal outcomes, as fixing will result in cell death. However, fixable viability dyes are also available, but they should always be titred and tested to make sure that there is no spectral overlap and are compatible with the cytometer.

15. This method can be used for other tissue types as well.
16. During FACS analysis, it is recommended to set-up a cut-off limit. For example, if there are less than ten dots in an initial gate, then it would be best to consider it as a negative (or a cut off), as very low event counts could lead to false positives.

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Chapter 9

Generation of Replication Deficient Human Adenovirus 5 (Ad5) Vectored FMD Vaccines

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Abstract

Adenovirus vectors offer a convenient platform for the expression of antigens and have become an attractive system for vaccine development. Currently, the most successful approach to the development of new foot-and-mouth disease (FMD) vaccines has been the production of a replication-defective human serotype 5 adenovirus that delivers the capsid and capsid processing coding regions of FMD virus (FMDV) (Ad5-FMD). A specific construct for FMDV serotype A24 has been fully developed into a commercial product fulfilling the requirements of the Center of Veterinary Biologics (CVB) of the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA), for commercialization in the USA. In this chapter, we describe a standard protocol for the generation and small-scale production of Ad5-FMDV serotype O1Manisa vaccines. We use directional cloning to introduce the FMDV O1Manisa capsid in the Ad5-Blue vector. This is followed by the linearization of the recombinant Ad5 with Pac I and transfection into HEK293 cells for rescue and propagation, and then by increased production and purification. Finally, purified recombinant virus is characterized by determining virus yield and expression of targeted antigen in specific cell type of interest.

Key words Adenovirus vectors, Ad5, Foot-and-mouth disease, FMDV, Vaccine production

1 Introduction

Foot-and-mouth disease virus (FMDV) causes a fast-spreading disease that can affect livestock production worldwide. For almost a century now, many labs have been developing vaccines and biotherapeutics to mainly protect cattle and swine against FMD. Currently, the disease is controlled by surveillance, strict sanitary policies, and in some geographic regions, the use of vaccines. Commercially available FMD vaccines consist of binary ethyleneimine (BEI) inactivated purified antigen (killed virus) formulated with adjuvants as monovalent or multivalent preparation [1]. Although very effective, these vaccines have several shortcomings including: (1) They require expensive high containment biosafety level 3 facilities for production; (2) Growth of vaccine in cell

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culture leads to virus adaptation with the potential of compromising antigenicity and efficacy; (3) Vaccine preparations are usually contaminated with viral nonstructural proteins affecting differentiation of infected from vaccinated animals (DIVA); (4) They require multiple doses and inclusion of adjuvants for relatively limited-term efficacy; (5) They prevent clinical signs, but they do not preclude virus persistence in ruminants; and (6) They are serotype specific and induce strong antibody responses, but induction of cellular immunity is very limited [2–5].

Over the last 20 years scientists have been intensively trying to develop novel FMD vaccines that address at least some of the limitations of the inactivated commercial vaccine. One candidate that has reached the level of licensing in the USA and potentially could be used worldwide, is a virus-vectored vaccine in which FMD virus like particles (VLPs) are delivered in vivo by a replication-defective human Adenovirus type 5 [6, 7]. Adenovirus vectors are highly efficient for gene transfer in many cell types and animal species [8]. Since the delivered antigen is expressed intracellularly in the vaccinated animal, Adenovirus-based vaccines induce innate and adaptive immunity including humoral and cellular responses. Moreover, they are excellent agents to infect and express transgenes in mucosal tissues. Adenoviruses display over 80 serotypes and are double-stranded DNA viruses with a genome of approximately 38 kb.

One of the most studied viral vectors for vaccine/transgene delivery in human and veterinary medicine is the human Adenovirus 5 (Ad5) of group A. Ad5 linear genome is flanked by inverted terminal repeats (ITRs), cis-acting elements with promoter activity, and has eight units for RNA polymerase II mediated transcription. The genome carries five early units (E1A, E1B, E2, E3, E4, and E5), two units that are expressed with a delay after initiation of viral replication (IX and IVa2), and one late unit (L) that is subdivided into L1–L5 [9]. For vaccine purposes Ad5 have been genetically manipulated by deleting E1A/B, and sometimes E2A, E3, and/or E4, giving room for transgene insertion and reducing toxicity. Expression cassettes usually contain heterologous promoters (i.e., cytomegalovirus [CMV] promoter) inserted in the deleted E1A region [10]. For amplification and packaging of these recombinant adenoviruses, special cell lines that complement for the missing genes are needed. For example, HEK293 provide the needed E1A/B activity required for replication [11], although other cell lines, such as PER.C6 may be needed depending on the modified Ad chosen as a vector [12].

As proof of concept, use of Ad vectors in the construction of FMDV vaccines potentially offered many advantages and addressed some of the limitations of the commercially available whole antigen FMDV vaccines: (1) Ad vectors can be grown in BSL2 labs; (2) Ad are DNA viruses and therefore less prone to spontaneous mutations during propagation (genetically stable, no need of virus

adaptation); (3) They allow for the expression of just the needed specific antigens, in case of FMDV, structural proteins (VP1–4, namely together P1) that compose the capsid, and the 3Cpro, a nonstructural viral protein required for P1 processing; (4) They induce humoral and cellular immune response; (5) They might prevent FMDV persistence in ruminants; (6) They might confer long term immunity. Based on these premises, approximately 20 years ago the first Ad5-FMD vaccine was developed [13]. The authors chose to use a replication-defective human Ad5 (E1A/E1B deleted), anticipated to work well in the FMDV host, cattle, and pigs, presumably lacking vector pre-immunity.

Although other cloning methodologies have been described to generate Ad5-FMD, Moraes et al. used an adapted Ad5 vector platform, pAd5-Blue, that allowed for direct insertional cloning of the genes of interest (GOI) into uniquely engineered specific restriction endonuclease sites positioned downstream of the CMV promoter, altogether replacing the E1A-E1B coding region of the Ad5 genome contained within the plasmid vector backbone (Fig. 1) [14]. The Ad5-Blue plasmid vector included the *lacZ* gene cloned at the specific restriction sites designed for the GOI,

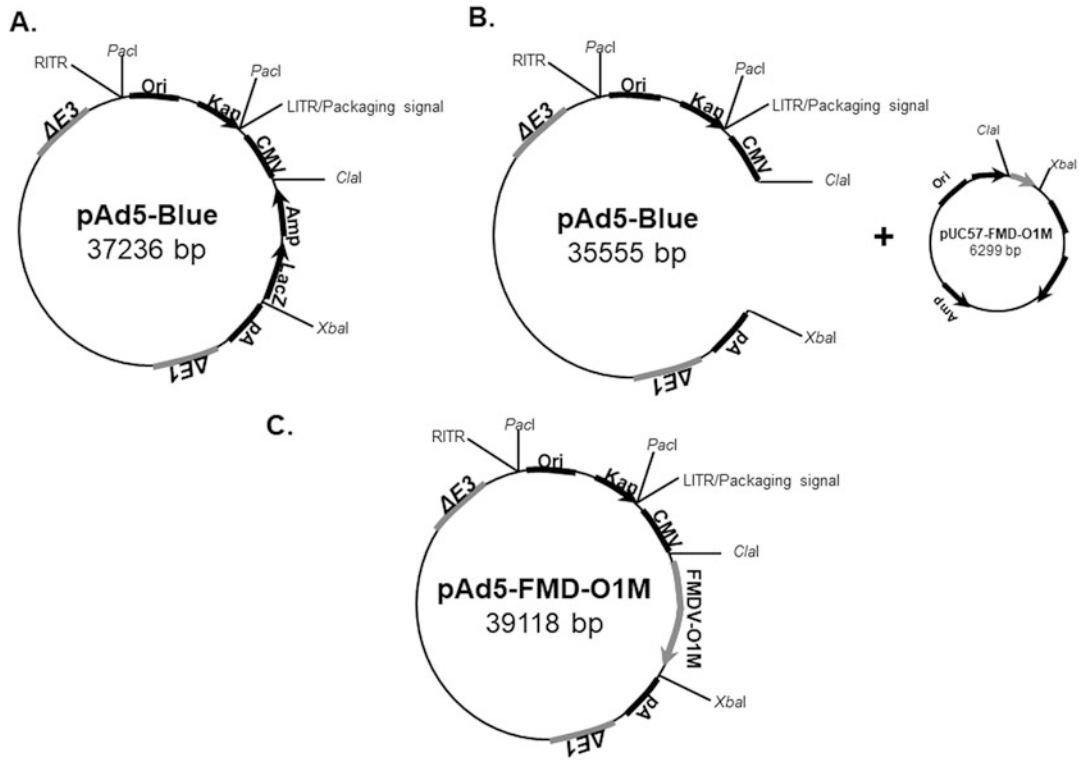

Fig. 1 Schematic representation of the construction of Ad5-FMD-O1M using directional cloning method. Vector Ad5-Blue (**a**) containing 37,226 bp and pUC57 encoding FMD-O1M (**b**) with 6299 bp were digested with restriction endonucleases ClaI and XbaI. Overhangs generated after digestion facilitate ligation of the insert (red) to the vector to generate pAd5-FMD-O1M now containing 39,110 bp (**c**)

thus allowing for white/blue color selection of recombinant clones in commercially available *E. coli* strains such as DH5α, Top10, or XL1-blue (Fig. 1). Replacement of the *lacZ* coding sequence by several GOI allowed for the expression of the FMD cassette. These plasmids were used to derive Ad5-FMD and ultimately were evaluated in pigs and cattle as vaccines and/or biotherapeutics against FMD [6].

Studies in vivo showed that Ad5-Blue derived FMD vaccines could protect pigs and cattle with one inoculation as early as 7 days post vaccination [7, 15–17]. Furthermore, the Ad5-FMD platform has been fully developed into a commercial product that uses a slightly modified Ad5 vector with proprietary rights (GenVec, Inc., MD) for large-scale production that fulfills the requirements of the Center of Veterinary Biologics (CVB) of the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA), for approval and commercialization in the USA. This vector passed all required tests complying with the safety profile and performance versatility against multiple FMDV serotypes [18, 19].

The Ad5-Blue platform has also proved very effective when used to deliver porcine and bovine cytokines in vivo. Treatment with an Ad5-Blue expressing porcine IFN alpha, blocked FMDV replication in pigs from one to approximately 4 days post inoculation [20]. In turn, an Ad5-Blue delivering bovine IFN lambda was effective to control FMD in cattle [21, 22]. Remarkably, combination treatment of Ad5-FMD with Ad5-poIFNalpha (in pigs) or with Ad5-FMD-Ad5boIFN lambda (in cattle) fully protected animals against FMD when challenged as early as 1 day and for at least 3–4 days post-inoculation [20, 23].

These results clearly demonstrate that the use of the replication-defective human Ad5 vector constitutes an excellent platform to deliver vaccines and biotherapeutics for the prevention and control of viral diseases of significance such as FMD.

2 Materials

2.1 Construction of Ad5-Vectored FMD

1. Restriction enzymes: ClaI and XbaI.
2. Shrimp Alkaline Phosphatase (SAP).
3. Plasmid Ad5-Blue (Addgene).
4. Custom synthetized FMDV P12A-2B-3C sequences with added ClaI/XbaI unique restriction sites at 5′ and 3′ ends, respectively, obtained from commercial sources.
5. Luria Bertani (LB) broth.
6. Kanamycin (Kan), stock solution of 50 mg/ml (1000×).
7. Kanamycin (Kan) (50 μg/ml) containing LB agar plates.

8. Kanamycin (Kan) (50 μg/ml) containing LB media prepared fresh the day of use.
9. Inoculating loops.
10. Commercial gel purification kits.
11. DNA Ligation kit.
12. S.O.C. media.
13. Electroporator (i.e., BTX Model 600).
14. Sterile disposable cuvettes fitted with electrodes and a gap of 1 mm BTX #620.
15. Commercial chemically and electro-competent *E. coli* cells (DH5α/Top10/XL1Blue).
16. Tris-acetate-EDTA (TAE) buffer pH 8.3.
17. 0.8% (W/V) agarose in TAE buffer gel.
18. Plasmid DNA purification kits, including mini and maxiprep kits.
19. Spectrophotometer (i.e. Nanodrop™).

2.2 Confirmation of Cloning

1. Restriction enzymes ClaI, XbaI, and Hind III.
2. 1× TAE buffer.
3. 0.8% agarose in 1× TAE gels.
4. Primers designed to bind to the ClaI (5′ end) or XbaI (3′ end) of the FMDV coding sequence insert.
5. PCR High Fidelity Polymerase kit.
6. Sequencing kit.
7. Software to create a Vector map and analyze sequences.

2.3 Production and Amplification of Recombinant Ad5-O1M-FMD Virus

1. Restriction enzyme Pac I.
2. Lipid-based transfection reagent (i.e., Lipofectamine™).
3. Human Embryonic Kidney (HEK) 293 cells from ATCC.
4. Dulbecco's Modified Eagle's Medium (DMEM).
5. Fetal bovine serum (FBS).
6. Non-essential amino acids (NEAA).
7. Antibiotic-Antimycotic (anti-anti).
8. Phosphate buffered saline (PBS) containing calcium (Ca^{2+}) and magnesium (Mg^{2+}): PBS++.
9. Cell culture 6-well plates.
10. Cell culture T-150 flasks.
11. 37 °C incubator with 5% CO_2.
12. Inverted microscope.

2.4 Purification of Recombinant Ad5-FMD Virus by Density Gradient Centrifugation

1. CsCl solutions to prepare gradient: Solution 1: 1.4 g/ml (53 g CsCl in 87 ml of 10 mM Tris–HCl buffer, pH 8.0) and Solution 2: 1.2 g/ml (26.8 g CsCl in 92 ml of 10 mM Tris–HCl buffer, pH 8.0 buffer).
2. Clear centrifuge tubes for either SW28 (Beckman Coulter, ref. # 344058) and SW41Ti rotors (Beckman Coulter ref.# 344059).
3. Ultracentrifuge: i.e. Beckman Coulter Optima XE-90.
4. 18G needles, 3–10 ml syringes, pipette-aid, 10 ml pipettes.
5. Gradient maker 15 ml (i.e., Cytiva SG).
6. 100,000 MWCO 3 ml capacity Dialyzer Cartridge (i.e., Slide-A-Lyzer™ Dialysis Cassette G2).
7. Dialyzing buffer:10 mM Tris–HCl pH 8.0, 2 mM $MgCl_2$, 5% sucrose.
8. Tris-EDTA (TE) buffer: 10 mM Tris–HCl pH 8.0 and 1 mM EDTA pH 8.0.

2.5 Measuring Titers for Ad5-01M-FMD

1. 96-well tissue culture plates.
2. HEK293 cells.
3. DMEM supplemented with 5% FBS, 1% antibiotics-antimycotics (anti-anti) and 1% non-essential amino acids (NEAA).
4. 37 °C incubator with 5% CO_2.
5. Inverted microscope.

2.6 Western Blotting

1. 6-well tissue culture plates.
2. HEK293 cells.
3. IB-RS2 cells.
4. DMEM supplemented with 5% FBS, 1% anti-anti and 1% NEAA.
5. 37 °C incubator with 5% CO_2.
6. Inverted microscope.
7. Cell Lysis buffer (i.e., RIPA buffer [10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 140 mM NaCl, diluted with dH_2O]) with protease inhibitors.
8. Protein loading buffer (Laemmli buffer).
9. PBS-T (PBS pH 7.4 + Tween 0.02%).
10. 10% SDS-PAGE electrophoresis gel.
11. Polyvinylidene difluoride (PVDF) membrane.
12. Gel electrophoresis apparatus.
13. Protein transfer apparatus.

14. Electrophoresis power supply.
15. PBS-0.05% Tween 20 (PBS-T).
16. 5% non-fat milk in PBS-T.
17. Antibodies to detect proteins.
18. Chemiluminescence detection kit.
19. Chemiluminescence detection imager device.

3 Methods

As mentioned in the introduction, this methodology describes a standard, old fashioned but reliable procedure for the cloning and purification of Ad5 encoding FMDV antigens that has been used successfully as a vectored vaccine in different efficacy studies in swine and cattle [13–16, 24].

3.1 Directional Cloning for the Construction of Ad5-O1M-FMD

1. Digest 20 μg of backbone plasmid (pAd5-Blue) and 10 μg of plasmid DNA encoding FMD cassette (i.e., pUC57-FMD-O1M) with 3 units (U) of ClaI and XbaI (*see* **Note 1**) in separate tube reactions each having a total volume of 100 μl. Incubate at 37 °C overnight using a water incubator. Digestion of the DNA can be done with both enzymes simultaneously since they work adequately in the same restriction buffer. At the end of the digestion, remove an aliquot (10 μl), add loading buffer and analyze by agarose gel electrophoresis to confirm the complete enzymatic digestion and proper DNA band sizes (analytical gel).
2. Dephosphorylate the ends of digested backbone pAd5-Blue vector (to prevent possible self-ligation of single cut molecules) using shrimp alkaline phosphatase (SAP).
3. Set up a preparative 0.8% agarose gel electrophoresis (containing wells that would fit the rest of the digested DNA in loading buffer, approximately 120 μl) and load the remanent of the digested previously analyzed DNA. Apply electrical current 60–80 V and run until the dye front is about 3/4 down in the gel. Stop the run, remove the gel and under safe, low DNA mutagenesis rate, UV light wavelength (approx. 300 nm, usually a setting in the transilluminator or lamp source), cut out the expected size DNA bands and transfer to microfuge tubes. Extract the DNA from the cut bands using a gel purification method of choice (i.e., Qiagen DNA gel extraction kit) following manufacturer directions. Measure backbone vector and insert purified DNA concentrations using a spectrophotometer (i.e., Nanodrop™). Calculate DNA concentration in molarity (mol/liter).

4. Set up ligation reactions following manufacturer's instructions and using a molar ratio 1:3 (vector:insert). After incubation of the ligation reaction, usually 10 min at 37 °C, the sample will be ready for transformation in bacteria. Dilute ligation mixture two- to fivefold in water. Run a control with the dephosphorylated vector alone at the same concentration used in the recombinant ligation mixture. Using water for dilution of transformation reaction should help to prevent arcing during electroporation.
5. Electroporate competent *E. coli* DH5α/Top10/XL1Blue with the ligation reactions. Mix 5 μl of ligation mixtures with 50 μl acteria and deposit in individual 1 mm electroporation cuvettes. Incubate on ice for 10 min. Set cuvette in the electroporator and apply electroporation pulse (usually under settings of 100 Ω, and 25 μF that give a typical time constant of about 2.6 ms). At the end of the electrical pulse quickly remove the cuvette from the apparatus and add 300 μl of S.O.C. medium kept at room temperature. Transfer to a 5 ml round bottom tube and incubate for 1 h at 37 °C with some rotation (220 rpm) to allow for plasmid amplification.
6. Plate each reaction onto a Kan-LB agar plate. Keep the plates at room temperature until the bacteria solution is absorbed, approximately 15 min. Invert plate and incubate at 37 °C overnight.
7. Examine the obtained colonies. None or few colonies are expected in the vector control alone. Detection of many colonies in this plate suggests the plasmid was not properly digested and dephosphorylated. If the number of colonies in the ligations containing the insert is larger than the number of colonies in the vector alone, pick up six colonies and inoculate each in 5 ml of LB + Kan. Grow overnight at 37 °C with shaking at 220 rpm.
8. Use each culture to isolate plasmid DNA using a DNA extraction kit (i.e., Qiagen miniprep kit). Quantitate the purified miniprep DNA and confirm the identity of the cloned pAd5-O1M-FMD by restriction digestion and DNA sequencing as described below in Subheading 3.2.
9. Amplify DNA of two positive correct clones using a large-scale DNA preparation kit (i.e., Qiagen DNA maxiprep kit) and use DNA for Adenovirus production as described below in Subheading 3.3.

3.2 Confirmation of Cloning by Restriction Enzyme Diagnostics and Sequencing

There are two main methods to confirm correct cloning. One implies the use of diagnostic restriction endonuclease digestions and the second one is based on Sanger sequencing.

1. For restriction analysis, digest 1 μg of purified plasmid miniprep DNA with 1 U each of ClaI and XbaI in a total volume of 20 μl using the manufacturer's suggested digestion buffer. Incubate 2 h to overnight at 37 °C in a water bath incubator.
2. Set up a 0.8% (W/V) agarose/1×TAE gel electrophoresis and load the ClaI/XbaI digested DNA next to a 1 μg aliquot of undigested pAd5-Blue control DNA. Confirm that the digest yielded two visible bands (35.5 kb and 3.563 kb), corresponding to the vector backbone band and the FMDV cassette insert. Although an additional band is expected, it will not be visible due to its small size (27 bp).
3. Set up another diagnostic digest with 1 μg of the putative cloned plasmid DNA with Hind III. Incubate 2 h to overnight at 37 °C using a water incubator. It is important to also digest pAd5-Blue vector alone for comparison.
4. Run a 0.8% agarose gel electrophoresis to analyze the Hind III restriction DNA pattern. Control pAd5-Blue restricted DNA will have the following fragments after Hind III digestion: 8010 bp, 6466 bp, 5322 bp, 4597 bp, 4241 bp, 3010 bp, 2937 bp, 2081 bp, 1423 bp, 75 bp, and 18 bp (The 3010 bp and 2937 bp fragments will co-migrate, and the 75 bp and 18 bp fragments will probably not be visible). A distinct Hind III restriction digest pattern will be clearly defined as the result of substituting a 1653 bp ClaI/XbaI fragment by the approximately 3500 bp FMDV cassette (Fig. 2a).
5. Use an aliquot of the recombinant plasmid DNA (pAd5-FMD-O1M) for Sanger sequencing. Primers annealing upstream of ClaI or downstream of XbaI, that may have been used for initial cloning, are used for sequencing, however, other primers annealing in the FMD cassette every 600–700 bp should be included to optimize the sequencing results.

3.3 Production and Amplification of Recombinant Ad5-O1M-FMD

Production of replication-defective Ad5 will require the digestion of pAd5-O1M-FMD with the restriction enzyme Pac I to yield the recombinant Ad5 linear genome followed by transfection in a cell line that complements in trans for the E1 gene products deleted in the vector [25]. HEK293 cells contain a stably integrated 4355 bp Ad5 genome fragment encoding for the full-length E1 region [11]. In fact, although at low rate, the presence of these sequences in the cells and in the pAd5-Blue vector could lead to recombination and generation of replication competent Ad5 (RCA). However, these events are rare in early passages of Ad5 [25]. In this manuscript we only describe a small-scale propagation method passaging the obtained recombinant Ad5 up to five times. In our hands, under these conditions, RCA detection is not evident by PCR or biological assays previously described [26].

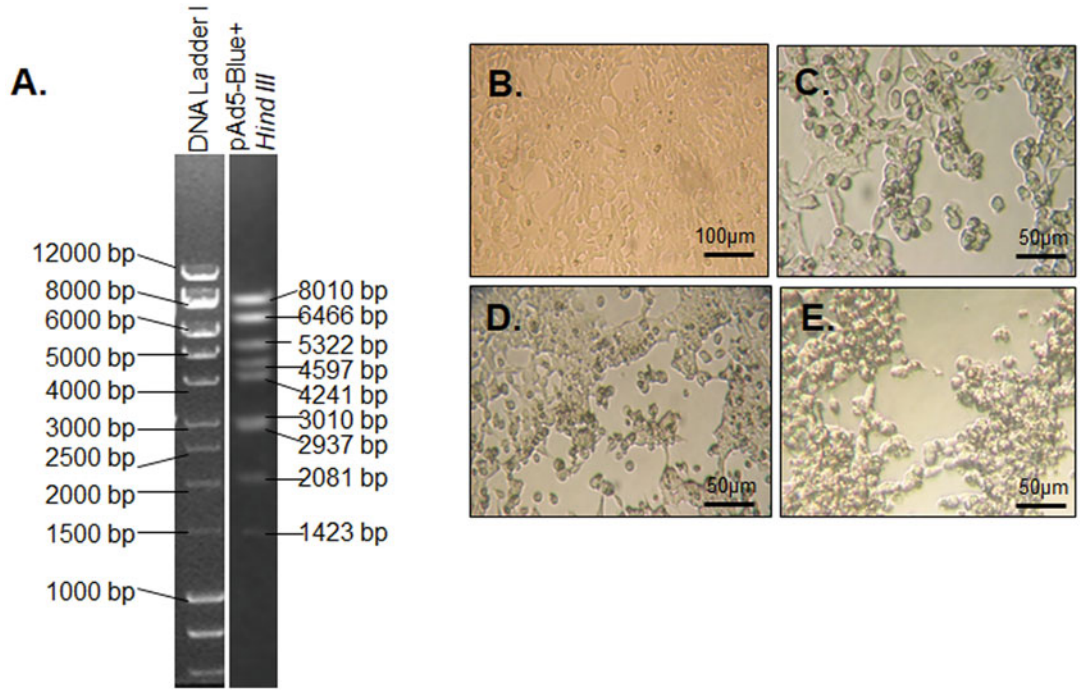

Fig. 2 Analysis of Ad5-Blue digestion with Hind III and infected HEK293 cells. (**a**) Distinct DNA band pattern of vector Ad5-Blue digested with Hind III is detected in 0.8% agarose gel. Numbers indicate the sizes (bp) of the DNA bands for the DNA marker and digested vector. HEK293 cells were mock transfected (**b**) or transfected with linearized Ad5-FMD-O1M and approximately 10 days later CPE were detected (**c–e**) indicating the presence of virus

It is worth to mention that for safety reasons, all laboratory work with adenoviral vectors requires biosafety level 2 (BSL-2) conditions and use of adequate PPE, coat and safety glasses/googles.

After transfection, in general, viral plaques become visible by 5–8 days. Harvesting of individual plaques, whenever possible, helps to isolate and identify real Ad clones.

3.3.1 Derivatization of Recombinant Ad5-O1M-FMD

1. Seed 1×10^6 HEK293 cells in a 6-well tissue culture plate 48 h before transfection and incubate at 37 °C under 5% CO_2.
2. Digest 20 µg of pAd5-O1M-FMD with 30 U of restriction enzyme Pac I. Incubate overnight at 37 °C. Remove an aliquot (10 µl) and analyze qualitatively in 0.8% agarose gel electrophoresis to confirm full digestion.
3. Transfect 293 cells using lipid-based transfection reagent of choice (i.e., Lipofectamine 2000™, ThemoFisher-Invitrogen). Always use media with no serum when preparing the DNA-lipid mixture. Consider that one digestion reaction will be sufficient for five transfections. About 4 µg of linearized

DNA should be applied to each well (5 wells). Make sure to have a mock transfected control where only the transfection reagent and no DNA is added. Incubate transfected cells at 37 °C with 5% CO_2.

4. Daily, examine the cells under the microscope and look for the appearance of cytopathic effect in the wells transfected with Pac I-digested pAd5-O1M-FMD DNA. Distinct viral plaques usually develop between 5 and 8 days post-transfection as cytopathic effects become evident (*see* **Note 2**). Mark plaques in the bottom of the tissue culture plate using an ink marker to distinctly identify their location. When obvious, pick individual plaques by aspirating a suspension of plaque forming cells in media using a pipette tip and suctioning approximately 500 µl directly from above the marked spot. The idea is to collect the apparently infected cells that form each plaque.
5. Transfer the cell suspension from each plaque to microcentrifuge tubes. Usually picking up 6 plaques is sufficient. Centrifuge samples at 1200 × *g* for 5 min. Remove and discard the supernatant and resuspend the cell pellet in 500 µl of PBS++ (*see* **Note 3**).
6. Store samples at −70 °C.
7. Prior to use for amplification, quick freeze and thaw three times to improve the release of recombinant Ad virus from the cell pellet.
   (*see* **Note 4**).

3.3.2 Amplification of Recombinant Ad5-01M-FMD

1. Seed 5 × 10^6 HEK293 cells in a T150 cell culture flask 48 h before the infection and incubate at 37 °C under 5% CO_2.
2. Take the plaque-specific recovered virus from previous step and mix in 5 ml of media (MEM, 1% anti-anti, 1% NEAA).
3. Remove supernatant from the HEK293 cells and add the 5 ml of virus/media mix. Incubate the flask at 37 °C with 5% CO_2 for 1 h, rocking every 15 min.
4. Add 20 ml of media containing 5% FBS. Incubate at 37 °C with 5% CO_2. Check cells under the microscope every day and look for CPE (Fig. 2c, d). Harvest all cells when most cells show signs of infection (i.e., grape-like clusters; Fig. 2e) but the monolayer remains attached to the flask (*see* **Note 5**).
5. Gently tap the flask to fully detach the cells from flask. The cell monolayer should come off very easily. Harvest all cells and media and transfer to a 50 ml conical tube. Spin samples at 1200 × *g* for 5 min. Supernatant can be saved as a low titer stock of Ad5-FMD. Resuspend pellet with 1 ml of PBS++ (this suspension will constitute the primary stock of recombinant Ad5).

6. Aliquot the 1 ml virus in PBS++ suspension into two aliquots, one of 850 μl (crude lysate) for later viral propagation and one of 150 μl for viral DNA extraction. Freeze at −70 °C. Perform three rounds of freeze and thaw in the 850 μl aliquot prior to use later (**step 8**) as inoculum for larger scale virus propagation. Use the remainder 150 μl aliquot for viral DNA extraction using a viral DNA extraction kit (*see* **Note 6**).
7. Crude lysates from **step 6** normally have a very low titer. Additional rounds of amplification are recommended before purification.
8. For large-scale viral propagation, seed at least 15× T150 cell culture flasks with HEK293 cells as described in **step 1**. Infect each flask with 5 μl of crude lysate (from **step 6**) diluted in 5 ml media (MEM, 1% anti-anti, 1% NEAA) as indicated in **step 2**.
9. Follow **steps 3–5** adjusting the volume of PBS++ to approximately 6 ml of PBS++ for further purification by density centrifugation.

3.4 Purification of Ad5-01M-FMD Recombinant Virus

Purification of Ad vectors is achieved by applying high *g*-forces to a solution of CsCl to generate a density gradient. Based on Ad5 buoyant density, ultracentrifugation facilitates the separation of empty, partially assembled, and fully assembled viral particles from cellular debris in the crude lysate [27]. In general, Ad5 has a buoyant density of 1.3349 g/cm^3 in a CsCl gradient [28], displayed as a single well-defined band after centrifugation. However, lighter densities of Ad5 have been observed and these may represent intermediate, immature, or defective forms of the virus [29]. Upon centrifugation, usually up to three bands become visible (Fig. 3). The bands on top reflect residual cell debris, and defective viral particles, while the lowest band contains infectious viral particles. The lowest band is harvested for a second round of purification in an additional CsCl gradient.

It is important to highlight that novel purification methods for adenovirus are available including tangential flow titration [30] and liquid chromatography [31]. Selection of the purification process is going to depend on the production scale needed.

1. Prepare two solutions of CsCl in Tris–HCl buffer (10 mM): Solution 1: 1.4 g/ml. Solution 2: 1.2 g/ml.
2. Pour 2.6 ml of solution 1 (1.4 g/ml) in a 13.2 ml ultra-clear SW41 centrifuge tube (*see* **Note 7**).
3. Carefully pour 3.3 ml of solution 2 (1.2 g/ml) on top of solution 1. Two phases (different refraction) should be visibly noted (*see* **Note 8**).

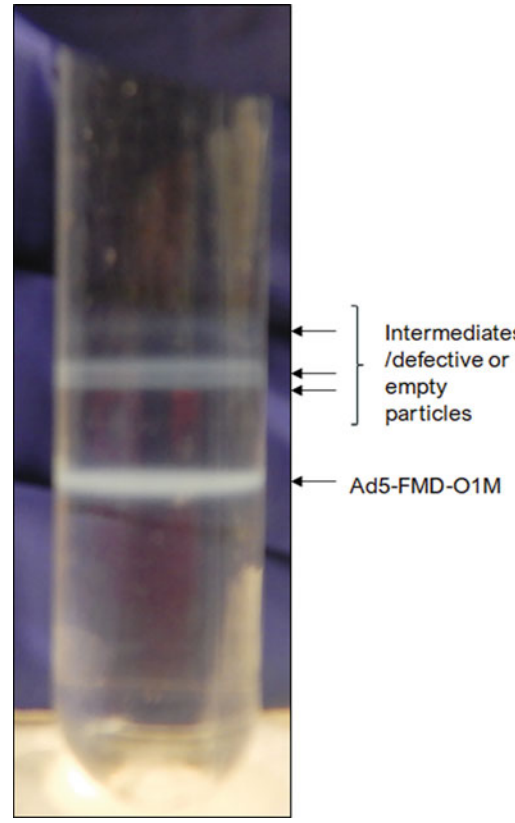

Fig. 3 Visualization of Ad5-FMD-O1M band and defective or empty particles after CsCl gradient centrifugation

4. Carefully add the 6 ml of crude lysate on top of the CsCl solutions. Be careful of not overflowing, leaving an inward meniscus or space of about 3 mm.
5. Place the gradient tube in a bucket of the SW41 Ti ultracentrifuge rotor. Prepare a balance tube, if necessary, by using an identical CsCl/buffer combination. Weigh in a scale for accuracy.
6. Centrifuge for 2 h at 23,000 × *g*, 15 °C with a brake setting of 5, in an ultracentrifuge (i.e., Beckman XL90).
7. Once the run ends, and carefully with the help of forceps, remove the tubes from the buckets and place in a rack. The presence of a white opaque band corresponding to the adenovirus, should be evident (placing the tube behind a dark sheet of paper should make the bands more visible). Normally the viral band is found at the interface between the two CsCl solutions (lowest band). More bands corresponding to residual cell debris and defective viral particles may be visible above the viral band, but they should be discarded (Fig. 3).

8. Carefully aspirate most of the solution from the top of the gradient also including the spurious bands mentioned in **step 7**.
9. Remove the lowest viral band by piercing the tube using a 3 ml syringe fitted with a 18G needle. Pierce the tube from the side just below the visible virus band and aspirate it all (white slurry) (*see* **Note 9**).
10. Remove the needle from the syringe and transfer the virus to a 15 ml tube (it is important to remove the needle to prevent virus shearing). Add at least 1 volume of TE. Since the approximate density of the viral band is 1.345 g/ml, it must be reduced before loading onto the continuous CsCl gradient.
11. Prepare the continuous gradient in a 13.2 ml ultra-clear SW41 centrifuge tube, by using a gradient maker loaded with 3.9 ml of 1.4 g/ml of CsCl and 4.5 ml of 1.2 g/ml of CsCl.
12. Load the virus obtained in **step 10** on top of the continuous gradient as indicated in **step 3**.
13. Centrifuge for 16 h at 23,000 × *g*, 15 °C with a brake setting of 5, in an ultracentrifuge (i.e., Beckman XL90).
14. At the end of the run, remove the tubes from the buckets and place in a rack. Identify the lowest band as the virus containing band. As with the discontinuous gradient, remove most of the gradient above the virus band and using a 3 ml syringe and 18G needle pierce the tube from the side and aspirate the intact virus band (lowest band) and put in a 5 ml tube.
15. Prepare 3 l of dialysis buffer.
16. Place the virus solution from **step 14** in a dialysis cassette and dialyze in 1 l of dialysis buffer prepared in **step 15** with at least three changes of buffer, gently stirring for 1 h in between the buffer changes.
17. Collect the virus from the dialysis cassette and fractionate into 50–100 μl aliquots. Freeze at −70 °C.
18. Take up 5 μl of the virus solution and dilute in 1 ml PBS. Read the OD_{260} to determine the virus particles (*see* **Note 10**).

$$1\ OD_{260} = 1.1 \times 10^{12}\ \text{virus particles.}$$

3.5 Measuring Titers of Recombinant Ad5-O1M-FMD

Virus titers could be expressed using different units, such as virus particles, fluorescence forming units, DNA copy numbers detected by qPCR, protein concentration, etc., but there are few techniques to quantitate infectious particles. These are plaque forming units (PFU), focus forming units (FFU), and 50% tissue culture infectious dose ($TCID_{50}$). Here we describe a titration method based on

the detection of CPE to determine $TCID_{50}$/ml, an endpoint dilution assay that determines what dilution of a viral sample is needed to infect 50% of inoculated cells [32].

1. Prepare a suspension of freshly trypsinized HEK293 cells at 1×10^5 cells/ml diluted in MEM + 2% FCS. Aliquot 100 μl in each well of two 96 well flat bottom plates (10^4 cells/well; two biological replicates).
2. Prepare 2 ml each of serial tenfold dilutions of the virus to be titered in MEM + 2% FCS. Dilute up to 10^{-13}. In this protocol dilutions ranged from 10^{-6} to 10^{-13}.
3. For each row of the 96-well plate (A-H), dispense 100 μl/well of each virus dilution (10^{-6} to 10^{-13}) in each of 10 wells. Use the last 2 wells of each row (positions #11 and 12) as control cells by adding just media (100 μl MEM + 2% FCS).
4. Incubate the plates at 37 °C in an incubator with 5% CO_2, for 10 days examining the development of CPE daily.
5. Mark the plates in the wells when CPE appear. Although the cells could be examined daily the final titer is based on the reading at 10 days.

   A well is counted as positive even if only a plaque or a few cells show CPE. The media only (negative control) should be used for comparison. The test is valid if the negative controls do not show any CPE or cell growth problems and the lowest dilution shows 100% infection (10/10) while the highest dilution show 0% infection (0/10).
6. Calculate $TCID_{50}$ using the following example as a guide:

   In this example, the dilutions that were plated were 10^{-3} through 10^{-10}. 100% of the wells at dilution 10^{-6} are positive (10/10), 6/10 wells at the 10^{-7} dilution are positive, 2/10 at 10^{-8}, and 0/10 of the wells at the 10^{-9} dilution are positive. The titer is determined using the Spearman–Kärber statistical method, i.e., for 100 μl of dilution, the titer is

$$T = 10^{[1+d(S-0.5])}$$

d = Log 10 of the dilution (= 1 for a tenfold dilution)

S = the sum of ratios (always starting from the first 10^{-1} dilution).

   If some of the lowest dilutions are omitted, like 10^{-1} through 10^{-2}, they still have to be included in the calculation as ratios of 1. Following the given example, $S = 1 + 1 + 1 + 1 + 1 + 1 + 0.6 + 0.2 + 0 + 0$.

$$TCID_{50} : T = 10^{[1+1(6.8-0.5)]} = 10^{7.3}$$

   Since only 100 μl of the virus dilution were plated on each well, an additional 10× dilution factor should be considered. Therefore, the virus titer is $10^{8.3}$ $TCID_{50}$/ml.

   (*see* **Note 11**).

3.6 Confirmation of the Expression of Capsid Proteins by Western Blot

Before any further use in animals, it is essential that some quality control techniques are run to verify that the Ad5-Vaccine is expressing the proteins needed for immunization. Detection of capsid expression in HEK293 cells confirms the presence of FMD antigens. Moreover, although recombinant Ad5 are known to have a broad tropism, transduction of Ad5-FMD in cells sharing the same species of the immunized organism is highly recommended. In this protocol, HEK293 cells and porcine IBRS-2 cells are infected and transduced, respectively with Ad5-FMD. As shown in Fig. 4, lysates from these cells should be analyzed for FMD capsid expression by immunoblotting (Western blot).

1. Seed either HEK293 or IBRS-2 cells at 2×10^6 or 1×10^6 cells/well respectively in two six-well tissue culture plates.
2. Infect HEK293 cells with the virus solution at a multiplicity of infection (MOI) of 0.5 and transduce IBRS-2 cells at a MOI of 20. Calculate the amount of virus (μl) required based on the infectious titers and add it to 500 μl of media (MEM + 1% anti-anti +1% NEAA) to be used in one well.
3. Remove media from either cell line; add the 500 μl of virus-media mix. Incubate the plate at 37 °C in an incubator with 5% CO_2 for 1 h, rocking every 15 min. Add 2.5 ml of extra media (MEM + 5% FBS + 1% anti-anti +1% NEAA) and return plate to incubator for 24 h.

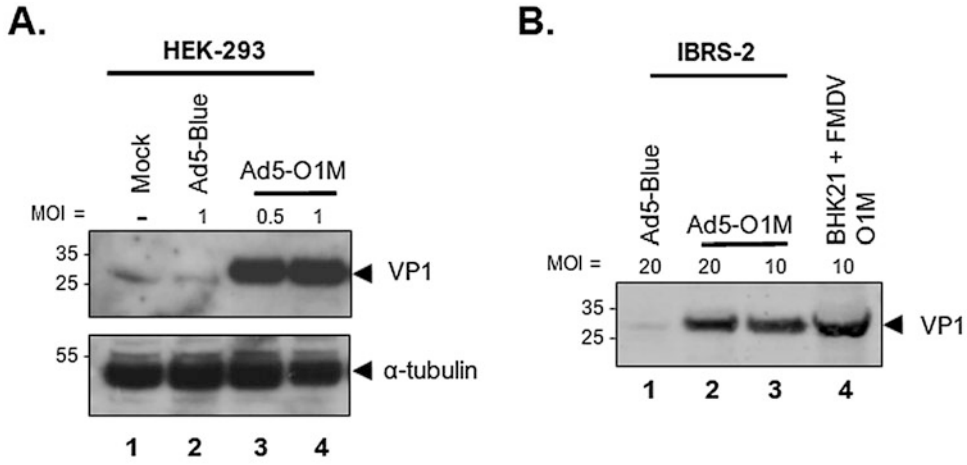

Fig. 4 Characterization of Ad5-FMD-O1M capsid expression in mammalian cells. (**a**) HEK293 cells were mock infected (lane 1) or infected using an MOI of 0.5 or 1 with either Ad5-Blue (lane 2) or Ad5-FMD-O1M (lane 3–4). 24 h later, cells were harvested using a lysis buffer (i.e., RIPA), and run in a protein gel to determine capsid expression using a polyclonal antibody against FMDV VP1. Detection of tubulin is used as a loading control. (**b**) Porcine kidney cells IBRS-2 were transduced with either Ad5-Blue or Ad5-FMD-O1M using MOI of 10 or 20 (lanes 1–3). Control in lane 4 indicates samples from cells infected with FMDV-O1M

4. Aspirate off the supernatant and add 1 ml of PBS. Scrape the cells using a cell scraper.
5. Transfer cell suspension in a pre-cooled micro-centrifuged tube and spin at 1200 × *g* for 5 min at 4 °C. Aspirate off supernatant and wash cells one more time with cold PBS.
6. After removing the washing buffer, resuspend cells in 300 μl of cell lysis buffer (i.e., RIPA buffer) and incubate on ice for 5 min. Centrifuge the lysate at 4000 × *g* for 5 min at 4 °C. Collect supernatant, transfer to a clean micro-centrifuge tube and discard pelleted debris. Store supernatant at −70 °C.
7. Take an aliquot of the lysate and add protein sample loading buffer (Laemmli buffer) containing DTT. Boil the lysate at 95–100 °C for 5 min before loading.
8. Run lysates in a 10–12% SDS-PAGE until buffer front reaches approximately the last 1/10th of the gel.
9. Transfer gel into a membrane (PVDF) using a semidry or wet transfer system.
10. Block membrane in 5% non-fat milk in PBS-T for 1 h at RT.
11. Add specific primary antibody (i.e., polyclonal rabbit anti FMDV VP1/VP3) and incubate overnight at 4 °C on a rocker (*see* **Note 12**).
12. Wash blot three times with PBS-T. Detect proteins of interest by incubating the blot with a secondary antibody (i.e., goat anti-rabbit IgG) conjugated to horseradish peroxidase (HRP) and incubate for 1 h at RT.
13. Wash blot three times with PBS-T and detect HRP activity by incubating with the appropriate substrate (i.e., Bio-Rad Clarity Max ECL).
14. Visualized capsid-antibody complexes by detecting chemiluminescence in a detector (i.e., Azure biosystems).

4 Notes

1. XbaI is blocked by overlapping dam methylation. If the XbaI recognition site is preceded by GA or followed by TC, the Dam methylase within most *E. coli* cloning strains will methylate the GATC site. For example, XbaI sites gaTˆCTAGA and TˆCTAGAtc will be blocked by methylation. To avoid *dam* methylation, use a *dam*-deficient strain such as *dam−/dcm−* Competent *E. coli*.
2. Feed the cells with complete medium if viral plaques are not detected 5 days post-transfection. Viral plaques have been detected up to 10 dpt. Occasionally it takes longer than 11 days to see plaques. If this is the case and the cells are

starting to get old, the cells can be harvested and passed into a T-25 flask. Within a couple of days, CPE should be evident. This will rescue a virus that might be in danger of being lost. However, this virus is now passage 2.

3. Save 100 μl for viral DNA extraction and PCR analysis using primers designed to anneal the flanking ClaI and XbaI regions. Use a viral DNA extraction kit of choice (i.e., Qiagen QIAmp DNA purification kit).
4. Failure to rescue virus or to expand the initial viral plaques could reflect that the HEK293 cell passage number is too high. Using freshly thawed cells with a lower passaging number can easily address this problem.
5. Complete infection depends on the MOI and may take up to 7 days.
6. Although quality of extracted DNA might not be sufficient to clearly detect it by agarose gel electrophoresis, it still may serve as template to amplify the insert by PCR and proceed to Sanger sequencing.
7. If bigger batches of Ad5 are needed, more flasks will be infected, therefore the gradient preparation needs to be adjusted to the volume of crude viruses. Tables 1 and 2 may be used as a guide for this purpose.
8. Attach to a 1 ml pipette tip a 200 μl pipette tip (without filter) to facilitate the release of the solution without disturbing the other layer. Alternatively attach a metal canula to a syringe and use it to do the overlay.
9. The area on top of the lower band may appear turbid. Avoid removing the turbid area.
10. The ratio of OD 260 over OD 280 reflects purity and should be close to 1.8 (optimal absorbance for DNA).

Table 1
Preparation of discontinuous gradients

# of flasks	Rotor type	Run conditions	# of gradients	Overlay volume per gradient	1.4 Cs volume	1.2 Cs volume
10 T-150 s	SW55	30 K, 2 h, 15 °C	1	2.8 ml	1 ml	1.5 ml
15 T-150 s	SW41	23 K, 2 h, 15 °C	1	6 ml	2.6 ml	3.3 ml
20 T-150 s	SW41	23 K, 2 h, 15 °C	2	6 ml	2.6 ml	3.3 ml
30 T-150 s	SW28	23 K, 2 h, 15 °C	1	18–20 ml	8 ml	6 ml

Table 2
Preparation of continuous gradients

# of flasks	Rotor type	Run conditions	# of gradients	Overlay volume per gradient	1.4 Cs volume	1.2 Cs volume
10 T-150 s	SW41	26 K, 20 h, 15 °C	1	4 ml	3.9 ml	4.5 ml
15 T-150 s	SW41	26 K, 20 h, 15 °C	1–2	4 ml	3.9 ml	4.5 ml
20 T-150 s	SW41	26 K, 20 h, 15 °C	2	4 ml	3.9 ml	4.5 ml
30 T-150 s	SW41	26 K, 20 h, 15 °C	2	4 ml	3.9 ml	4.5 ml
>30 T-150 s	SW28	23 K, 20 h, 15 °C	1	8–10 ml	12 ml	14 ml

11. To determine the pfu/ml, a relative accurate approximation could be done by subtracting 0.7 from the $TCID_{50}$ titer. Therefore, $10^{7.6}$ pfu/ml or 4×10^7 pfu/ml.
12. Antibody dilution may require optimization. In this protocol we have used a polyclonal antibody against FMDV VP1 at 1:2500 dilution.

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Chapter 10

Recombinant Modified Vaccinia Virus Ankara Development to Express 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 abortive 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 wide range 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. rMVAs encoding proteins of Bluetongue virus (BTV), an orbivirus that infects domestic and wild ruminants through transmission 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 BTV. The included protocols cover the cloning of VP2, NS1, and VP7 BTV-4 genes in a transfer plasmid, the construction of rMVAs, the titration of virus working stocks, and the protein expression analysis by immunofluorescence and radiolabeling of rMVA infected cells as well as virus purification procedure.

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 (CEF). After this extensive propagation, the viral genome suffered several major deletions and numerous small mutations, with the majority of ORFs in the N-terminal region and some others of the central and C-terminal region mutated, deleted, fragmented or truncated, resulting in replication defects in human and most other mammalian cells as

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well as profound attenuation of pathogenicity [2–4]. This is why these viral vectors have shown 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 [4]. MVA has intrinsic adjuvant capacities and it has been widely investigated as a safe smallpox vaccine and as an expression vector to produce vaccines against other infectious diseases and cancer [3]. In this sense, rMVAs expressing immunogenic viral proteins has been shown to induce both humoral and cell mediated immunity [1, 6, 7].

Type-I interferons may act as a link between the innate and adaptive immune system, including humoral and cellular responses [8, 9]. Poxviruses have the ability to induce the expression of type-I and type-II interferons and to express soluble receptors capable of interacting with host antiviral mechanisms [10–12]. 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. MVA has been used to construct many vectored vaccines expressing different proteins from different kind of orbiviruses [4, 13–16]. The transfer plasmid pSC11 [17] was designed to place the genes of interest (in our case from BTV) under the control of the vaccinia virus (VV) early/late promoter p7.5 into the vaccinia *TK* locus. 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 type-I interferon receptor defective mice (IFNAR(−/−)) on a 129 Sv/Ev background and sheep [18–27]. 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 [24]. MVAs have also been found to be protective in combination with other vaccine platforms, such as antigen presenting protein microspheres (μNS) carrying VP2, VP7, and NS1 [28] or the viral vector chimpanzee adenovirus Oxford 1 (ChadOx1) [18].

In this work, we detail the methodology applied to generate the rMVAs encoding the proteins VP2, VP7, and NS1 of BTV-4. In addition, this chapter describes the protocols followed 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

2.1 Cells and Viruses

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 derived from an African green monkey, *Chlorocebus* sp. (Vero cells) (ATCC-CCL-81).
4. Chicken embryo fibroblast (DF-1 cells) (ATCC-CRL-12203).

2.2 Cell Culture Reagents

1. Serum and antibiotic-free Dulbecco's modified Eagle's medium (DMEM).
2. Complete DMEM: DMEM with 2 mM glutamine, 10% fetal bovine serum (FBS), MEM Non-Essential Amino Acids Solution 1×, and 1% Penicillin/Streptomycin.

2.3 Construction of Recombinant MVAs

1. TRI Reagent Solution.
2. 10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT.
3. Reverse Transcriptase (i.e., SuperScript® II).
4. RNAse solution (i.e., RNaseOUT).
5. 10× PCR Buffer II.
6. 10 mM dNTPs.
7. Specific primer (VS and RS) (Table 1).

Table 1
Primer used for amplification of BTV genes

Gene		Sequence
VP2	VS	5′-CGCCCGGGATGGAGGAGTTTGTCATTCC-3′
	RS	5′-CGCCCGGGCTAAACGTTGAGTAATTTCG-3′
NS1	VS	5′-CGCCCGGGATGGAGCGCTTTTTGAGAAAATAC-3′
	RS	5′-CGCCCGGGCTAATACTCCATCCACATCTG-3′
VP7	VS	5′-CGCCCGGGATGGACACTATCGTCGCAAG-3′
	RS	5′-CGCCCGGGCTACACATAGCGCGCGCGTGC-3′

SmaI-restriction site underlined

8. PCR DNA Polymerase (i.e., AmpliTaq).
9. 1% Agarose gel (Tris-acetate-EDTA buffer (TAE) and 1% agarose).
10. DNA staining solution (i.e., Midori green).
11. Gel Extraction Kit (i.e., Qiaex).
12. pSC11 plasmid (kindly provided by Professor Francisco Rodríguez, Centro Nacional de Biotecnología, CSIC, Madrid, Spain).
13. SmaI restriction endonuclease, Shrimp Alkaline Phosphatase (SAP) and T4 ligase enzyme.
14. Luria-Bertani (LB) agar plates and media.
15. Ampicillin sodium salt.
16. Plasmid Miniprep Kit I (i.e., E.Z.N.A.® Q-spin).
17. DNA transfection reagent (i.e., Lipofectamine 3000® and P3000 reagent).
18. Noble agar (i.e., Difco Noble Agar (DB)) and distilled water.
19. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).
20. Complete DMEM-agar-X-Gal: Complete DMEM-0.6% Noble agar with X-Gal (0.4 μg/μL).
21. 10%.Formaldehyde.
22. Crystal violet in 80% methanol.

2.4 Confocal Microscopy

1. 4% paraformaldehyde solution (diluted in PBS).
2. Phosphate buffered saline 1× (PBS 1×).
3. Blocking solution: PBS, 20% fetal calf serum, 0.2% saponin.
4. Mouse polyclonal antibody against BTV-4.
5. Alexa Fluor® 488 conjugated anti-mouse IgG (H + L) antibody.
6. Antifade reagent (i.e., ProLong™ Gold).
7. DAPI solution (1:10,000 dilution in PBS).

2.5 Immunoprecipitation Analysis

1. RIPA buffer: 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, protease inhibitors.
2. Methionine-free DMEM cell culture medium.
3. [^{35}S] Methionine (800 Ci/mmol).
4. Magnetic Protein G beads (i.e., Dynabeads® system).
5. 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.

2.6 Purification of rMVAs

1. 1 mM Tris•HCl, pH 9.0.
2. 36% sucrose cushion and sucrose gradient.

3. SW 28 type centrifuge tubes and rotor (50 mL).
4. Ultracentrifuge.

3 Methods

These methods describe the generation of the rMVAs 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 are amplified from total RNA of BTV-4 infected cells (*see* Fig. 1). To generate the MVA transfer plasmids pSC11-VP2, pSC11-NS1, and pSC11-VP7, the restriction site SmaI must be introduced into the 5′ and 3′ ends of the PCR products, unique restriction site contained into pSC11 (*see* Fig. 2).

1. Infect confluent Vero cell 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 24 h at 37 °C, 5% CO_2.
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 of 10× RT buffer, 4 μL of 25 mM $MgCl_2$, 2 μL of 0.1 M DTT, 1 μL of RNaseOUT™ (40 U/μL), and 1 μL of SuperScript® II Reverse Transcriptase (200 U/μL).
7. Incubate 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.

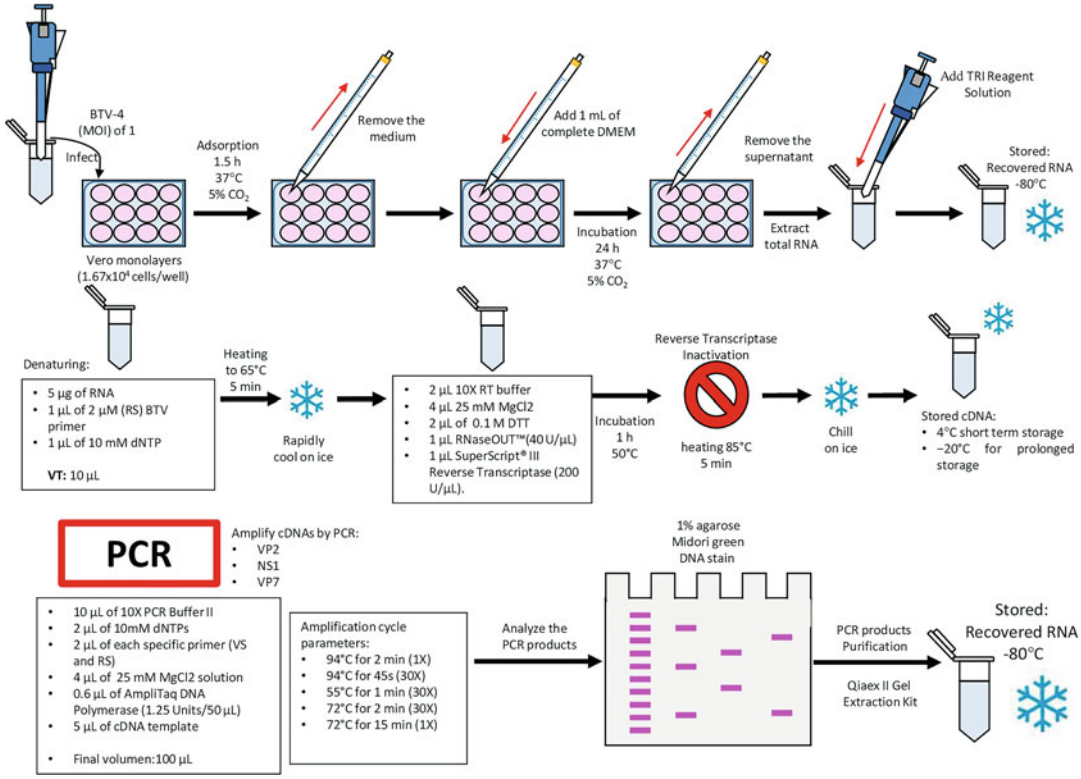

Fig. 1 Amplification of VP2, NS1, and VP7 genes from total RNA of BTV-4 infected cells (*see* **Note 1**)

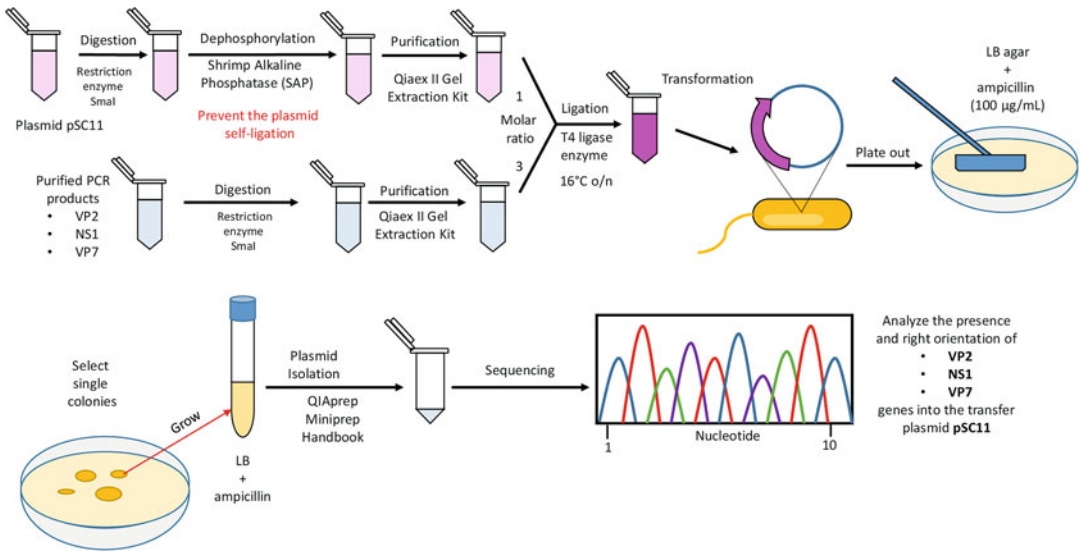

Fig. 2 Cloning of VP2, NS1, and VP7 BTV-4 genes into the pSC11 transfer plasmid (*see* **Note 2**)

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 SmaI site (Table 1), 4 μL of 25 mM $MgCl_2$ solution, 0.6 μL of AmpliTaq DNA Polymerase (1.25 Units/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×); 72 °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 SmaI into the 5′ and 3′ ends) with the restriction enzyme SmaI 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 JM109 Competent 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 E.Z.N.A.® Plasmid Mini Kit I 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. 3). The MVA transfer plasmids pSC11-VP2, pSC11-NS1, and pSC11-VP7 should contain the VP2, NS1, and VP7 BTV genes, respectively, 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 wild type (wt) and subsequent transfection with pSC11 plasmids will lead to the generation of recombinant viruses.

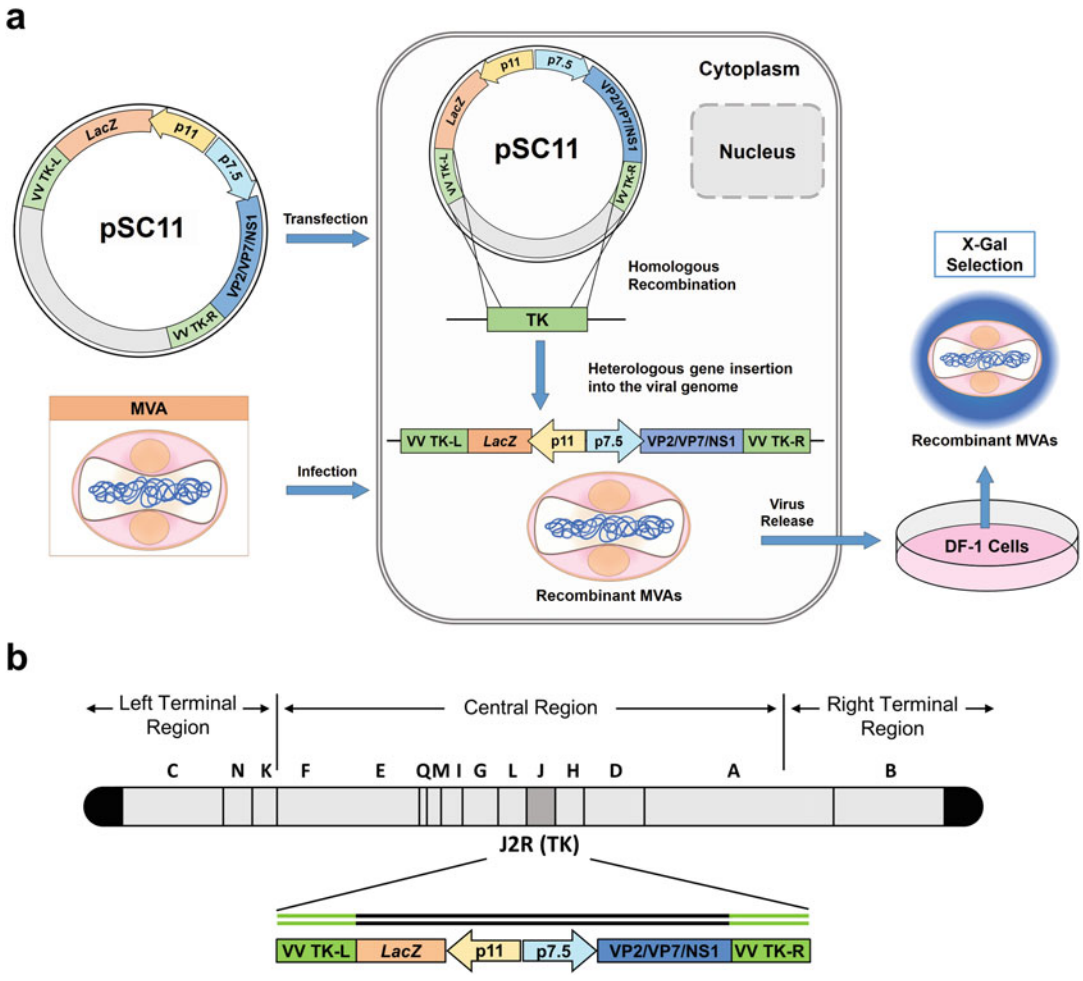

Fig. 3 General procedure for the generation of recombinant MVAs. Genes VP2, NS1, and VP7 of BTV-4 were cloned into the vaccinia transfer plasmid pSC11 downstream of the p7.5 vaccinia promoter. DF-1 cells were infected with MVA virus (MOI = 0.01 p.f.u./cell). After virus adsorption, cells were transfected with pSC11-VP2, pSC11-NS1, or pSC11-VP7 plasmids. (**a**) Recombinant MVA viruses were generated by homologous recombination at the *Thymidine kinase* locus, allowing the analysis by using the *LacZ* gen marker. (**b**) Genome map of MVA strain. The heterologous BTV gen was cloned in the *TK* locus, located within the central region of the MVA genome

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

1. Plate DF-1 cells in p35 or six-well plates 1 day prior to infection in a 2 mL volume of complete DMEM. DF-1 cells that are 60–80% confluent are needed for infection and transfection.
2. Infect DF-1 cells with 100 μL of MVA-wt in serum and antibiotic-free DMEM at a MOI of 0.1.
3. Incubate the cells at 37 °C and air-5% CO_2 atmosphere during 1.5 h. After virus adsorption, DF-1 infected cells are transfected with pSC11-VP2, pSC11-NS1, or pSC11-VP7.

4. For each well of a six-well plate, mix 2 μg of plasmid with 5 μL of P3000™ Reagent in 125 μL of serum and antibiotic-free DMEM. Add to this mixture 5 μL of Lipofectamine 3000® reagent diluted in 125 μL of serum and antibiotic-free DMEM or Opti-MEM™ and incubate at room temperature for 15 min after vigorously vortexing the mixture.
5. Add the mixture to the infected cells.
6. Incubate at 37 °C and air-5% CO_2 atmosphere, shaking the plate every 30 min during the first 90 min of transfection.
7. When the cytopathic effect (CPE) is apparent (~3 days), harvest cells and supernatants by disruption of the monolayer (*see* **Note 3**). Carry out 3 cycles of thawing/freezing and sonicate for 2 min to disrupt the cells and release viruses.
8. 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 will try to find clear, well-separated plaques for isolating and screening recombinant viruses (*see* Fig. 4).

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^{-6} (*see* **Note 4**).
3. Allow the viruses to adsorb at 37 °C for 1.5 h.
4. Incubate infected cells at 37 °C and air-5% CO_2 atmosphere during 72 h.
5. Remove the medium and add complete DMEM-agar-X-Gal (*see* **Note 5**) over the monolayer.
6. Allow the overlay to solidify.
7. Incubate at 37 °C for 4 h.
8. Pick only well-separated blue plaques, about 6 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.
9. Carry out 3 cycles of thawing/freezing and sonicate for 2 min.
10. Repeat this cloning procedure (**steps 8** and **9**) for six extra rounds to get a highly purified clone.

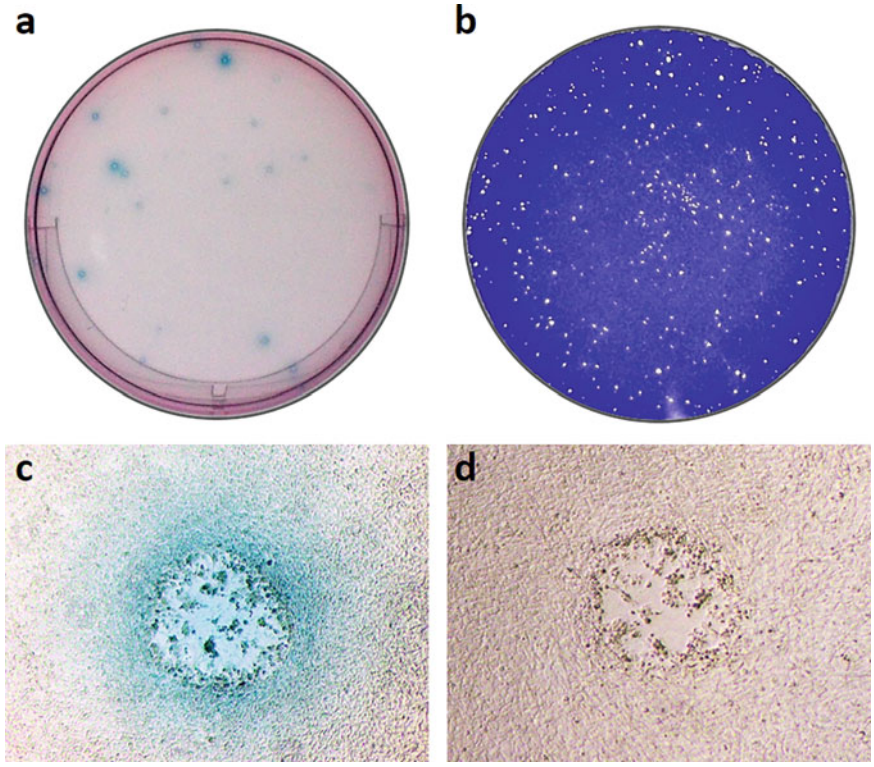

Fig. 4 Plaque purification assay of rMVA-VP2 in avian DF-1 cells. DF-1 cells were infected with 50 μL of tenfold dilutions of supernatants from the infected/transfected cells. Representative plaque assay plate (**a**) stained with X-gal or (**b**) fixed with 10% formaldehyde and stained with crystal violet. (**c**) Blue plaque: rMVA-VP2. (**d**) White plaque: MVA-wt

3.3 Amplification, Escalation, and Titration of Virus Working Stocks

In this step, we will amplify and scale the cloned plaques using DF-1 cells to procure virus working stocks.

1. Starting from P35 plates, P60, P100 and finally 175 cm^2 flasks, passage DF-1 to 80% confluency. The general procedure for each plate round is detailed in **steps 2–4**.
2. Remove the old medium, add fresh medium and inoculate 1 mL of seed virus stock and incubate DF-1 cells at 37 °C for 2–3 days until all cells show clear CPE; oftentimes, most cells will be floating.
3. Remove part of the medium (*see* **Note 7**), 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 the tube in water bath following three cycles of 30 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 viral plaque assays of virus 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. Let viruses adsorb 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 8**).
10. To generate more rMVA stocks, use 80% confluent DF-1 cells in 175 cm^2 flask, using 0.1 MOI from previously titered stocks (*see* **Note 9**).

3.4 Protein Expression Analysis

There is a variety of 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. 5) and the immunoprecipitation of proteins in radiolabeled infected cells.

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 a MOI of 1.
3. After 24 h of infection, wash cells once with 1 mL of PBS 1×, fix the infected cells with 4% paraformaldehyde solution (diluted in PBS) and store the plate at room temperature during 20 min.
4. Remove 4% paraformaldehyde solution and wash once with 1 mL of PBS 1× (*see* **Note 10**).
5. Incubate the fixed cells with 1 mL of blocking solution for 1 h.
6. Remove the blocking solution and add the primary antibody. We use sheep 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 at room temperature for 3 h.
7. Remove the polyclonal antibody and wash with PBS 1× three times during 10 min, preferably with shaking.

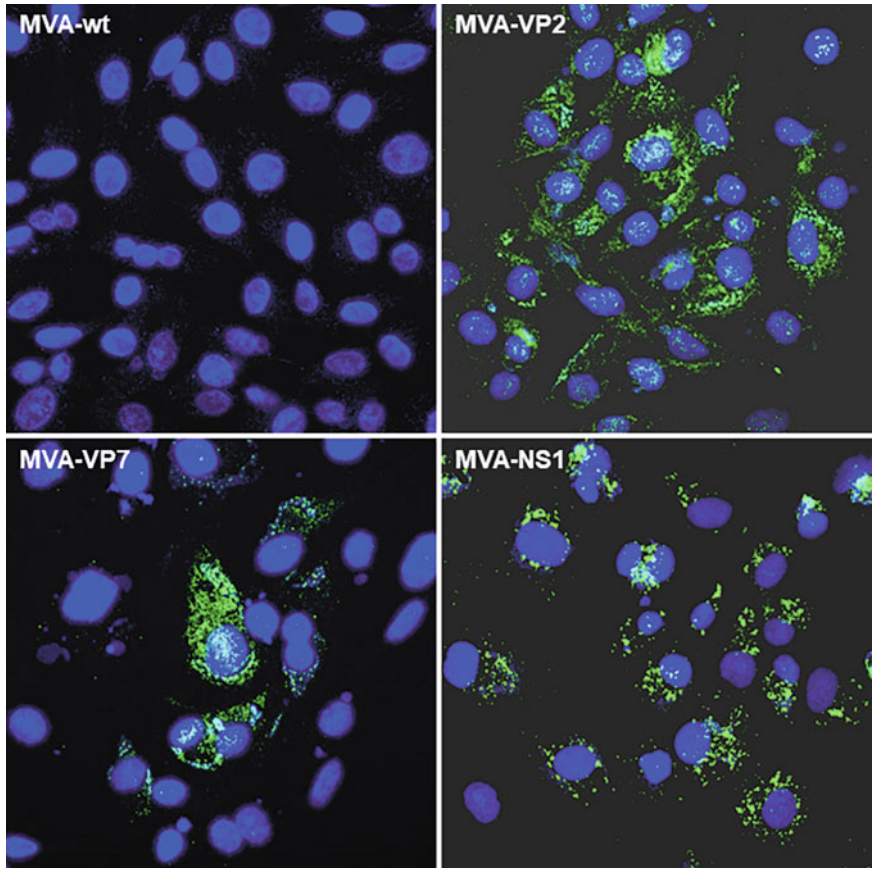

Fig. 5 Analysis of BTV-4 VP2, NS1 and VP7 expression by immunofluorescent staining. DF-1 cells were infected with recombinant MVA containing BTV-4 VP2, NS1, or VP7 genes. At 24 h.p.i, cells were fixed and analyzed by immunofluorescence assay using a sheep polyclonal antisera specific of BTV-4. Specific secondary antibody conjugated to Alexa Fluor 488 was used for the assay. Nuclei were visualized by using DAPI. Laser scanning confocal microscopy images were acquired with an inverted Zeiss Axiovert LSM 880 microscope

8. Add the conjugated secondary antibody (Alexa Fluor® 488 donkey anti-sheep IgG (H + L)) specific of sheep primary antibody diluted 1:5000 in blocking solution over the cells. Incubate 30 min at room temperature in dark conditions, preferably with shaking.
9. Remove the secondary antibody and wash with PBS 1× three times during 10 min with shaking preferably.
10. After washing, incubate with DAPI 1:10,000. We usually use 250 μL/well.
11. Mount the coverslips on slides using ProLong™ Gold antifade reagent, air dry in dark conditions 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. 6).

1. Infect DF-1 cells in 35-mm dishes with MVA-VP2, MVA-NS1, or MVA-VP7 at a MOI of 1.
2. After 90 min of virus adsorption, remove the culture medium and rinse 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) (*see* **Note 12**).
5. Incubate the cells for 16 h at 37 °C.
6. At the end of the incubation, remove the labeling medium in appropriate radiocontainment vessels 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.

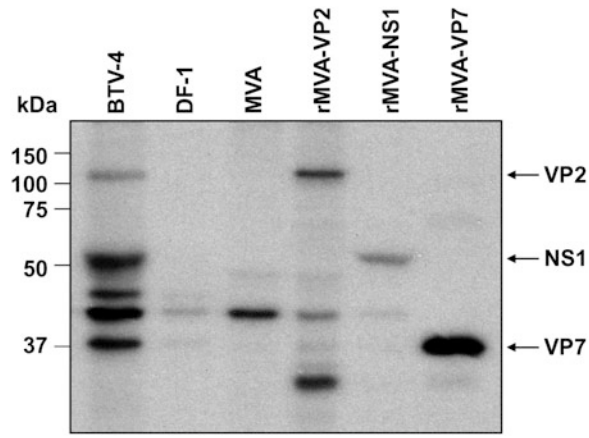

Fig. 6 Analysis of BTV protein expression by radiolabeling, immunoprecipitation, and SDS-PAGE. [^{35}S] methionine-labeled BTV proteins were isolated by immuneprecipitation using polyclonal antibody specific of BTV-4. The expression of BTV proteins in DF-1 cells infected with rMVA-VP2, rMVA-NS1, or rMVA-VP7 was then analyzed by SDS-PAGE

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 9391 × *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.
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 is a variety of forms to purify and 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. Lay 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 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 13**).
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 50 min at 26,000 × *g* (12,000 rpm in 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 14**).

8. Centrifuge 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 SmaI restriction site: **pSC11-A(VS): GTGGTGATTGTGAC TAGCGTAG**.
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. We usually maintain 4 mL of medium/flask.
8. It is convenient to count the plaques of the intermediate dilutions, because it is easier to count (the size of MVA plaques are small).
9. In order to obtain a high viral titer is convenient to use low MOI to avoid a prompt damage in the cells.
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. Along the following steps care should be taken to segregate appropriately all radio contaminated media and plastic ware.
13. At this stage, the virus may be sufficiently pure for some purposes—e.g., isolation of DNA.

14. The total volume should be ~60 mL, which is enough to fill two SW 28 centrifuge tubes. If less volume is obtained, fill the tubes with 1 mM Tris·HCl, pH 9.0.

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Chapter 11

Cloning Strategies for the Generation of Recombinant Capripoxvirus Through the Use of Screening and Selection Markers

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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 commonly used to generate capripoxvirus knockout viruses (KO), and is based on the targeting of 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. An alternative step for the removal of both the EGFP and gpt cassettes and an optional selection step using CRISPR technology are also described.

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 [1, 2]. 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,

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increased heart rates, increased nasal, and mucosal discharge, as well as the formation of skin macules affecting the majority of the skin surface in severe cases [3–5]. Mortality rates vary but can reach over 90% in outbreaks with sheep and goat pox, whereas 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 [6]. Attenuated vaccines have been developed for sheep and goat pox and LSD [4, 7, 8]; while effective in preventing disease outbreaks, the mechanism of attenuation is not known. Recombinant viruses generated which disrupt or inactivate genes suspected of being virulence factors could be used as vaccines once they have been demonstrated to be effective and approved by regulatory agencies. To date, the complete genomes of several LSDV, SPPV, and GTPV isolates have been sequenced and annotated [1]; and several putative virulence factors have been identified (*see* Table 1). Furthermore, the use of viral gene deletions has also been shown to be a valuable tool in the elucidation of the role of individual genes of poxvirus genomes [9]. The ability to manipulate poxvirus genomes has been previously demonstrated for capripoxvirus through the creation of a gene knockout to sheep pox virus kelch-like gene SPPV-019, which was then subsequently shown to affect virulence [10]. The principal method to generate gene knockouts using homologous recombination is the same for all poxviruses [9] (*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 capripoxvirus vaccines, the past decade has seen the use of recombinant capripoxviruses being 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) [11, 12], Rift Valley fever virus (RVFV) [13, 14], peste des petits ruminants virus (PPRV) [15], and rinderpest virus (RV) [16–18]. 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 capripoxvirus. Other capripoxvirus targets have also been evaluated for recombinant gene insertion, including a G-protein-coupled chemokine receptor in the attenuated KS-1 strain of LSDV [19], where the cDNA of Rift Valley fever glycoprotein Gn was inserted to allow expression [20]. A recent example of gene knockout studies in capripox involved the 005 ORF (an IL-10 homologue) of the virulent Warmbaths strain of LSDV [21]; when this gene is inactivated, the virulent strain was found to be attenuated in sheep and goats.

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/homolgue
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]

In this chapter, the protocol to generate recombinant capripoxvirus, targeting the viral thymidine kinase gene will be described (Fig. 1), and will incorporate the use of a reporter gene such as enhanced green florescent protein (EGFP) as well as a positive selection marker [9]; in this case, the *E. coli* guanine phosphoribosyl transferase (gpt) [22].

The selective media to be used in this protocol include mycophenolic acid and aminopterin, inhibitors of purine metabolism. While this would normally inhibit the de novo synthesis of DNA, the recombinantly expressed gpt enables for the generation of guanine from xanthine [23]. In this protocol, the reader will have the option to either: (a) retain the genes for screening and selection (i.e., gpt and EGFP, respectively) (Fig. 2a) or (b) removing both the gpt and EGFP genes through a method described by Wallace et al. [24]. For the removal of gpt and EGFP, the design of the

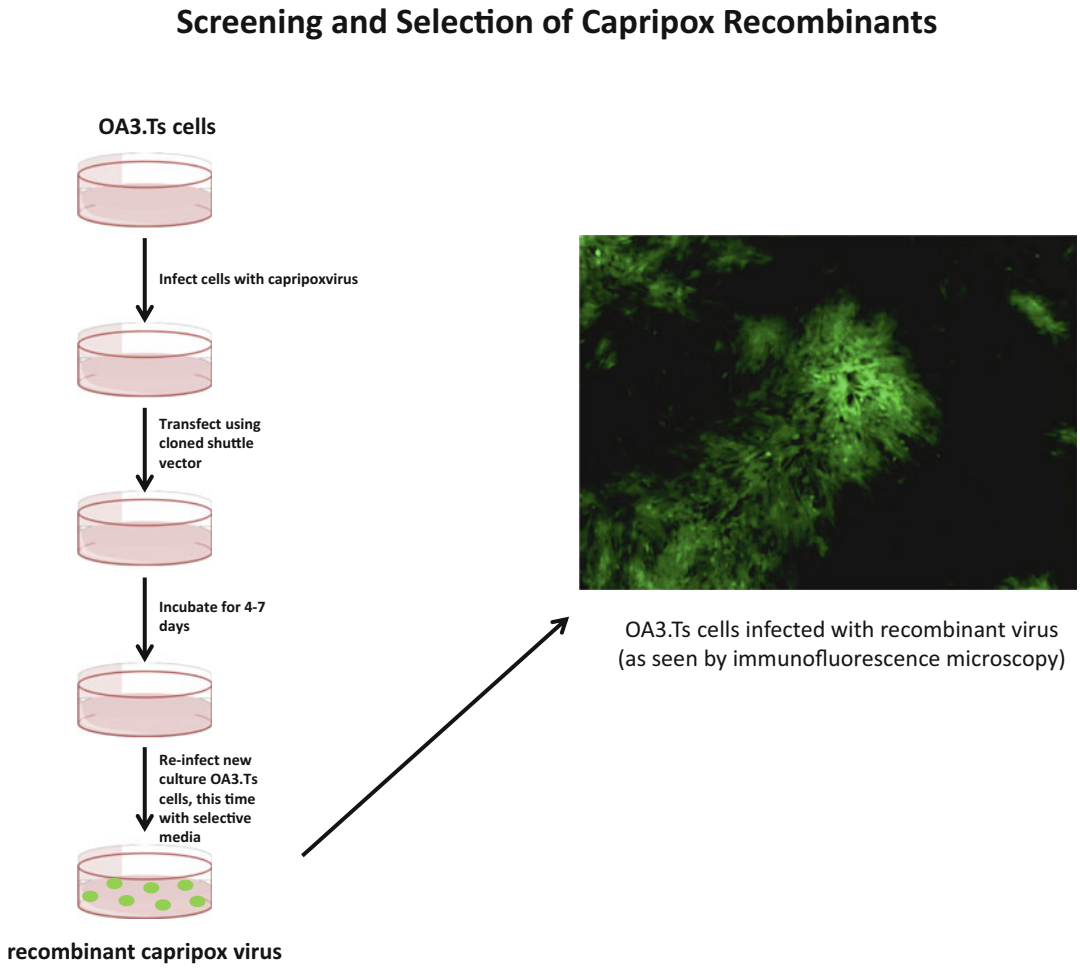

Fig. 1 Schematic of the process of generating recombinant capripoxvirus. OA3.T cells are transfected with a transfer vector containing the gene of interest, as well as the elements needed for recombination (*see* this figure). Upon transfection of the transfer vector, cells are then cultured in selective 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

transfer vector will differ in that both genes will be flanked by two vaccinia virus P11 promoters (Fig. 2b). The first P11 promoter will be upstream from the EGFP ORF, while the second will be located downstream from the gpt gene (which would have its own unique promoter). Removal of selective pressure will enable for its removal through recombinant deletions of the repeated regions (in the example provided in Fig. 1b, the recombination of the p11 promoters).

Additionally, through the use of CRISPR technology, the selection process can be enhanced. Gowripalan et al. [25] have recently shown that CRISPR/Cas9 can be exploited to counteract the

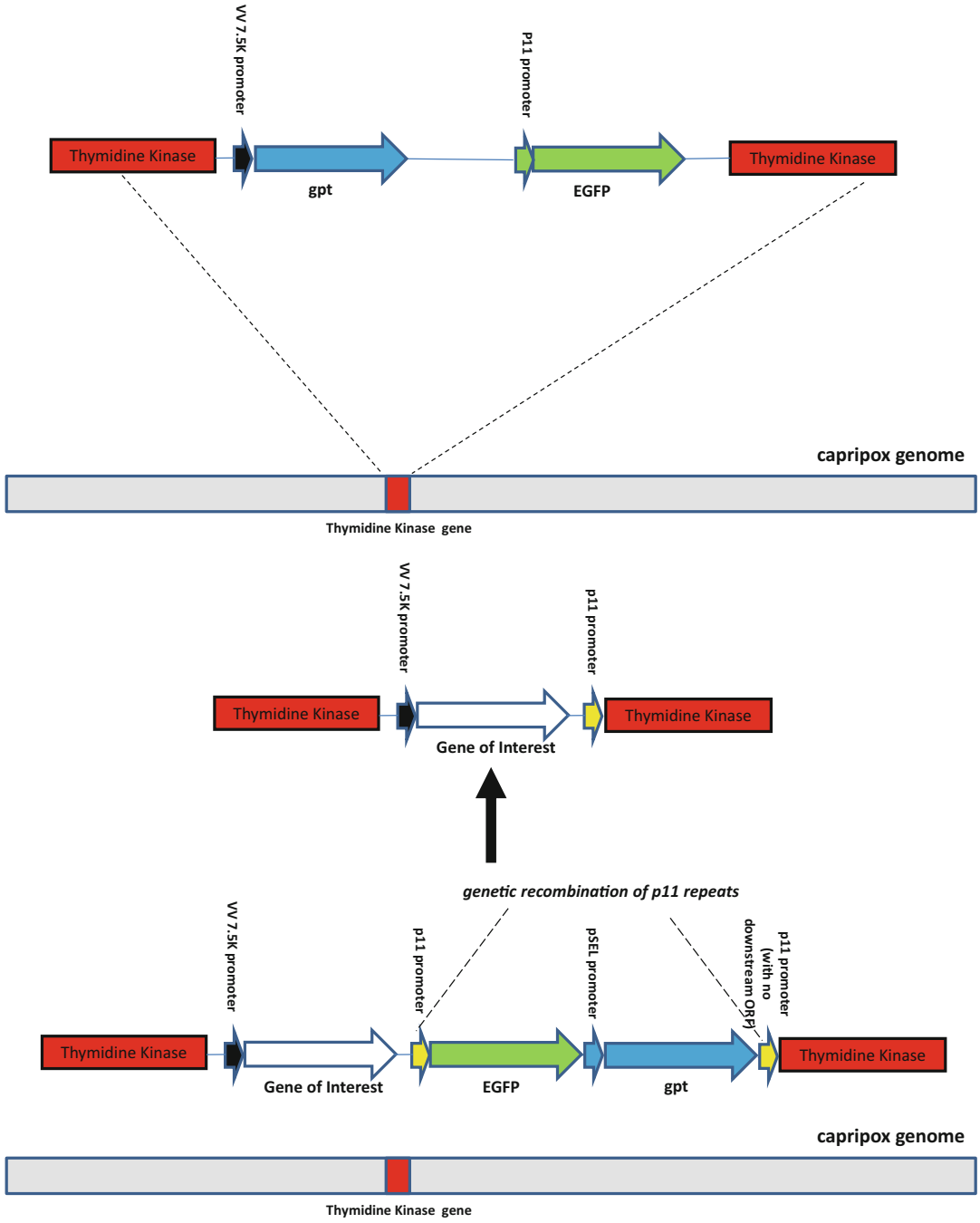

Fig. 2 The insert of a shuttle vector for generating recombinant capripox virus. A viral gene is targeted for gene knockout (Panel **a**) by cloning an insert containing the following: (1) selection and reporter genes (in this case, gpt and EGFP, selectively); (2) vaccinia early/late promoter 7.5K, enabling for the expression of gpt and p11 promoter for the expression of EGFP; (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 be designed, such that a flanking sequence (in this case, p11 promoter is flanking the region containing EGFP and gpt. Upon removal of selective pressure, recombination of the two p11 promoters results in the removal of both gpt and EGFP (Panel **b**)

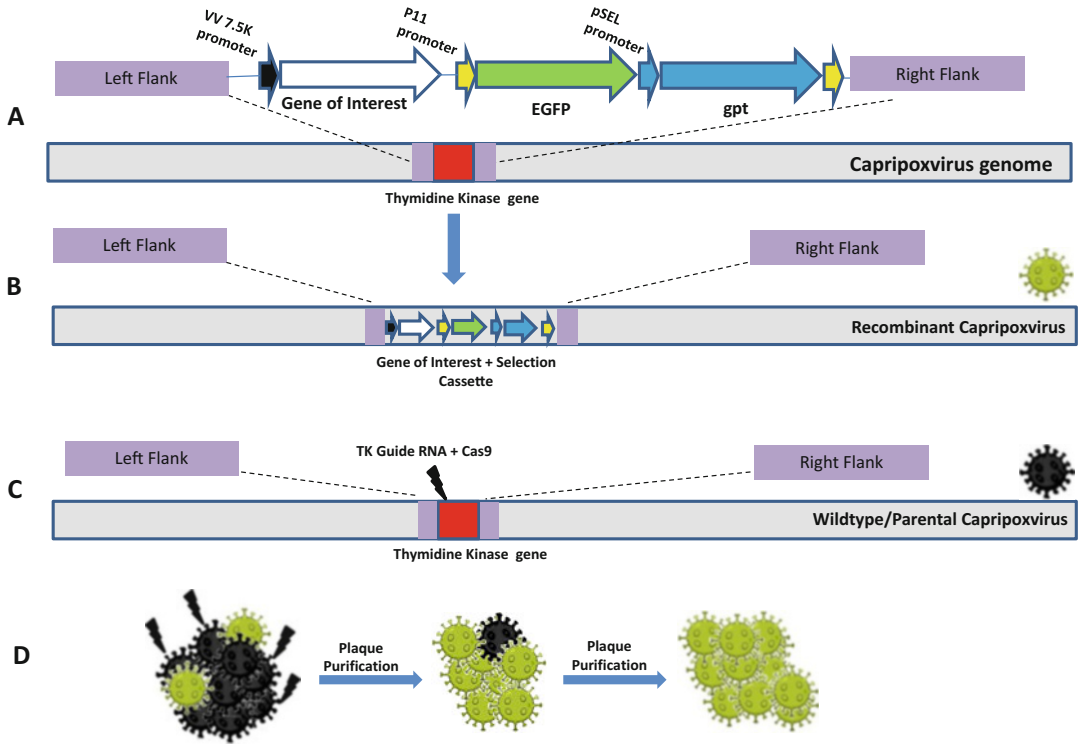

Fig. 3 Schematic of the use of CRISPR in the plaque purification of recombinant capripoxvirus. Upon homologous recombination (**a**), there are two likely outcomes, recombinant capripoxvirus that has taken up the gene of interest and selection cassette (**b**) or wildtype/parental capripoxvirus (**c**) that has not undergone recombination. The presence of a significant amount of wildtype capripoxvirus lengthens the overall selection process, therefore, the use of a guide RNA to target the gene of interest would substantially lower the survival of wildtype virus containing the TK gene. Especially, in the interest of creating gene knockout mutants, an additional step to target a specific gene will greatly enhance selection and presence of mutant or recombinant capripoxvirus (**d**)

increased number of parental virus in contrast to recombinant virus during the selection of recombinant virus by targeting a sequence unique to the parental virus. This new insight can result in drastically improved times in the process of recombinant virus selection (Fig. 3).

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.

1. OA3.T cells (ATCC CRL-6546).
2. Cell culture media: Dulbecco's Modified Eagle Medium (DMEM) supplemented with10% Fetal bovine serum (FBS), 1× Penicillin/Streptomycin (Pen-Strep) and 1× Non-Essential Amino Acids (NEAA).
3. 6-well cell culture plates, Eppendorf tubes, and cryotubes.
4. Lumpy skin disease virus (either the Neethling strain or the KS-1 vaccine strain are commonly used—*see* **Note 2**).
5. A plasmid containing the elements described in Fig. 2a, b (*see* **Notes 3** and **4**). A list of potential LSDV targets for functional knockout and/or attenuation is described in Table 1.
6. Cell transfection reagent. We used X-tremegene HP DNA Transfection Reagent (Roche Diagnostics). Also *see* **Note 5**.
7. Calcium and magnesium free Phosphate Buffered Saline (PBS).
8. Opti-MEM (Invitrogen/Life Technologies) (*see* **Note 6**).
9. Gpt selection agent containing 500× mycophenolic acid solution and 100× aminopterin.
10. Commercially available semi-solid cell culture medium or DMEM/carboxymethyl cellulose (CMC) medium may also be used.
11. For the optional step of the CRISPR/Cas 9 targeting of parental virus, CRISPR reagents (i.e., Lipofectamine CRISPRMAX Transfection reagent, Cas9 Protein, guide RNAs) can be obtained commercially (Life Technologies) and performed according to supplied protocols.
12. For the targeting of the capripoxvirus Thymidine Kinase (TK) gene, the following guide RNAs (gRNAs) has been used:

    5′GACUAUGGAUAUAUACAUUU3′

    5′GACUAUGGAUAUAUACAUUU3′

    5′UAUGGAUAUAUACAUUUAA3′.
13. Primers for detection of gpt selection marker:
    (a) Primer 1 (5′-ATGAGCGAAAAATACATCGTCACC-3′).
    (b) Primer 2 (5′-TTAGCGACCGGAGATTGGCGGGA-3′).

3 Methods

3.1 Infection of Transfected OA3.T Cells with LSDV

1. Seed OA3.T cells in a 6-well plate, at 60–70% confluency 24 h prior to transfection. Prior to addition of LSDV, verify the condition of the cells, and ensure that the confluency is between 70 and 80%.
2. Remove medium, and add 1 mL of fresh medium (DMEM/ / 1× Pen-Strep—without serum) to each well.

3. Add 1000 $TCID_{50}$ (100 μL of a 10^4 $TCID_{50}$/mL stock) of capripoxvirus (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/5% FBS) to each well.

3.2 Transfection of OA3.T Cells

1. While the cells are being infected, warm up a tube of X-tremegene HP transfection reagent to room temperature, 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 6 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 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. Freeze the plate, and perform three freeze-thaw cycles (i.e., freezing the culture plate at −80 °C and thawing at RT/37 °C). This step will liberate viral particles into the resulting cell lysate.
2. Remove the lysate and centrifuge at 3000 × *g* for 15 min.
3. Aliquot the supernatant into sterile cryotubes and store at −80 °C.
4. 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**–**7**, this time using 100 μL of the supernatant.
5. 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* **Notes 7** and **8**).
6. CRISPR/Cas 9 targeting of parental/wildtype DNA can be performed according to the manufacturer's protocol following

the selection protocol to increase presence of recombinant DNA. A set of three possible guide RNAs (gRNAs) are described in Subheading 2 (**item 12**).

3.4 Isolation of Individual Recombinant Plaques

1. On a new culture plate, seed OA3.T cells, as described in Subheading 3.1 (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 semi-solid medium (*see* Subheading 2 **item10**) at 37 °C.
5. After incubating for 4 h, remove the supernatant from all the wells of the culture plate. Add 3 mL of semi-solid 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 5 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 semi-solid 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 3.1.
12. Remove 100 μL of the contents of the medium in the tube and use to re-infect the cells, as described in Subheading 3.3.
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** of Subheading 3.3 (*see* **Note 11**).

15. The recombinant virus can then be analyzed by PCR following DNA extraction, using primers specific to the 5′ and 3′ of the targeting viral (i.e., knockout) gene and the flanking sequences (*see* **Note 12**).

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 4 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 with the highest dilution where plaque formation is observed.
8. Remove the plate, and perform three freeze-thaw cycles (i.e., freezing the culture plate at −80 °C and thawing at RT/37 °C). This step will liberate viral particles into the resulting cell lysate.
9. Remove the lysate and centrifuge at 3000 × *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. Usually non-fluorescent plaques will start to be visible by the third round of screening. 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. 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 poxvirus of interest, and designing a plasmid that uses the DNA sequences for insertion for the specific poxvirus 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, or GTPV 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 a 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 (it should be noted that promoters from vaccinia appear to readily function when inserted in capripoxviruses).
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 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 should be noted that, as of this submission, the authors have found the manufacturers of the gpt selection media (provided by EMD) to be in very limited supply. Should this be the case, the reader could substitute these reagents with HAT medium (50×), mycophenolic acid (adjusted to 12.5 mg/mL), and xanthine (adjusted to 25 mg/mL), supplied by Sigma-Aldrich. The HAT medium should be resuspended in cell media; the mycophenolic acid should be prepared as a 500× stock solution in ethanol; and xanthine should be prepared as a 100× stock in PBS (with the xanthine being dissolved using sodium hydroxide).
8. 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

capripoxvirus polymerases. Recombinant viruses represent only a small fraction of observed EGFP, and are only amplified following additional gpt selection steps.

9. 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.
10. 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.
11. It should be stressed that all steps should be performed in a biosafety cabinet, using sterile technique.
12. 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 12

Using RVFV as a Vector Platform for the Expression of Ruminant Disease Antigens

Sandra Moreno, Belén Borrego, and Alejandro Brun

Abstract

Live attenuated viruses remain as vaccine agents with unparalleled performance in terms of duration, magnitude, and breadth of induced immune responses. As the yellow fever-attenuated vaccine strain Y17D, attenuated Rift Valley fever virus shares features suitable to be used as a viral vector for heterologous antigen expression and bivalent vaccine development. Current reverse genetics technology showed the successful rescue of RVFV carrying foreign antigens with little immunogenicity loss in experimental animal models. We show here the basic experimental protocol to achieve the expression of candidate vaccine antigens from other important diseases of ruminants using RVFV as a vector platform as well as preliminary steps for the characterization of immunogenicity in vivo.

Key words Reverse genetics, Recombinant Rift Valley fever virus, Ruminant disease virus, Viral-vectored vaccine, *Neospora caninum*

1 Introduction

The Rift Valley fever virus (RVFV) replicates in ruminant species, mosquitoes, and humans. Deletion of the NSs gene from the virus genome results in a high grade attenuation of the virus in immunocompetent hosts but still retaining residual virulence in immunocompromised animals or upon intranasal administration in mice [1]. Upon deletion, the NSs gene can be replaced by either reporter genes or by other genes of interest (antigens) making RVFV a putative vaccine vector platform [2]. Recently, we obtained hyper attenuated RVF virus variant encoding mutations related with a marked loss of virulence, even in immunocompromised animals [3]. Inclusion of some of these mutations in a heterologous RVFV backbone (ZH548) reduced virulence in susceptible mouse models ([4], and unpublished data] and therefore could be used to provide additional safety features to a potential RVFV-based virus vector. On the other hand, the Rift Valley fever virus targets antigen

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presenting cells (APCs) such us dendritic cells and macrophages [5, 6]. Being both cell types key players in the development of immune responses because of their high antigen presentation capabilities, the use of this virus as a carrier to provide antigen presentation and processing is of great interest in modern vaccinology. Since the virus can also infect many cell types it should be presumed the occurrence of other indirect ways to facilitate or increase recombinant antigen presentation. Additionally to a replication-competent phenotype in ruminant species RVFV may act as a replication-deficient vector in non-RVFV-susceptible species as recently suggested [7]. An earlier demonstration of the RVFV vector capabilities was reported by the expression of influenza H5 hemagglutinin [8, 9] which proved immunogenic in laboratory mice. In our hands the expression of two bluetongue virus BTV-4 antigens led to the induction of specific immune responses both in mouse and sheep [10]. These encouraging results warranted more research to test the use of a RVFV expression platform with other non-viral antigens (i.e., bacterial or parasitic) from ruminant pathogens. Neosporosis is an infectious disease of cattle and dogs caused by *Neospora caninum*, an apicomplexan (*O. Eucoccidiorida*) intracellular parasite closely related to *Toxoplasma* spp. It is a major animal health problem since it causes abortions and neonatal mortality [11]. As an approach to the development of vaccines against *N. caninum*, novel candidate antigens are being used, based on selection of rhoptry (ROP) proteins. Specifically, immunization with recombinant NcROP2 and NcROP40 proteins increased significantly the survival rate in the offspring of pregnant mice challenged with *N. caninum* tachyzoites [12, 13]. Using both ROP proteins as model antigens, we describe in this protocol the essential steps for the rescue and characterization of recombinant RVF viruses. Both ROP proteins were C-terminally tagged including the V5 tag epitope [14] and a sequence corresponding to VP1 amino acid residues 133–156 of foot and mouth disease virus (FMDV) isolate C-S8c1, a major B cell antigenic site of VP1 (site A) [15]. Besides acting as a detection tag, this short sequence provides the system with an immunization marker that constitutes a practical tool to confirm a successful immunization.

2 Materials

2.1 Cell Culture

1. Vero cells (ATCC-CCL-81). These are kidney epithelial cells derived from *Chlorocebus* spp. (African green monkey).
2. HEK-293 cells (ATCC- CRL-1573). These are human embryonic kidney cells.
3. BHK-21 cells (ATCC- CRL-12071). Derived from baby hamster kidney.

4. Serum and antibiotic free Dulbecco's modified Eagle's medium (DMEM).

   DMEM 10% FBS: DMEM with 2 mM glutamine, 10% fetal bovine serum (FBS), MEM Non-Essential Amino Acids Solution 1×, and 1% Penicillin/Streptomycin.
5. DMEM 5% FBS: DMEM with 2 mM glutamine, 5% FBS, MEM Non-Essential Amino Acids Solution 1×, and 1% Penicillin/Streptomycin.
6. DMEM-1% carboxy-methyl-cellulose (CMC).
7. 10% Formaldehyde.
8. 2% Crystal violet in 80% methanol.
9. Foot and mouth disease virus (FMDV) isolate C-S8c1 [16].

2.2 Plasmid Generation

1. RVFV rescue plasmids: pHH21 plasmid containing genomic (negative sense) RVFV-ZH548 strain L, M and S segment sequences and pI.18 expression plasmids containing RVFV RdRp polymerase and nucleoprotein N sequences (kindly provided by Dr. Friedemann Weber's laboratory, Giessen University, Germany) (*see* **Note 1**).
2. Expression plasmid, to express the genes of interest (e.g. pcDNA 3.1).
3. AarI, XhoI and NcoI restriction endonucleases.
4. 0.8% Agarose gel made in Tris-acetate-EDTA (TAE) buffer.
5. Nucleic acid gel stain (we use GelRed staining solution from Biotium).
6. DNA recovery kit from agarose gels or similar.
7. T4 DNA ligase enzyme and reaction buffer.
8. Competent bacterial cells.
9. Luria-Bertani (LB) agar plates and media.
10. Ampicillin sodium salt solution (100 mg/mL).

2.3 Virus Rescue and Growth

1. Eukaryotic cell transfection reagent (i.e., Lipofectamine 3000®).
2. 1.2% Noble agar in water.
3. 0.01% Neutral red solution in PBS 1×.
4. 10× RT buffer; 25 mM $MgCl_2$; 0.1 M DTT.
5. Reverse Transcriptase (i.e., SuperScript® IV).
6. 10× Standard reaction buffer with $MgCl_2$.
7. 10 mM dNTPs.
8. Specific primers (VS and RS, *see* Table 1).
9. PCR-grade DNA polymerase.

Table 1
Primers used for the analysis of the genetic stability

Oligo name	Sequence 5′-3′	Function
RTss1	CTATTACAATAATGGACAACTATCAAGAGC	Complementary first strand cDNA synthesis
ss1 NΔ9 fwd	AGCCACTTAGGCTGCTGTCTTGT TCGAAAGAAGGCAAA	N nested gene amplification
NcROP2 fwd NcROP2 rev	CGAATCCTATGTATTATCATGGCAG GGATACAAAAAGTATCGGTTCACG	ROP2 gene amplification
NcROP40 fwd NcROP40 rev	GCCACTGTCTTTTCACACTACAT GGAGAAGCCACCAAAATAAAGA	ROP40 gene amplification

10. RNA virus extraction kit.
11. Cup sonicator and general purpose plasticware for cell culture.
12. Class-II biosafety cabinet for all procedures involving virus infections and growth.

2.4 Expression Analysis

1. Acetone-methanol solution (40%/60% vol).
2. Dulbecco's Phosphate buffered saline 1× (PBS 1×).
3. IFA wash solution (PBS-T): PBS 1× with 0.05% Tween-20.
4. IFA blocking solution: PBS-T supplemented with 20% FBS.
5. TBS (Tris-buffered saline): 200 mM Tris-HCl pH 7.6, 1.5 M NaCl.
6. Western blot blocking solution: TBS-T supplemented with 5% skim milk.
7. Western blot wash solution (TBS-T): TBS 1× with 0.05% Tween-20.
8. Anti-V5tag mouse monoclonal antibody.
9. SD6 mouse monoclonal antibody specific to FMDV CS8c1 [15].
10. HRPO-conjugated anti-mouse IgG antibody.
11. Alexa Fluor® 594-conjugated goat anti-mouse IgG (H + L).
12. Alexa Fluor® 488-conjugated goat anti-mouse IgG (H + L).
13. DAPI nuclear staining reagent (i.e., ProLong™ Gold).
14. 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.
15. Nitrocellulose membranes.
16. Electrophoresis and western blotting equipment.

2.5 In Vivo Analysis in Mice

1. BALB/c mice.
2. Sterile insulin syringes (i.e., BD Microfine).
3. System for capillary blood collection (i.e., Sarstedt Microvette).
4. Microfuge.

3 Methods

The following methods describe (1) the general procedure for the generation of recombinant viruses (rRVFVs), in this example carrying *N. caninum* NcROP2 and NcROP40 coding sequences C-terminally tagged as model antigens, (2) the analysis of recombinant protein expression by immunofluorescence assay (IFA) and western blot (WB), (3) the up growth and quantification of virus stocks, and (4) a formal in vitro and in vivo characterization of the recombinant viruses.

3.1 Cloning of Heterologous Genes for Generation of Recombinant RVFVs

pCDNA 3.1 plasmids encoding *N. caninum* sequences NcROP2 (pCDNA 3.1-NcROP2) and NcROP40 (pCDNA 3.1-NcROP40) are obtained through gene synthesis from a commercial supplier. Specific ROP2 and ROP40 gene sequences (NcLIV 001970 and NcLIV 012920, respectively) were retrieved from the ToxoDB database (https://toxodb.org). Restriction sites *XhoI* and *NcoI* were introduced at the 5′ and 3′ ends, respectively. As mentioned previously, the V5 tag and the FMDV Cs8c1 site A (recognized by SD6 mAb) were added at the 3′ end of each ROP sequence (Fig. 1a).

1. Check by transfection the correct expression of the plasmids that carry the heterologous genes of interest (in this example pCDNA 3.1-NcROP2 and pCDNA 3.1-NcROP40). Transfect semi-confluent layers of BHK-21 cells seeded in 96 well-plates with 100 ng of the plasmids using 0.1 μL of Lipofectamine 3000. If possible transfect cells with a plasmid bearing an unrelated antigen as a negative expression control.
2. 24 hours post-transfection fix cells by adding ice-cold 1:1 methanol-acetone solution and incubate for 20 min at −20 °C.
3. Remove methanol-acetone and add blocking solution. Incubate for 1 h at room temperature. Add the primary antibodies specific for the two tags included in the sequence, a mouse anti-V5tag monoclonal antibody and the mAb SD6. The working dilution for each antibody has to be previously determined or may be recommended in the case of a commercial antibody source. Incubate primary antibodies for 1 h at 37 °C or 4 °C overnight.

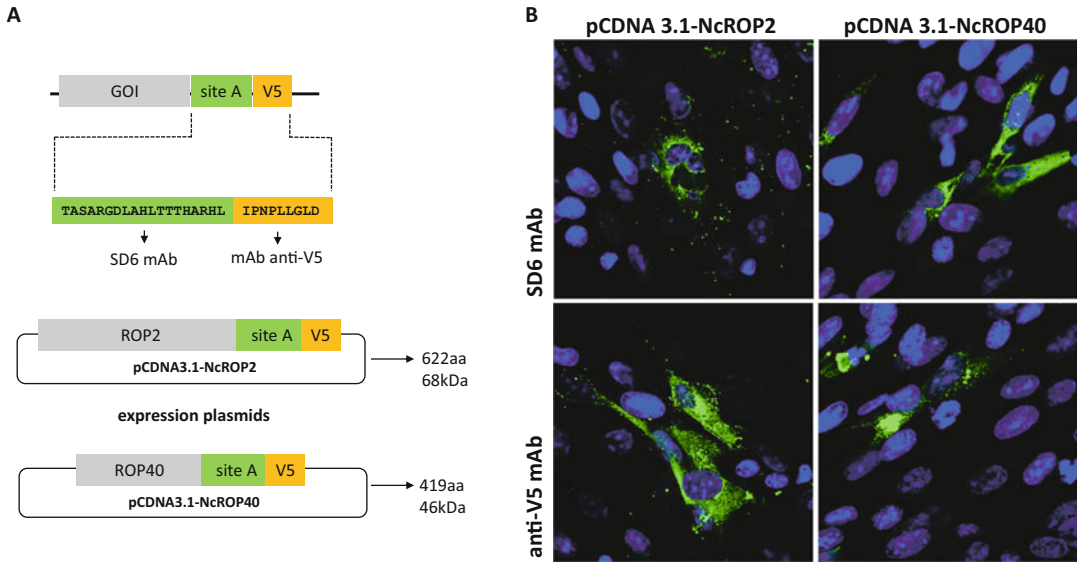

Fig. 1 (**a**) Schematic representation of the C-terminal tags fused to the gene of interest (GOI). Both tags are linear epitopes for mAbs SD6 and anti-V5. Two tagged ROP genes are directionally cloned into the expression plasmid pCDNA3.1. The estimated size and molecular weight for each fusion protein is indicated. (**b**) Indirect immunofluorescence of BHK-21 cells transfected with pCDNA 3.1-NcROP2 and pCDNA 3.1-NcROP40. In the upper panel transfected cells are detected with the SD6 monoclonal antibody while the lower panel shows transfected cells detected with an anti-V5tag monoclonal antibody

4. After incubation with the primary antibody, wash cells three times with PBS-T and incubate for 30 min at room temperature with goat anti-mouse Alexa Fluor 488 followed again by three consecutive PBS-T washes. Add DAPI staining solution to wells.
5. Check expression by immunofluorescence (Fig. 1b) and if everything is correct (*see* **Note 2**) proceed to next step.
6. Digest the plasmids pCDNA 3.1-NcROP2 and pCDNA 3.1-NcROP40 with the restriction enzymes *XhoI* and *NcoI* to extract the corresponding NcROP2 and NcROP40 DNA fragments.
7. Digest plasmid pHH21_RVFV_N_TCS with the restriction enzyme *AarI*. This cut generates compatible cohesive ends for restriction enzymes *XhoI* and *NcoI*.
8. Separate restriction fragments in a TAE-agarose gel. Purify the digested fragment(s) from the gel using a DNA gel recovery kit or similar technique.
9. Ligate the purified *XhoI*/*NcoI* digested, NcROP2 and NcROP40 fragments with the purified *AarI* digested plasmid pHH21_RVFV_N_TCS using T4 DNA 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.

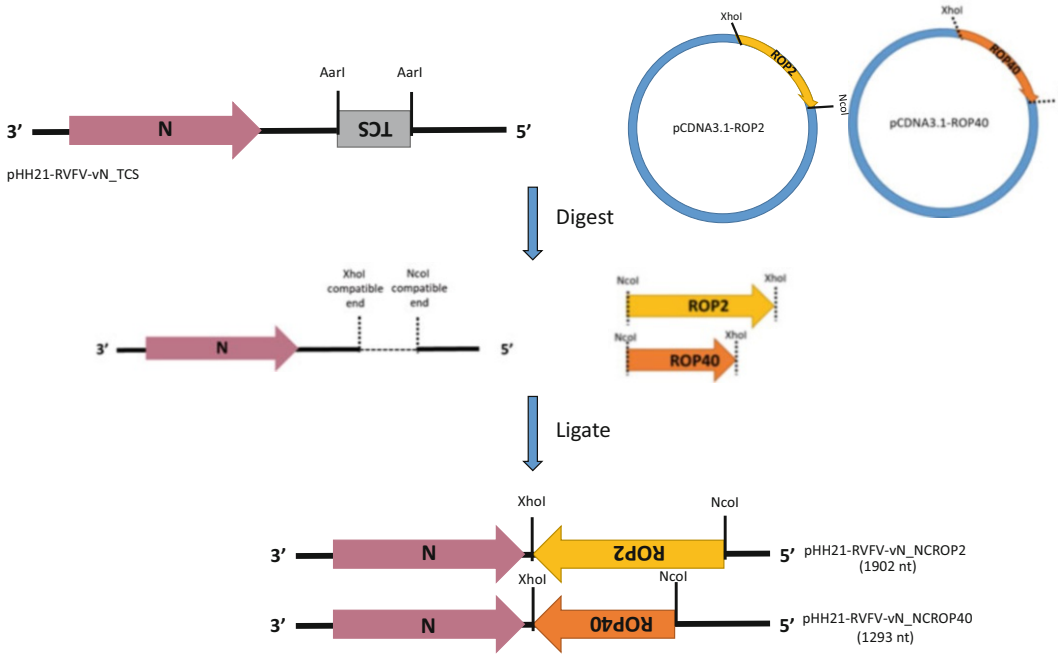

Fig. 2 Generation of plasmids for viral rescue. The plasmid pHH21-RVFV-vN_TCS contains a cloning site (TCS) with two AarI sites for the insertion of heterologous genes. pCDNA 3.1-ROP2 and pCDNA3.1-ROP40 contain *NcROP2* and *NcROP40* sequences flanked by XhoI and NcoI sites for digestion and subsequent insertion into *AarI* linearized pHH21-RVFV-vN_TCS. Both genes are cloned in the opposite orientation with respect to the nucleoprotein gene to maintain the same coding arrangement of a wild-type virus

10. Transform the ligation products into competent bacterial cells and plate out the transformants on LB agar with selection in the presence of ampicillin (100 μg/mL). Incubate at 37 °C overnight.
11. The next day select single colonies, perform a PCR with specific primers to identify positive colonies and then grow selected clones in LB with ampicillin. A midi size plasmid prep should yield enough amounts of purified plasmid for performing the next steps (Fig. 2).

3.2 Generation of Recombinant rRVFV

The next step of the procedure is the rescue of recombinant viruses by means of reverse genetics. To recover recombinant RVFV (rRVFV) an RNA pol I/II-based rescue system was used [2]. The plasmids pHH21 provide templates for the generation of the three RVFV RNA viral segments (in negative sense), under the control of the human RNA pol I promoter sequence. Plasmids pI.18 encode N or L sequences, under transcriptional control of an eukaryotic promoter, for expression of the viral nucleoprotein and RdRp polymerase, which, in turn, allow the packaging and replication of the viral genomic RNAs.

3.2.1 Transfection of HEK-293 and BHK-21 Cells with pHH21 and pI.18 Plasmids

1. Seed HEK-293 and BHK-21 cells in a 1:1 ratio in six-well plates (*see* **Note 3**).
2. Transfect semi-confluent layers of co-cultured cells with 1 μg each of pHH21-RVFV-vL, pHH21-RVFV-vM (and either pHH21-RVFV-vN_GFP or pHH21-RVFV-vN-ROP2 or pHH21-RVFV-vN-ROP40), and with 0.5 μg of pI.18-RVFV-L and pI.18-RVFV-N using 13.5 μL of Lipofectamine 3000 (Fig. 3a) (*see* **Note 4**).
3. Harvest the supernatants of transfected cells on day 3, 5, and 7 post-transfection and add fresh medium (*see* **Note 5**).
4. Screen for the presence of recombinant virus by inoculating Vero cells, grown in six-well plates, with 500 μL of each supernatant from transfections.
5. Check for cytopathic effect (CPE) to identify the presence of recombinant virus infection. If CPE is not evident, an immunofluorescence assay with specific anti-RVFV antibodies can be used to verify the presence of virus. As a positive control it is convenient to rescue a rZH548 virus encoding GFP.
6. If CPE is confirmed in **step 5**, propagate rescued virus in Vero cell cultures. Amplify the virus starting from a T25 flask. Inoculate with 500 μL of supernatants from the cultures from **step 5** wait until CPE is evident remove part of the medium, 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.
7. Sonicate the tube in a water bath following three cycles of 30 s, aliquot the virus in small volumes. We usually prepare aliquots of 0.5 and 1 mL each. Store at −80 °C.

3.2.2 Plaque Purification of Recombinant Viruses and Working Stock Preparation

This step is necessary to ensure that the viral stock is homogeneous. The rescued virus should be plaque purified twice and only then a viral stock should be prepared.

1. Seed six-well plates with Vero cells and infect them with serial dilutions starting from dilution 10^{-2}.
2. After 1 h of adsorption, remove the medium and add fresh complete DMEM.
3. Incubate at 37 °C for 72 h.
4. Remove the medium and add DMEM with 0.6% of agar over the monolayer.
5. Allow the overlay to solidify and add 1 mL per well of 0.01% neutral red in PBS 1×.
6. Incubate cells for 1 h at 37 °C (*see* **Note 6**).
7. Remove neutral red and incubate for 30 additional minutes at 37 °C.

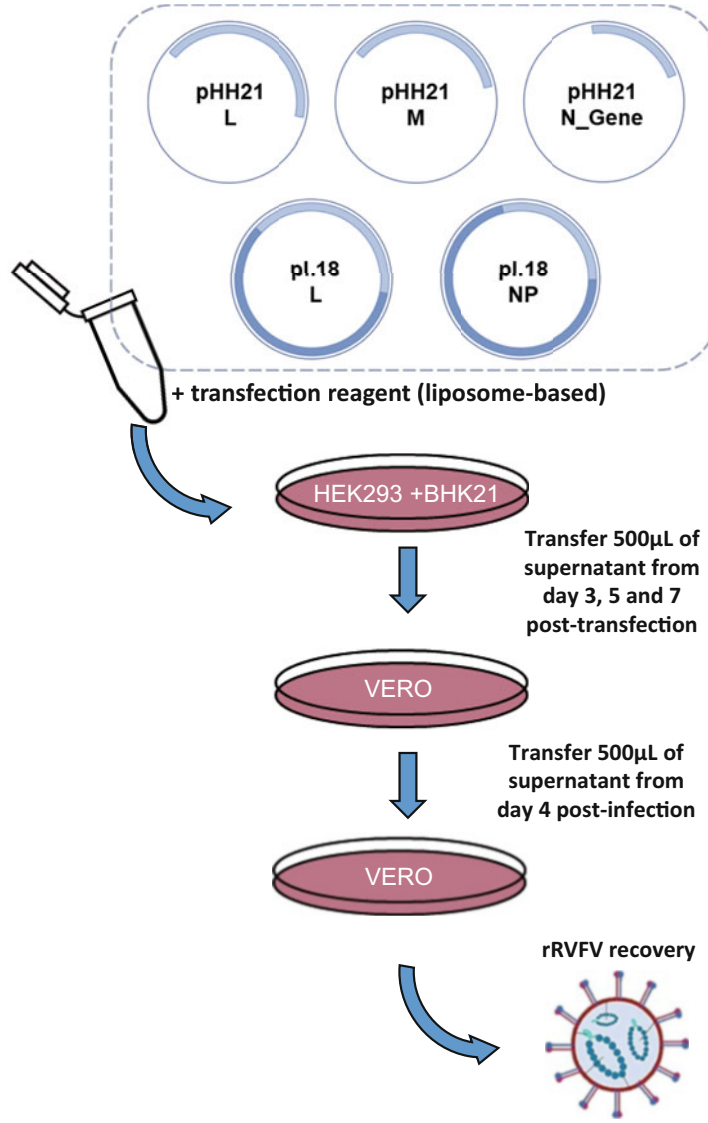

Fig. 3 Schematics of recombinant rRVFV generation. A co-culture of HEK293 and BHK-21 cells is transfected with a mixture of 5 plasmids containing sequences from the three viral genomic segments (*L*, *M* and recombinant *S [N_Gene of interest]*) and the nucleocapsid protein (NP) and viral polymerase RdRp (L) coding sequences. Post-transfection supernatants are collected at different times and used for blind serial passages on Vero cell cultures until CPE appears

8. Pick only well-separated plaques, about 5–6 per clone. Insert a 1 mL pipette tip into the agar overlay right over the plaque, aspirate with the pipette so that the agar plugs into the tip.
9. Transfer to a tube with 500 µL of complete DMEM and make sure the plug leaves the pipette tip.

10. Carry out three cycles of thawing/freezing and sonicate for 1 min.
11. Check the selected clones through IFA and PCR. Positive clones are subjected to a new plaque purification cycle. Repeat this cloning procedure two or three times to get a highly purified clone.
12. Finally, grow positive clones in Vero cells to generate the working stock. Inoculate a T25 flask with 500 μL of supernatants from selected positive clones. When CPE is evident remove part of the medium, 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.
13. Titrate the virus obtained in **step 15** and then infect a T75 flask at MOI of 0.1. When CPE is evident proceed as in **step 15**.
14. Sonicate the tube in a water bath following three cycles of 30 s, aliquot the virus in small volumes. We usually prepare aliquots of 0.5 and 1 mL each. Store at −80 °C.

3.3 Protein Expression Analysis

Once the viruses are generated it is necessary to check whether they express or not the heterologous protein and if the expression level are adequate. This step also helps to identify if the viral population is homogeneous or, on the contrary, it is a mixed population that needs to be plaque purified as explained in Subheading 3.2.2. Protein expression can be analyzed by immunofluorescence or western blot.

3.3.1 Immunofluorescence Assay

1. Infect Vero cells with each recombinant virus at a MOI of 1.
2. 48 h post-infection fix cells with ice-cold 1:1 methanol-acetone solution and incubate for 20 min at −20 °C.
3. Remove methanol-acetone and add blocking solution. Incubate for 1 h at room temperature. Add the primary antibody (diluted 1:200 in PBS-FCS 20% for serum and 1:500 for monoclonal antibody).
4. Incubate primary antibodies for 1 h at 37 °C or 4 °C overnight.
5. After incubation with the primary antibody, wash cells three times with PBS-T and incubate for 30 min at room temperature with goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594, followed again by three PBS-T washes.

3.3.2 Western Blot Analysis

1. Infect monolayers of Vero cells with rZH548-ΔNSs::NcROP2 or rZH548-ΔNSs::NcROP40.
2. After 24 h, wash cells twice with PBS and lyse in SDS-PAGE sample buffer.
3. Analyze samples by SDS-PAGE in reducing conditions.

4. Transfer proteins to a nitrocellulose membrane.
5. Incubate the membrane for 1 h with 5% skimmed milk in Tris-buffered saline (TBS).
6. Upon blocking, add the primary anti-V5 epitope mAb or an anti-RVFV-N mAb diluted 1:1000 in 5% milk-TBS-T (TBS with 0.05% Tween-20) and incubate for 1 h at RT with shaking or at 4 °C overnight.
7. Wash the membrane three times with TBS-T and incubate for 1 h RT with an anti-mouse IgG-HRPO conjugated antibody.
8. Wash the membrane again three times with TBS-T before adding luminescent substrate.
9. Visualize the results using an appropriate imaging System (Fig. 4).

3.4 Phenotypic Characterization of the Recombinant Viruses

A phenotypic characterization of the rescued viruses is important since they had the NSs gene removed and a heterologous protein is expressed instead. It is also important to study their growth kinetics to see if the insertion of the protein has any deleterious/negative effect on the ability of the virus to grow in high titers. The absence of NSs expression usually results in viruses with more diffuse (less cytolytic) lysis plaques but sometimes the insertion of a heterologous protein alters this phenotype again, sometimes they become clearer and some other times they become even more diffuse indicating differences in viral yields.

3.4.1 Growth Curve

1. Seed 12-well plates with Vero or HEK-293 cells and infect them with the rRVFVs at a MOI of 0.01 (*see* **Note 7**).
2. Collect supernatants at 24, 48, and 72 hpi.
3. To determine viral titers by plaque assay, infect 12-well plates with tenfold serial dilutions of the harvested supernatants.
4. Incubate for 1 h at 37 °C. Remove the inoculum, wash cells with medium and add fresh semi-solid DMEM-1% CMC medium.
5. Incubate for the appropriate amount of time at 37 °C (*see* **Note 8**).
6. Fix cells with 10% formaldehyde and stain with 2% crystal violet.
7. Calculate titers from the plaque numbers obtained for each dilution.

3.5 Analysis of Stability in Cell Culture

It is important to monitor the stability of the recombinant viruses upon serial passages in cell culture. The nature of the transgene will condition the viability of the recombinant virus and their ability to propagate in cell culture up to certain number of passages. This can

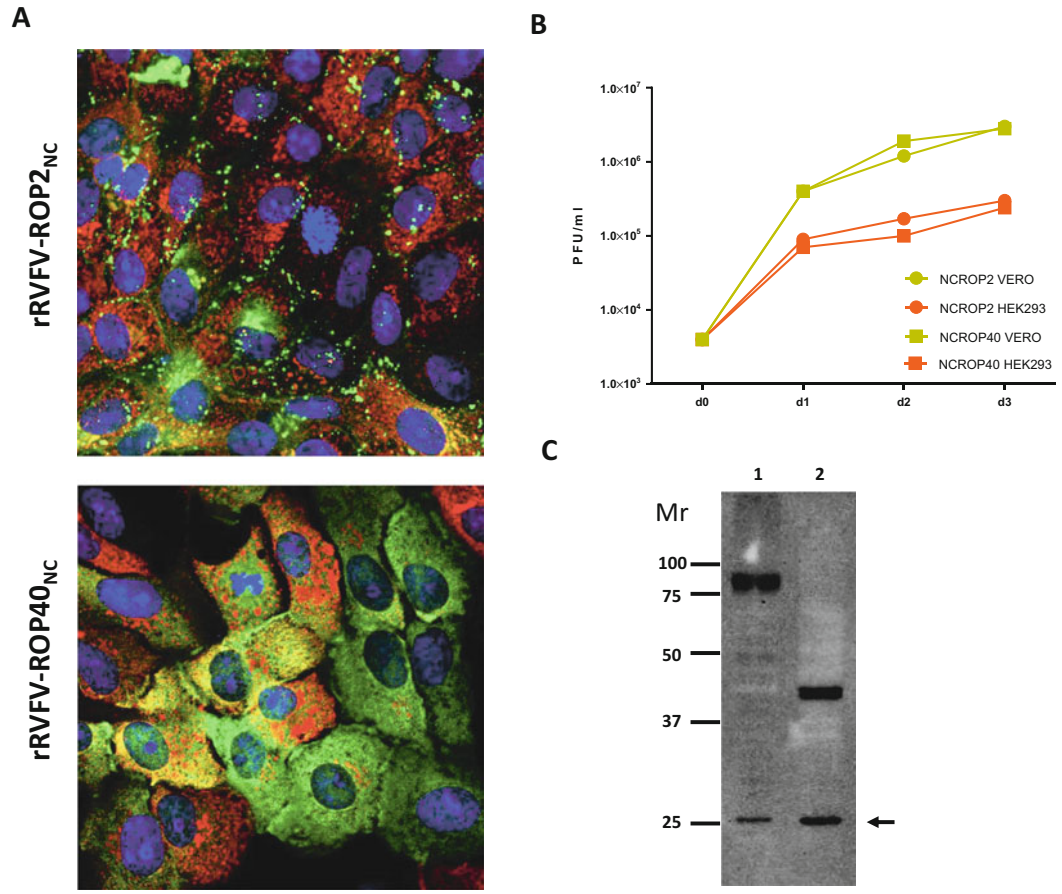

Fig. 4 (**a**) Indirect immunofluorescence analysis of rRVFV infected Vero cells (merged images). A rabbit anti-RVFV polyclonal serum was used for the detection of RVFV antigens (red fluorescence). A mouse anti-V5tag monoclonal antibody was used to detect the expression of either NcROP2 or NcROP40 (green fluorescence). DAPI staining was used to visualize cell nuclei. (**b**) Growth kinetics of rRVFVs. Vero and HEK293 cells were infected with a MOI of 0.01 and supernatants were harvested at the indicated time points. Viral titers were determined after infection of Vero cells with serially diluted supernatants. (**c**) Western blot analysis of rZH548-ΔNSs::NcROP2 (lane 1) and rZH548-ΔNSs::NcROP40 (lane 2) infected Vero cell extracts. An anti-V5tag mAb was used for detection of NcROP2 and NcROP40 expression. As a control of productive RVFV infection, nucleoprotein N (arrow) was detected using 2B1 mAb. Mr.: relative molecular mass in kDa

be checked at the phenotypic level (monitoring the transgene expression) (Fig. 5) or also at the genetic level (detecting the presence of the transgene by RT-PCR) (*see* **Note 9**).

3.5.1 Phenotypic Stability

1. Seed Vero cells in a T25 flask and infect them with your viral stock at a MOI of ≤0.1.
2. 72 hours later (when CPE is extensive) collect the monolayer and most of the supernatant. Immediately disrupt the cells with three cycles of thawing/freezing at −80 °C. Combine the rest of the supernatant and the disrupted cells in a new tube.

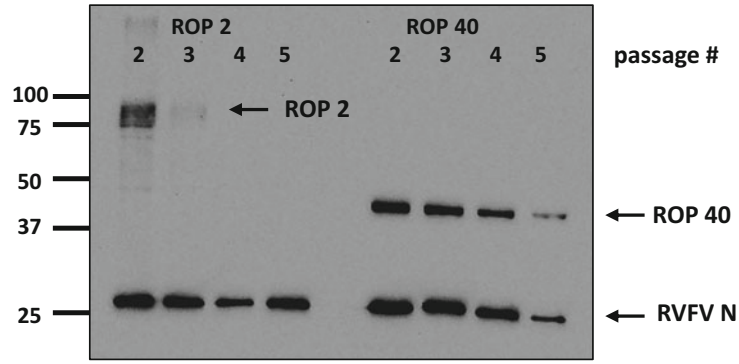

Fig. 5 Detection of NcROP2 and NcROP40 transgenes by western blot of extracts from Vero cells infected with serially passaged (p2 to p5) recombinant RVF viruses. The anti-V5tag mAb was used to detect the expression of both tagged NcROP2 and NcROP40. As a control of productive RVFV infection, nucleoprotein N was detected using 2B1 mAb. Note that in this experiment ROP2 signal waned after the third serial passage. Mr.: relative molecular mass in kDa

3. Titrate this passage and repeat **steps 1** and **2**.
4. **Steps 1–3** must be repeated until reaching at least a fourth passage.
5. Using the first, second, third, and fourth serial passages of each recombinant virus infect Vero cell monolayers using a MOI $\geq$1.
6. 24 hours later collect and clarify the supernatants using maximum speed centrifugation and save for use in Subheading 3.5.2 **step 1**. Collect remaining cells in PBS and lyse in SDS-PAGE sample buffer.
7. Proceed as explained in Subheading 3.3.2.

3.5.2 Genetic Stability

1. For this analysis we can use the supernatants collected in Subheading 3.5.1 **step 6**.
2. Extract RNA from those supernatants using a RNA virus extraction kit.
3. Use Superscript IV (or similar retrotranscriptase) for reverse transcription of RNA purified from infection clarified supernatants. For cDNA generation use a genomic S segment specific primer (*see* Table 1).
4. Amplify the resulting cDNA from **step 3** by PCR using primer pairs for both the nucleoprotein N gene and the heterologous gene (*see* Table 1).

3.6 Analysis of Immunogenicity In Vivo

Ideally the immune responses elicited upon inoculation should be checked using the adequate target host. The type of immunological analysis to perform should to be adapted according to the nature of the recombinant antigens and/or the correlates of immunity

observed upon natural infection, if known. Since in most parasitic infections T-cell responses (both CD8 and CD4) play an essential role to control chronic infections [17], their specific activation needs to be assessed to evaluate the performance of a vaccine. However, cellular immunological assays are complex and time consuming so, ideally, they should be carried out once confirmed that the in vivo immunization worked properly. In our example, the simplest way to confirm a successful immunization is to check whether the recombinant viruses induced an immune response against the inserted FMDV epitope tag. To do so, BALB/c mice are inoculated with both rRVFV. Three weeks later blood samples are retrieved to evaluate the presence of serum anti-FMDV antibodies by an immunofluorescence assay on FMDV infected cells.

1. Inoculate intraperitoneally BALB/c mice with 10^5 pfu of the viruses (rZH548-ΔNSs::NcROP2 and rZH548-ΔNSs::NcROP40).
2. Three weeks later collect blood from the submandibular plexus (or any other IACUC approved method) of mice in a blood collection tube.
3. Maintain blood at 37 °C for 1 h to speed the clotting process and then centrifuge the tubes at 4 °C for 10 min at 8000 rpm in a microfuge (*see* **Note 10**).
4. Collect the serum in a clean tube and discard the pellet.
5. Infect BHK-21 cells with FMDV.
6. The following day (after an overnight incubation) fix the cells with methanol-acetone solution and proceed as in Subheading 3.3.1. Use mice sera collected in **step 5** to detect FMDV infected BHK-21 cells. Start in a dilution 1:50 and then proceed with twofold dilutions. Figure 6 show the results of a IFA cell staining with sera from mice inoculated with rRVFV on FMDV infected cells.

4 Notes

1. Different RVFV reverse genetic systems may be available from other laboratories. Alternatively, these can be developed for a particular virus strain, using gene synthesis services, according to published methodology.
2. It is advisable to check if the plasmids correctly express the antigens before starting the whole protocol. The easiest way to do this is by transfecting BHK-21 or HEK-293 cells with the expression plasmids and carrying out an immunofluorescence assay. We found that rarely happens that cells seem to not express the protein of interest when transfected, but then the

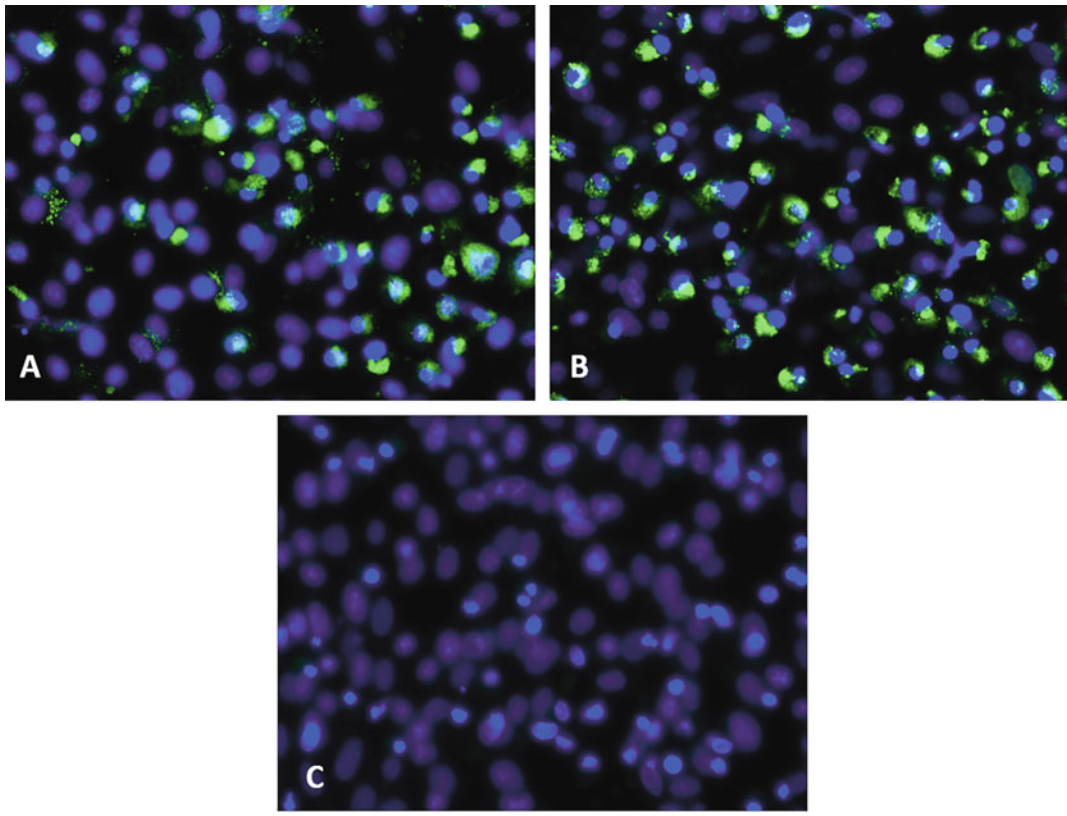

Fig. 6 Indirect immunofluorescence analysis of BHK-21 cells infected with FMDV isolate CS8c1. Infected cells were incubated with sera from mice vaccinated with 10^5 pfu of rZH548-ΔNSs::NcROP2 (**a**) or rZH548-ΔNSs::NcROP40 (**b**). A mock-infected cell culture is shown as a control (**c**)

rescued viruses do express it. So in our experience, it is worth trying to rescue the virus even if the plasmids don't seem to express the protein, provided the sequence of the transgene is correct.

3. Cells should be at a confluence of 80% at the moment of starting the rescue experiment. A higher cell confluency may lead to cell death. Cells should be kept in 5% FBS-DMEM after transfection so that cells do not overgrow.
4. We have tested different transfection agents and we strongly recommend using Lipofectamine 3000 or a similar liposome-based reagent. Among different cheaper possibilities we tried, for example, polyethylenimine (PEI). In our hands this reagent did not work as efficiently for virus rescue, maybe because it causes too much damage to cells reducing viability and affecting virus production.
5. Be careful when collecting cell supernatants and when adding fresh DMEM. It is important not to disturb the cell monolayers since this could affect the virus generation.

6. Try not to exceed the staining time since neutral red is mutagenic.
7. Since the recombinant viruses lack NSs expression is very interesting to check their growth kinetics both in an interferon deficient (Vero) and interferon competent cell lines. We use HEK-293 but A549 cells could be used as well.
8. The incubation time might vary between recombinant viruses, from 72 h to 5 days. 72 hours is the standard but some viruses require more time to produce CPE. It is advisable to check the size of the plaques before fixing the plate the first time you do this assay with a virus.
9. For a proper characterization of the genomic structure of the recombinant viruses a Northern blot analysis using RNA extracted from recombinant virus particles should be performed in comparison with control/parental virus. Specific labeling probes for each viral RNA segment would allow to confirm the expected genomic sizes of the RNA along the different number of serial passages to be analyzed. However since this is a much more laborious method we recommend first to perform an RT-PCR assay.
10. The incubation at 37 °C helps with the clotting process but it is not necessary if using the system for capillary blood collection aforementioned in Subheading 2. On the contrary, if that system is not used, after the incubation step at 37 °C it is advisable to keep the samples refrigerated for at least 1–2 h before proceeding with the centrifugation step. This allows the retraction of the coagula, thus maximizing the recovery of serum. To avoid hemolysis do not maintain the blood samples refrigerated more than 24 h.

Acknowledgements

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Chapter 13

Generation and Characterization of Single-Cycle Infectious Canine Influenza A Virus (sciCIV) and Its Use as Vaccine Platform

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Abstract

Influenza A viruses (IAVs) infect a broad range of hosts, including multiple avian and mammalian species. The frequent emergence of novel IAV strains in different hosts, including in humans, results in the need for vigilance and ongoing development of new approaches to fighting or prevent those infections. Canine influenza is a contagious respiratory disease in dogs caused by two subtypes of IAV, the equine-origin H3N8 canine influenza virus (CIV), and the avian-origin H3N2 CIV. A novel approach to influenza vaccination involves single-cycle infectious influenza A viruses (sciIAVs), which are defective for an essential viral gene. They are propagated in complementing cell lines which provide the missing gene in trans. As sciIAV cannot complete their replication cycle in regular cells they are limited to a single round of viral replication. Because of their safety profile and ability to express foreign antigens inside infected cells, sciIAVs have served both as live-attenuated vaccines and as vaccine vectors for the expression of heterologous antigens. Here, we describe experimental procedures for the generation of a single-cycle infectious CIV (sciCIV), where the viral hemagglutinin (HA) gene was exchanged for the gene for green fluorescent protein (GFP). Complementation of the viral HA protein is provided in trans by stable HA-expressing cell lines. Methods for the in vitro characterization of HA deficient but GFP-expressing sciCIV (sciCIV ΔHA/GFP) are described, as well as its use as a potential vaccine.

Key words Reporter virus, Viral vectors, Single-cycle influenza virus, Influenza vaccine, Influenza HA-expressing MDCK cells (MDCK-HA), Reverse genetics, Reporter gene, Canine influenza virus, Single-cycle infectious influenza A virus, Green fluorescent protein, Humoral response

1 Introduction

Influenza A viruses (IAV) belong to the *Orthomyxoviridae* family and are enveloped viruses which contain genome formed by eight single-stranded negative-sense RNA segments [1]. The eight RNA segments of IAV encode at least 12 proteins, using several mechanisms of protein expression, such as alternative open reading frames

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(ORF), alternative splicing or ribosomal frame-shifting [1–3]. Two major surface glycoproteins are located in the viral envelope: hemagglutinin (HA), which mediates receptor binding and fusion events; and neuraminidase (NA), which mediates nascent virion release from infected cells [1, 4, 5]. The antigenic diversity of HA and NA glycoproteins is used to classify IAV, of which there are 18 HA and 11 NA antigenic variants or subtypes [4–7].

IAV can infect a broad range of animal hosts, including multiple avian and mammalian species [5, 8, 9]. Canine influenza is a contagious respiratory disease of dogs caused by two subtypes of canine influenza virus (CIV). CIV H3N8 originated around 1999, due to the transfer of H3N8 equine influenza virus to dogs, while CIV H3N2 is an avian-origin virus that likely adapted directly to infect dogs [8, 10, 11]. Because of the wide distribution of dogs and their close association with humans, it has been suggested that dogs could act as intermediate or "mixing vessel" hosts for genetic reassortment of IAV, and therefore CIV pose a zoonotic risk with pandemic-potential [5, 11–13].

Vaccination is the most effective strategy for the prevention of IAV infections, and to date only inactivated influenza vaccines (IIVs) have been commercially available to prevent CIV H3N8 or H3N2 infections. However, several live-attenuated influenza vaccines (LAIV) prototypes have been reported [14–18]. Notably, single-cycle infectious influenza A viruses (sciIAV) have gained considerable attention in the last years as potential vaccines, or vaccine vectors, as alternatives to the use of IIV or LAIV. sciIAV combine the safety of IIV and immunogenicity of LAIV, while avoiding concerns about possible reduced immunogenicity of IIV or safety of LAIV [19–25]. Moreover, sciIAV have been successfully used as vaccine candidates in several animal models, where they induce protective humoral and cellular immune responses [22, 23, 25].

SciIAVs are defective for one or more essential functions due to the removal of key viral genes from the viral genome. These genes may include those involved in viral entry, replication, assembly or in the release of new viral particles, among others. The missing viral gene or its function is typically provided in trans using complementing cell lines to allow sciIAV propagation, while no infectious viral progeny can be produced [25–29]. Therefore, with a variety of essential genes that may be deleted, the generation of sciIAV can be accomplished using multiple approaches [25, 26]. Moreover, the deleted viral gene(s) can be replaced by other foreign sequences, including antigens from other pathogens or reporter genes for the use of sciIAV as vaccine vectors or for tracking viral infections, respectively [21, 22, 25, 30–32]. Therefore, sciIAV can be used as valuable tools for studying IAV infections or for the identification of antivirals or neutralizing antibodies [24, 25, 29].

Here we describe approaches for using reverse genetics techniques for the generation and characterization of a single-cycle infectious canine influenza virus (sciCIV) [7, 33–35]. In this approach the viral segment encoding CIV HA was genetically modified to express the green fluorescent protein (GFP). The sciCIV was propagated in MDCK cells stably expressing CIV HA, and replication of the sciCIV was limited to HA-expressing MDCK cells and was easily tracked by GFP expression. Importantly, using a mouse model of infection, we demonstrate that the HA deficient sciCIV, which was pseudotyped with CIV HA, was safe and immunogenic, and a single intranasal dose was able to confer protection after challenge with a wild-type (WT) CIV, suggesting the feasibility of using this sciCIV as a vaccine to prevent CIV infection.

2 Materials

2.1 Viruses

This protocol is described for the rescue of sciCIV ΔHA/GFP based on the backbone of influenza A/canine/NY/dog23/2009 H3N8, which can be performed under BSL-2 laboratory conditions (*see* **Note 1**). All material used with infectious wild-type (WT) virus should be fully decontaminated before disposal, following the appropriate institutional biosafety committee (IBC) recommendations.

1. PR8 sciIAV ΔHA/GFP: This virus was generated in the backbone of influenza A/Puerto Rico/8/1934 H1N1 (PR8) and HA-pseudotyped using Madin-Darby canine kidney (MDCK) PR8 HA-expressing cells [22, 27, 29], and it is used in this protocol as a positive control.
2. Influenza A/canine/NY/dog23/2009 H3N8 WT: This virus was used in vivo as control to evaluate protection efficacy after vaccination with sciCIV.
3. Influenza A/canine/NY/dog23/2009 H3N8 reverse genetics plasmids [36]. The pDZ ambisense plasmids [37] containing influenza A/canine/NY/dog23/2009 H3N8 genes driving expression of influenza viral RNAs based on the use of human polymerase I (Pol-I) promoter have been previously described [36]. Activity of Pol-I promoters has been shown to be species specific [7, 35, 38, 39] and, therefore, these plasmids require the use of a human cell line.
4. pPolI HA(45)GFP(80) plasmid to generate sciCIV have been previously described [27].

2.2 Tissue Culture Media and Reagents

1. Dulbecco's Modified Eagle's Medium (DMEM).
2. Fetal bovine serum (FBS).
3. 35% Bovine Serum Albumin (BSA).

4. Penicillin/Streptomycin (100 μg/mL)-2 mM L-Glutamine (PSG).
5. Trypsin–EDTA.
6. Phosphate buffered saline (PBS) 1×: To prepare PBS 10×, mix 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4, 2.4 g of KH_2PO_4. Adjust pH to 7.4. Add ddH_2O up to 1 L. To prepare PBS 1×, dilute 1:10 PBS 10× with ddH_2O. PBS 10× and 1× need to be sterilized by autoclaving and can be stored at room temperature.
7. Transfection reagent, i.e., Lipofectamine 2000 (LPF2000; Invitrogen).
8. OptiMEM I medium (Invitrogen).
9. Tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma).
10. Hygromycin B.
11. Cloning rings.
12. Double-distilled water (ddH_2O).
13. DMEM 10% FBS, 1% PSG: This media is used for maintenance of cell lines. Mix 445 mL DMEM, 50 mL of heat inactivated FBS, and 5 mL of 100× PSG. Store at 4 °C. Where indicated, media was supplemented with 200 μg/mL of Hygromycin B for selection.
14. DMEM 0.3% BSA, 1% PSG: This post-infection media is used after viral infections. Mix 490.7 mL of DMEM, 4.3 mL of 35% BSA, and 5 mL of PSG. Store at 4 °C. Just before use, add TPCK-treated trypsin to a final concentration of 1 μg/mL (1:1000 dilution of the TPCK-treated trypsin stock at 1 mg/mL).
15. PBS 1×, 0.3% BSA, 1% PSG: This media is used for viral dilutions and during viral infections. Remove 13 mL from a 500 mL sterile PBS 1× bottle. Add 5 mL of 100× PSG and 3 mL of 35% BSA. Mix and add 5 mL of CaMg 100× for a total of 500 mL. Make 100 mL aliquots and store at 4 °C.
16. CaMg 100×: Mix 1.327 g of $CaCl_2{\cdot}2H_2O$ with 2.133 g of $MgCl_2{\cdot}6H_2O$. Add ddH_2O up to 100 mL. Sterilize by autoclaving and store at room temperature.
17. Crystal violet: Mix 20 mL of methanol and 80 mL of ddH_2O. Then, dissolve 0.5 g of crystal violet.
18. Laboratory equipment: Centrifuge, hemocytometer or automatic cell counter, laboratory 5% CO_2 tissue culture humidified incubators at 37 °C, cell culture plasticware.

2.3 Mammalian Cell Lines

1. Human embryonic kidney 293T (HEK293T) cells (American Type Culture Collection, ATCC, CRL-11268) (*see* **Note 2**). HEK293T cells have been shown to have high transfection

efficiency and thus, optimal for initial amplification of IAV vRNAs from ambisense plasmids [7, 33–35]. Although highly transfectable, HEK293T cells appear not to be ideal for producing infectious virus stocks.

2. MDCK cells (ATCC CCL-34) (*see* **Note 2**): This cell line is less efficient for transfection than HEK293T cells. However, MDCK cells produce higher levels of influenza virus replication for producing high viral titers [40].
3. MDCK cells constitutively expressing influenza HA from influenza PR8 (MDCK PR8-HA) (*see* **Note 2**): This cell line was previously described [22, 27, 29].
4. MDCK cells expressing the HA protein from influenza A/canine/NY/dog23/2009 H3N8 strain, were generated as described in Subheading 3.2.

All cell lines are maintained with DMEM 10% FBS, 1% PSG at 37 °C in a 5% CO_2 atmosphere tissue culture humidified incubator. MDCK HA-expressing cell lines were maintained with 200 μg/mL of Hygromycin B. For virus rescue and amplification, cells are maintained in DMEM 0.3% BSA, 1% PSG with 1 μg/mL of TPCK-treated trypsin at 37 °C in the 5% CO_2 atmosphere tissue culture humidified incubator.

2.4 Cloning A/canine/NY/dog23/2009 H3N8 HA

1. PCR reagents (i.e., Expand™ High Fidelity PCR System from Roche).
2. dNTPs.
3. Specific primers to amplify A/canine/NY/dog23/2009 H3N8 HA: CIV HA/ClaI/F: 5′-AATT ATCGATAAC*ATGAAAACAACCATTATTTTAATACT-3′* and CIV HA/XhoI/R: 5′-AATTCTCGAG*TCAAATGC AAATGTT GCATCT -3′*. Restriction sites are underlined and CIV HA sequences are indicated in cursive.
4. ClaI and XhoI restriction enzymes.
5. Shrimp Alkaline Phosphatase (SAP).
6. T4 DNA Ligase.
7. ddH_2O.
8. Bacterial competent cells (i.e., DH5α, Invitrogen).
9. Luria-Broth (LB) liquid media: To prepare LB liquid media, mix 10 g of Bacto Tryptone, 5 g Bacto Yeast extract, and 10 g NaCl, and adjust the volume of the solution to 1 L with ddH_2O. Autoclave and store at 4 °C. For growing DH5α competent cells transformed, add ampicillin (final concentration 100 μg/mL) to LB media.

10. LB agar plates: Prepare 500 mL LB liquid media and, just before autoclaving, add 15 g/L of Bacto Agar. After autoclaving, equilibrate at 55 °C for 30 min and add ampicillin (final concentration 100 μg/mL). Add 20–25 mL to Petri dishes. Store LB agar plates at 4 °C.
11. Ampicillin powder.
12. PCR cleanup system (i.e., Promega's Wizard SV Gel).
13. SeaKem LE agarose (Lonza).
14. SOC medium.
15. Laboratory equipment: Microcentrifuge, heat block, an electrophoresis system, a power supply, NanoDrop or similar spectrophotometer, microbiological incubator.

2.5 Components for Immunofluorescence-Assay

1. 10% Formaldehyde.
2. Triton X-100.
3. BSA.
4. PBS 1×.
5. Monoclonal antibody HB-65 against IAV nucleoprotein, NP (ATCC: H16-L10-4R5).
6. Monoclonal antibody PY102 against IAV PR8 HA [41].
7. Polyclonal antibody NR-3103 against A/canine/NY/dog23/2009 H3N8 HA (BEI Resources, NR-3103) [15] (*see* **Note 3**).
8. Anti-mouse or anti-rabbit IgG-FITC conjugate.
9. DAPI (4′,6-diamidino-2-phenylindole).
10. Fix and permeabilization solution: Mix 400 mL of 10% formaldehyde, 5 mL of Triton X-100, and 595 mL of ddH_2O. This solution is used to fixation and permeabilization of MDCK cells in one step.
11. Blocking solution: Dissolve 25 g of BSA in 1 L of PBS 1×.
12. Antibody dilution solution (PBS 1% BSA): Dissolve 1 g of BSA in 99 mL of PBS 1×. Dilute the primary or secondary antibody at 1 μg/mL (HB65 and PY102) and 1:1000 (NR3103), respectively; and the secondary antibody 1:200.
13. Fluorescence microscope.

2.6 SDS-PAGE and Immunoblotting Reagents

1. SDS polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGEs are prepared by using the protocol described by Laemmli [42] or can be purchased from multiple suppliers.
2. Nitrocellulose membranes (i.e., Whatman®).
3. SDS-PAGE running buffer pH 8.3: 125 mM Tris-HCl, 960 mM Glycine, 0.5% SDS.

4. Transfer buffer: 20% methanol (v/v), 24 mM Tris-HCl, 194 mM Glycine.
5. PBS 1×.
6. Blocking and antibody dilution solutions: 5% (w/v) fat-free milk in PBS 1× containing 0.1% of Tween 20 (PBS-T).
7. Western blotting detection reagent: (i.e., ECL™ GE healthcare).
8. Monoclonal antibody PY102 against IAV HA [41].
9. Polyclonal antibody NR-3103 against A/canine/NY/dog23/2009 H3N8 HA (BEI Resources, NR3103) [15].
10. Monoclonal antibody anti-actin.
11. Anti-mouse or anti-rabbit IgG horseradish peroxidase (HRP) linked whole antibody.
12. RIPA buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS).
13. 4× Laemmli's sample buffer.
14. Laboratory equipment: Microcentrifuge, heat block, an electrophoresis system, a power supply, Western blot imaging system.

2.7 Mouse Immunization and ELISA Components

1. C57BL/6 mice (*see* **Note 4**).
2. Anesthetic solution: 2,2,2-tribromoethanol (TBE). Animals are inoculated with 250 mg/kg of (*see* **Note 5**).
3. 4 mm lancet [43, 44] for mouse bleeding by submandibular puncture (*see* **Note 5**).
4. Mouse surgical equipment.
5. PBS 1×.
6. ELISA Microtiter plates.
7. BSA.
8. Mouse sera collected from immunized animals.
9. Anti-rabbit IgG horseradish peroxidase linked whole antibody.
10. Tetramethylbenzidine (TMB) substrate.
11. 0.2 N H_2SO_4. To prepare 1000 mL of a 0.2 N solution of H_2SO_4, dilute 5.56 mL of 96% H_2SO_4 to a final volume with ddH_2O.
12. Blocking and antibody dilution solutions: These solutions are prepared as indicated in Subheading 2.5.
13. Laboratory equipment: ELISA microplate reader, Mineralight UV lamp UV S-68 (Ultra-Violet Products) or similar.

3 Methods

3.1 Cloning of CIV H3N8 HA in pCAGGS

1. For the PCR amplification of CIV H3N8 HA, we use the Expand High Fidelity PCR system (Roche) (*see* **Note 6**). Primers used in this protocol are indicated in Subheading 2.4. Forward and reverse primers contain sequences complementary to CIV HA ORF together with restriction sites (ClaI and XhoI) for cloning purposes in the plasmid pCAGGS (*see* **Note 7**).
2. Prepare the PCR mix (50 μL reaction volume) and perform the PCR reaction as indicated in Table 1.
3. Separate the PCR products by standard agarose gel electrophoresis, remove an agarose slice containing the CIV H3N8 amplified product, and use a gel extraction kit to isolate the DNA from the agarose slice.
4. Quantify the nucleic acid concentration of the PCR product using a NanoDrop or similar spectrophotometer. The amplified PCR product can be stored at 4 °C or −20 °C until further use.

Table 1
PCR conditions for the amplification of CIV H3N8 HA

PCR mix	
Template (*see* **Note 8**)	2 μL (100—200 ng)
dNTPs (10 mM)	2 μL
Forward primer (10 μM)	2.5 μL
Reverse primer (10 μM)	2.5 μL
10× reaction buffer	5 μL
$MgCl_2$ (25 mM)	6 μL
High fidelity polymerase	0.75 μL
ddH_2O	29.25 μL
PCR cycles	
Number of cycles	Temperature/time
1	94 °C/2 min
30	94 °C/30 s 50 °C/30 s 72 °C/2 min
1	72 °C/10 min

5. Digest the PCR product (0.5–1 μg), and the pCAGGS plasmid (2 μg) by adding 2 μL of the indicated restriction enzymes (ClaI and XhoI) and 5 μL of the 10× recommended restriction buffer in a 50 μL reaction. Incubate the reaction mix at 37 °C for 2–4 h.
6. Identify the digested products by agarose gel electrophoresis. Cut the gel slice containing the digested products and purify using a commercial gel agarose cleanup kit. Quantify the nucleic acid concentrations using a NanoDrop or similar spectrophotometer.
7. Dephosphorylate the digested pCAGGS vector to prevent self-ligation by treating with SAP (1 U/μg DNA) at 37 °C for 60 min. Inactivate SAP by heating at 65 °C for 15 min. The dephosphorylated pCAGGS vector can be purified using a commercial PCR cleanup kit. Quantify the DNA concentration using a NanoDrop or similar spectrophotometer. Insert and vector may be used immediately for ligation or stored at −20 °C for later use.
8. Ligate the digested PCR product into the digested pCAGGS vector by adding the following: 100 ng of digested and SAP-treated pCAGGS vector, appropriated ng of DNA insert (1:3 molar ratio is recommended), 2 μL 10× ligation buffer, 1 μL (5 U) T4 DNA ligase, and ddH_2O up to 20 μL. Incubate the ligation mixture at room temperature for 1 h.
9. Transform DH5α competent cells (*see* **Note 9**) by adding 10 μL of the ligation mixture to 100 μL competent DH5α cells on ice (4 °C). Incubate for 5 min on ice (4 °C). Then, heat-shock for 45–60 s at 42 °C in a water bath, and transfer them back to ice (4 °C) for 5 min. Add 400 μL of SOC medium. Incubate for 45 min in a 37 °C shaking (200 rpm) incubator. Plate of bacteria in LB agar plates with 100 μg/mL of ampicillin (*see* **Note 10**). Incubate the plates, inverted, overnight at 37 °C (*see* **Note 11**).
10. Next day, transfer individual bacterial colonies from the LB agar plates to tubes containing 4 mL of LB liquid media with 100 μg/mL of ampicillin and incubate for 16–18 h in a 37 °C shaking (200 rpm) incubator.
11. After overnight incubation, purify the plasmid DNA using a miniprep kit, following the manufacture's recommendations (*see* **Note 12**). Determine plasmid DNA concentration using a NanoDrop or similar spectrophotometer.
12. Analyze the purified plasmids by restriction analysis and confirm by sequencing (*see* **Note 13**).

3.2 Generation of CIV HA Protein-Expressing MDCK Cells

Numerous methods have been reported for the generation of protein-expressing cell lines, leading to the transient or stable expression of proteins [45, 46]. We will focus on the generation of MDCK cell lines stably expressing HA from influenza CIV H3N8 (MDCK CIV HA) by co-transfecting the pCAGGS CIV H3N8 HA-expressing plasmid (Subheading 3.1) and the plasmid pCB7 (7:1 ratio) for stably expression of CIV H3N8 HA and hygromycin B resistance, respectively [22, 29, 47] (Fig. 1a). Alternatively, a single pCAGGS plasmid encoding CIV H3N8 HA and the hygromycin B resistance gene can be used [27].

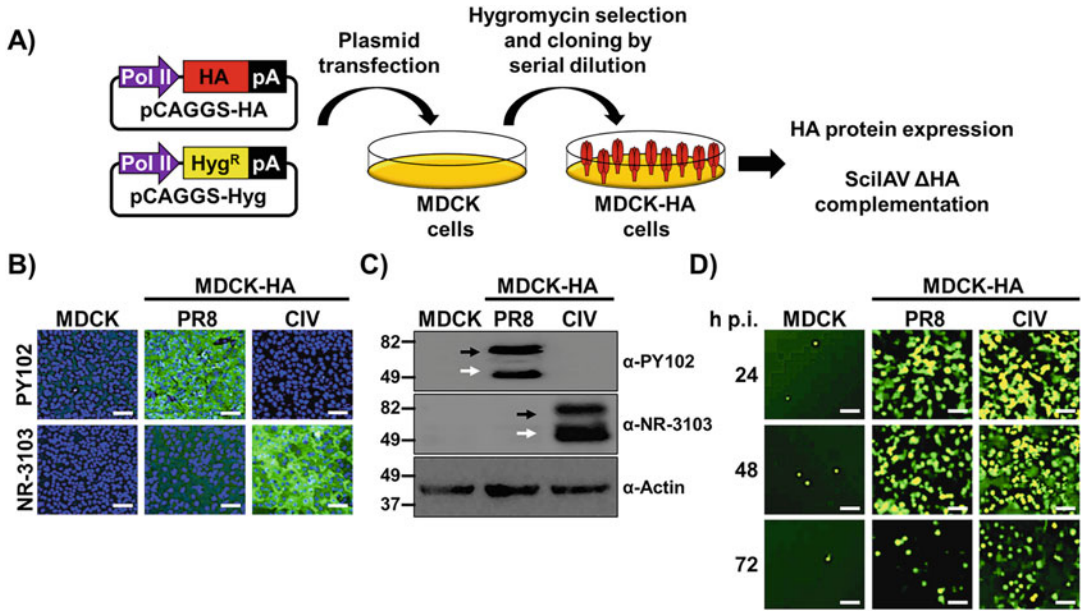

Fig. 1 Generation and characterization of CIV H3N8 HA-expressing MDCK cells. (**a**) Schematic representation to generate CIV H3N8 HA-expressing MDCK cells: Polymerase II (Pol II) driven pCAGGS plasmids encoding CIV H3N8 HA (top) and hygromycin B resistance (Hyg^R; bottom) are co-transfected (ratio 7:1) into parental MDCK cells. After transfection, cells are seeded at low density (cloning dilution) and hygromycin-resistant clones are individually selected in the presence of hygromycin. CIV H3N8 HA-expressing MDCK clones are screened for HA protein expression using specific antibodies by IFA (**b**) and Western blot (**c**), and by complementation of the sciCIV ΔHA/GFP by GFP expression using a fluorescent microscope (**d**). (**b**) IFA analysis: PR8 and CIV H3N8 HA-expressing MDCK cells were fixed and stained with the PR8 specific HA monoclonal antibody PY102 or with the CIV H3N8 HA specific polyclonal antibody NR-3103. Cells were counterstained with DAPI to visualize the cell nuclei. Parental MDCK cells were used as negative control. Representative images are shown. Scale bars, 50 μM. (**c**) Western blot analysis: Whole-cell lysates from PR8 AND CIV H3N8 HA-expressing MDCK were stained with the PR8 specific HA monoclonal antibody PY102 or the CIV H3N8 specific HA polyclonal antibody NR-3103. A monoclonal antibody against actin was used as a loading control. Whole-cell lysates from parental MDCK cells were used as negative control. The HA0 and HA1 subunits of PR8 and CIV H3N8 HA are indicated with black or white arrows, respectively. Molecular markers are indicated on the left. (**d**) Complementation of sciCIV ΔHA/GFP: Confluent monolayers of parental MDCK cells or MDCK cells expressing PR8 and CIV H3N8 HA were infected (MOI of 0.001) with PR8 sciIAV ΔHA/GFP. At 24, 48, and 72 h post-infection (h p.i.), GFP expression was analyzed using a fluorescence microscope. Scale bars, 50 μM

1. *Day 1*. Prepare transfection mix A and B in different tubes. Mix *A*: Prepare 250 μL of OptiMEM I media and 4 μL of LPF2000 and incubate for 5–10 min at room temperature (*see* **Note 14**). Mix *B*: Add 3.5 μg of pCAGGS CIV H3N8 HA and 0.5 μg of pCB7 to a tube containing 50 μL of OptiMEM I media.
2. Add mix A into the tube containing the plasmid DNAs (mix B) and incubate for 20–30 min at room temperature. Meanwhile, prepare the MDCK cells for transfection.
3. Wash a 100 mm confluent dish plate of MDCK cells twice with 5 mL of PBS 1×. Remove the last PBS 1× wash and add, slowly, 2 mL of a 0.25% Trypsin-EDTA. Incubate at 37 °C until cells detach from the 100 mm dish plate.
4. Resuspend the cells in 10 mL of cell culture media, transfer to a 15 mL centrifuge tube and centrifuge the cells for 5 min at 200 × *g*.
5. Carefully remove the media and resuspend the cells in 8 mL of fresh cell culture media by gentle pipetting up and down. Determine the concentration of the MDCK cells using a hemocytometer.
6. Dilute the cells so that 1 mL of media contains ~7×10^5 cells. Add 1 mL of MDCK cells (~7×10^5 cells) to the tube containing the transfection mix and incubate for 2 min at room temperature.
7. Add the 1.3 mL (1 mL of cells and ~300 μL transfection mix) into individual wells in a 6-well tissue culture plate. Gently shake the 6-well plate and let the transfection incubate for 6 h or overnight in the 37 °C, 5% CO_2 humidified tissue culture incubator.
8. *Day 2*. After 12 h incubation, change the transfection media for fresh cell culture media.
9. *Day 3*. After 24 h post-changing media, cells are seeded at a low density so that hygromycin-resistant cells can be clonally isolated. The cells in the 6-well tissue culture plate of MDCK cells are washed twice with 1 mL of PBS 1×. Remove the last PBS 1× wash and add, slowly, 0.5 mL of a 0.25% Trypsin-EDTA. Incubate at 37 °C until the cells detach from the well.
10. Resuspend the cells in 3 mL of cell culture media, transfer to a 15 mL centrifuge tube, and centrifuge the cells for 5 min at 200 × *g*. At this point, cells can be frozen down as a polyclonal line.
11. For monoclonal selection, remove the media and resuspend the cells in 10 mL of fresh cell culture media by gentle pipetting up and down.

12. Prepare 5 × 15 mL tubes containing 9 mL of cell culture media to perform tenfold dilutions and add the cells from each tube to a 100 mm plate.
13. *Day 4*. Remove the media and add fresh cell culture media containing 200 μg/mL of hygromycin B and incubate at 37 °C until individual and discernible clones are visualized (*see* **Note 15**). Change media containing selection hygromycin B every 2–3 days.
14. Using cloning rings, trypsinize the individual cell clones and transfer them to 12-well tissue culture plates and allowed to expand for monoclonal expansion selection with media containing hygromycin B. We recommend expanding and characterizing a minimum of 30 individual cell clones and maintaining the hygromycin B selection during cell growth (*see* **Note 16**).
15. Once the 12-well tissue culture plate cells clones have expanded to high confluence, each can be passage into a 6-well tissue culture plate well. A small portion of the cells should be assessed for expression of the HA protein (Subheading 3.3).
16. Once expression is verified, CIV H3N8 HA-expressing clones of interest can be scaled up to larger volumes and freeze down cell stocks using appropriate freezing medium lacking the selection antibiotic.
17. Upon establishing the monoclonal stable CIV H3N8 cell line, a lower amount of hygromycin B (e.g. 100 μg/mL) can be used for maintenance.

3.3 Characterization of MDCK CIV H3N8 HA Cells

Immunofluorescence (IFA) and Western blotting (WB) can be used to evaluate the distribution and relative quantity of CIV H3N8 HA protein in individually selected MDCK clones (Fig. 1b, c, respectively). Positive (e.g. other previously generated MDCK HA-expressing cell lines) and negative (parental MDCK cells) controls should be included in these assays. Moreover, to analyze for functional HA expression, it is important to assess the ability of the CIV H3N8 HA-expressing MDCK cells to complement a sciIAV ΔHA/GFP (Fig. 1d). Based on the results from these different analysis, the best clone or clones of HA-expressing MDCK cells can be selected for further studies.

3.3.1 Immunofluorescence Analysis (IFA)

1. Seed approximately 4×10^4 CIV H3N8 HA (for each isolated individual clone), PR8 HA (positive control) or parental (negative control) MDCK cells/well, in 48-well plates and place the plates at 37 °C in the 5% CO_2 humidified incubator.
2. Next day, check the cells under a light microscope to confirm the presence of a cell monolayer, remove the media and add

250 μL/well of the fix/permeabilization solution. Incubate at room temperature for 20 min.

3. Remove the fix/permeabilization solution and wash once with 0.5 mL/well of PBS 1×.
4. Incubate the cells with blocking solution for 1 h at room temperature.
5. Dilute the primary monoclonal (1 μg/mL of PY102 for PR8 HA detection) or polyclonal (1:1000 of NR-3103 for CIV HA detection) antibodies in blocking solution. Add sufficient antibody to completely cover the cell monolayer to prevent drying (~200 μL/well). Incubate for 1 h at 37 °C.
6. Carefully remove the primary antibodies and wash the cells with 0.5 mL/well of PBS 1× three times.
7. Dilute the fluorescently labeled secondary monoclonal or polyclonal antibodies and DAPI in blocking solution to a predetermined optimum working dilution. Add sufficient secondary antibody to completely cover the cell monolayer to prevent from drying (~200 μL/well). Incubate for 30 min at 37 °C.
8. Carefully remove the secondary antibodies and wash the cells with PBS 1× three times. Remove the last PBS 1× wash and add 250 μL of PBS 1×.
9. Evaluate HA expression and distribution using a fluorescent microscope (Fig. 1b). All MDCK CIV H3N8 HA-expressing cells from the isolated clones should be labeled (e.g. clonal population of cells homogeneously expressing HA). If not all the cells express CIV H3N8 HA, clones should be re-cloned or discarded. It is important to use a clonal population of MDCK cells that homogenously express CIV H3N8 HA.

3.3.2 Western Blot Analysis

1. Preparation of lysates from cell cultures. Seed approximately 2×10^5 MDCK CIV H3N8 HA (for each isolated individual clone), PR8-HA (positive control) or parental (negative control) MDCK cells/well, in 12-well plates and place the plates at 37 °C in the 5% CO_2 humidified incubator.
2. Next day, check the cells under a light microscope to confirm the presence of a cell monolayer, remove the media, and wash the cells with 0.5 mL/well of PBS 1× twice.
3. Place the cell culture dish on ice (4 °C) and then add 250 μL/well of ice-cold RIPA buffer. Incubate the cells for 20 min in ice (4 °C). Scrape cells off the dish using a cell scraper and transfer them to a microcentrifuge tube (*see* **Note 17**).
4. Centrifuge the tubes in a microcentrifuge at 4 °C for 15 min at 10,000 × *g*. Collect the supernatants in a new tube and discard the pellets. Lysates can be aliquoted and stored at −20 °C until future use.

5. Western Blotting assay. Determine how much protein to load and add 4× Laemmli sample buffer. Reduce and denature the samples at 90 °C for 5 min.
6. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with a molecular weight marker. Run the SDS-PAGE gel for 1–2 h at 60 mA.
7. Transfer the proteins on the gel to a nitrocellulose membrane at 100 V for 1 h.
8. Block the membrane at room temperature with blocking solution for 1 h.
9. Incubate the membrane with the primary PY102 (1 μg/mL for PR8 HA), NR-3103 (1:1000, for CIV HA detection) or anti-actin (1 μg/mL) monoclonal or polyclonal antibodies in blocking buffer. We recommend overnight incubation at 4 °C.
10. Wash the membrane three times with PBS-T, 5 min each wash.
11. Incubate the membrane with the manufacture's recommended dilution of conjugated secondary antibodies in blocking buffer at room temperature for 1 h.
12. Wash the membrane three times with PBS-T, 5 min each wash.
13. Follow the kit manufacturer's recommendations for signal development (peroxidase activity), using appropriate equipment for chemiluminescence detection (Fig. 1c).

3.3.3 sciIAV Complementing Assay

1. Seed approximately 2×10^5 CIV H3N8 HA (for each individual isolated clone), PR8 HA (positive control) or parental (negative control) MDCK cells/well, in 12-well plates and place the plates at 37 °C in the 5% CO_2 humidified incubator.
2. Next day, before viral infection, check the cells under a light microscope to confirm the presence of a confluent cell monolayer.
3. Aspirate the cell culture media and wash cells, twice, with 1 mL of PBS 1×.
4. Infect the parental and HA-expressing MDCK cells with 250 μL of PR8 sciIAV ΔHA/GFP at a low multiplicity of infection (MOI, 0.001). Put the plate on a rocking platform for 1 h at room temperature to allow viral adsorption.
5. After 1 h of viral absorption, remove the infection media and add 1.5 mL of post-infection media containing 1 μg/mL of TPCK-treated trypsin. Incubate the plates at 37 °C in a 5% CO_2 humidified incubator.
6. At indicated times post-infection (24, 48, and 72 h), monitor GFP expression under a fluorescent microscope (Fig. 1d).

3.4 Generation of sciCIV Δ HA/GFP

Overall, rescue of sciCIV ΔHA/GFP is performed using experimental approaches similar to those previously described with PR8 [7, 33–35] but using CIV H3N8 plasmid-based reverse genetics techniques [36] . To increase the likelihood of successful rescuing sciCIV ΔHA/GFP, we recommend three independent transfections. The following transfection and infection protocols is established for 6-well plates. A schematic representation of the protocol to generate recombinant sciCIV ΔHA/GFP is illustrated in Fig. 2.

1. Preparation of transfection mix. OptiMEM I-LPF2000 mix: Prepare 250 μL of OptiMEM I media and 9 μL of LPF2000 per transfection and incubate for 5–10 min at room temperature. Meanwhile, prepare the plasmid transfection mixture.

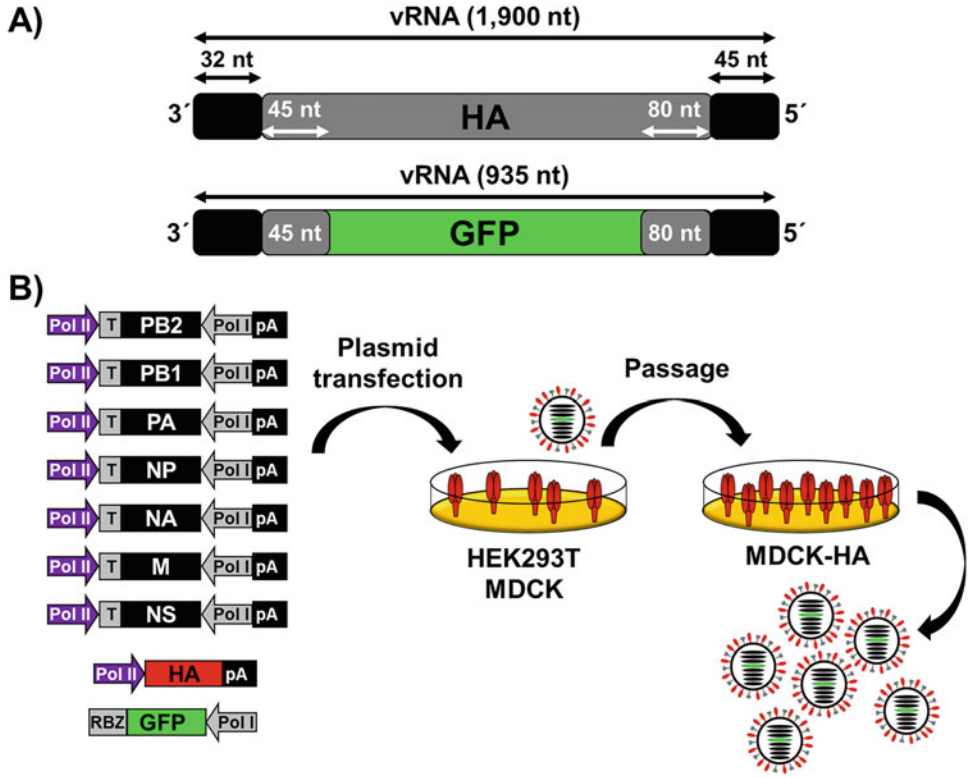

Fig. 2 Rescue of CIV H3N8 sciCIV ΔHA/GFP. (**a**) Schematic representation of the ΔHA/GFP vRNA segment: Top, WT IAV HA vRNA segment. Bottom, HA(45)GFP(80) vRNA. Non-coding regions (NCR) are indicated with black boxes at each vRNA termini. Nucleotide lengths of the 3′ and 5′ vRNA HA NCRs, packaging signals (white numbers and arrows) and vRNAs are also indicated. (**b**) Plasmid-based reverse genetics to generate CIV H3N8 sciCIV ΔHA/GFP: Ambisense pDZ plasmids encoding CIV H3N8 PB2, PB1, PA, NP, NA, M, NS as well as a hpol-I-driven plasmid encoding GFP flanked by the IAV HA NCR and packaging signals (**a**) and the pCAGGS plasmid expressing CIV H3N8 HA (Fig. 1) are co-transfected into co-cultures of HEK293T/MDCK CIV H3N8 HA cells. Virus-containing tissue culture supernatants are subsequently passage into fresh monolayers of CIV H3N8 HA-expressing MDCK cells for sciCIV ΔHA/GFP amplification. *Pol II* polymerase II promoter. *Pol I* polymerase I promoter. *pA* polyadenylation signal. *T* polymerase I terminator. *RBZ* hepatitis delta virus ribozyme

2. Plasmid transfection mixture: Add 1 μg of each influenza A/canine/NY/dog23/2009 H3N8 pDZ plasmids (PB2, PB1, PA, NP, NA, M, and NS) together with 1 μg of the previously described pPolI-ΔHA/GFP [27] (*see* **Note 18**) and pCAGGS CIV H3N8 HA used to generate the MDCK HA-expressing cell lines (Subheading 3.1) to a tube containing 50 μL of OptiMEM I media.
3. OptiMEM I-LPF2000-plasmid DNA transfection mixture: Add 250 μL of the OptiMEM I-LPF200 mix (**step 1**) into the plasmid DNA transfection mixture (**step 2**). The final volume is 300 μL. Incubate for 20–30 min at room temperature. Meanwhile, prepare the human HEK293T and CIV H3N8 HA-expressing MDCK cells generated in Subheading 3.3 for transfection (*see* **Note 19**).
4. Preparation of HEK293T/MDCK-HA co-cultures. Usually, one confluent 100 mm dish of each cell line (HEK293T and MDCK CIV H3N8 HA) can be used for approximately 10–12 viral rescues.
5. Wash the cell monolayers twice with 4 mL of PBS 1×. Remove the last PBS 1× wash and add 2 mL of a 0.25% Trypsin-EDTA. Incubate at 37 °C until cells completely detach from the plates.
6. Carefully resuspend individually each cell line in 10 mL of cell culture media and transfer to a 15 mL centrifuge tube. Centrifuge both cell lines for 5 min at 200 × *g*. Remove the cell culture media and resuspend the HEK293T and MDCK CIV H3N8 HA cells, separately, in 6 mL of cell culture media.
7. Mix equal volumes of HEK293T and MDCK CIV H3N8 HA cells, based on the number of transfections, in a separate tube.
8. After 20–30 min incubation at room temperature, add to each of the OptiMEM I-LPF2000-plasmid DNA transfection tubes (**step 3** in this section) 1 mL of the HEK293T/CIV H3N8 HA MDCK cell co-culture and incubate for 5 min at room temperature.
9. Add the 1.3 mL (1 mL of cell and 300 μL of transfection mixture) into individual wells in a 6-well tissue culture plate. Gently shake the 6-well plate and let the transfection incubate for 6 h or overnight in the 37 °C, 5% CO_2 humidified tissue culture incubator.
10. After 6–12 h post-transfection, change the transfection media for post-infection media containing 0.5 μg/mL of TPCK-treated trypsin and incubate for 48–72 h at 37 °C.
11. After 48–72 h incubation, collect the tissue culture supernatants into microcentrifuge tubes and centrifuge for 1–2 min at 13,000 × *g* to remove cells and cell debris. Transfer supernatants to new microcentrifuge tubes. Tissue culture

supernatants can be used immediately to infect fresh monolayers of CIV H3N8 HA-expressing MDCK cells or stored at −80 °C for later use.

12. Infection of CIV H3N8 HA-expressing MDCK cells to confirm viral rescue and prepare stocks. CIV H3N8 HA MDCK cells are infected as previously described in Subheading 3.3.3, but scaling the volumes to the appropriate plates.
13. The day before infection plate approximately 5×10^5 CIV H3N8 HA-expressing cells/well (6-well plate) to reach confluence (1×10^6 cell/well) by the next day. Before infection, check the cells under the microscope to confirm the presence of a cell monolayer.
14. Aspirate the cell culture media and wash cells, twice, with 4 mL of PBS 1×. Infect the CIV H3N2 HA-expressing MDCK cells with 250 μL of virus-containing tissue culture supernatants (**step 11**) for 1 h at room temperature. Gently rock the plates every 10 min to prevent the cells from drying.
15. After 1 h of viral absorption, remove the infection media and add 2 mL of post-infection media containing 1 μg/mL of TPCK-treated trypsin. Incubate the plates at 37 °C in a 5% CO_2 humidified incubator.
16. At 3–4 days after infection, monitor the cells for cytopathic effect (CPE), which is indicative of virus rescue. Other assay (s) will need to be performed to confirm the presence of rescued virus (e.g. hemagglutination assay or IFA).
17. Harvest the tissue culture supernatants from infected cells when approximately 75% of the cells show CPE. Transfer the tissue culture supernatants to 15 mL tubes and centrifuge at 13,000 × *g* for 5 min at room temperature to remove cells and cell debris. Collect the supernatants to fresh centrifuge tubes and store them at 4 °C (short-term storage) or at −80 °C (long-term storage).
18. Supernatants from **step 17** can be used to generate a working stock preparation by infected 100 mm dishes of CIV H3N8 HA-expressing cells at low MOI (0.01–0.001).

3.5 Multicycle Growth Analysis of sciCIV Δ HA/GFP

1. Seed approximately 2×10^5 MDCK CIV H3N8 HA (from selected individual isolated clone), PR8 HA (positive control) or parental (negative control) MDCK cells/well, in 12-well plates and place the plates at 37 °C in the 5% CO_2 humidified incubator.
2. Next day (before infection), check the cells under a light microscope to confirm the presence of a confluent cell monolayer.

3. Aspirate the cell culture media and wash cells, twice, with 1 mL of PBS 1×.
4. Infect (MOI, 0.001) the parental and HA-expressing MDCK cells with 250 μL of the generated sciCIV ΔHA/GFP (Subheading 3.4). Put the plate on a rocking platform for 1 h at room temperature to allow viral adsorption.
5. After 1 h of viral absorption, remove the infection media and add 1.5 mL of post-infection media containing 1 μg/mL of TPCK-treated trypsin. Incubate the plates at 37 °C in a 5% CO_2 humidified incubator.
6. Assess GFP expression by fluorescence microscopy at the indicated times post-infection (24, 48, and 72 h), (Fig. 3a).
7. At the same times post-infection, also collect the tissue culture supernatants to determine viral titers as indicated below (Fig. 3b).
8. Seed approximately 2×10^4 CIV H3N8 HA-expressing MDCK cells/well in 96-well plates the day before the infection to reach confluence after 1 day. Before infection, check the cells under a light microscope to confirm the presence of a cell monolayer.

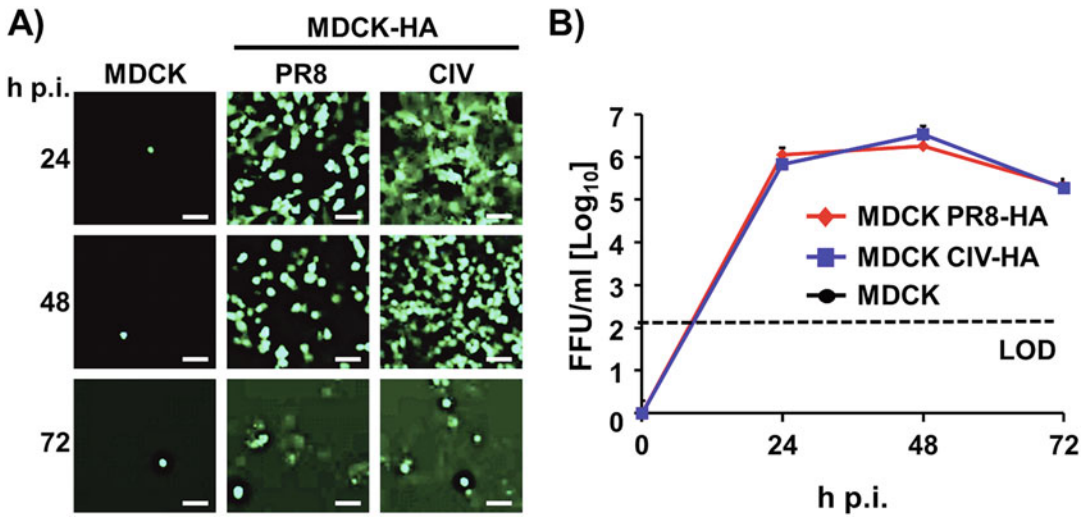

Fig. 3 sciCIV ΔHA/GFP complementation with HA-expressing MDCK cells. (**a**) Multicycle growth analysis of sciCIV ΔHA/GFP in parental and HA-expressing PR8 and CIV H3N8 MDCK cells: Confluent monolayers of parental and HA-expressing MDCK cells were infected (triplicates) with sciCIV ΔHA/GFP at a low MOI (0.001). At the indicated h post-infection (h p.i.), GFP expression was visualized using a fluorescence microscope. Representative images are shown. Scale bars, 50 μM. (**b**) Viral titrations: Tissue culture supernatants from cells infected in panel A were collected for titration in CIV H3N8 HA-expressing MDCK cells. Data represent the means ± SDs of the results determined in triplicate. Dashed black line indicates the limit of detection (LOD) of the assay (200 FFU/mL)

9. Make tenfold serial dilutions of the virus-containing tissue culture supernatants (*−1* to *−8*). Starting with the most diluted sample, add 50 μL/well of the virus dilution to each of the wells in the 96-well plate (*see* **Note 20**).
10. After 1 h of viral absorption, remove the virus inoculum and add 100 μL of post-infection media containing 1 μg/mL of TPCK-treated trypsin.
11. Place the plates at 37 °C in the 5% CO_2 humidified incubator for 8–12 h (*see* **Note 21**).
12. Remove the infectious media from the 96-well plates, and add 100 μL/well of PBS 1×.
13. Because GFP expression, foci can be visualized and enumerated using a fluorescence microscope (*see* **Note 22**).
14. Calculate the viral titer in focus forming units per milliliter (FFU/mL) using the following formula: FFU/mL = number of positive fluorescent cells × virus dilution × 1/volume of inoculum (mL). Mean value and standard deviation can be calculated using Microsoft Excel software.

3.6 In Vivo Characterization of sciCIV Δ HA/GFP

All animal protocols described here were approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) at the University of Rochester School of Medicine and Dentistry, and comply with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. The facilities and programs of the Vivarium and Division of Laboratory Animal Medicine of the School of Medicine and Dentistry are accredited by AAALAC International and comply with state law, federal statutes and National Institutes of Health (NIH) policies. Similar requirements should be applied at each institution to adhere to the animal protocols described in this manuscript. In this report, the IAVs used to infect mice (sciCIV ΔHA/GFP or CIV H3N8 WT) can be used under BSL-2 conditions. A detailed protocol to perform IAV studies in mice can be found in [44].

3.6.1 Intranasal (i.n.) Infection

1. Place the female 6- to 8-week-old C57BL/6 mice under specific pathogen-free conditions. Organize and label the mouse cages with the virus and dose that will be used (*see* **Note 23**). Identify the mice in each cage using an ear punch code or with another approved method such as painting of the tails.
2. Prepare the dilution of 1×10^5 FFU of CIV WT or sciCIV ΔHA/GFP in a total volume of 30 μL/mouse in sterile PBS 1×. Maintain the virus inoculum on ice (4 °C).
3. Weigh the mice with a scale. Anesthetize the mouse intraperitoneally (i.p.) with 250 mg/kg of 2,2,2-tribromoethanol (TBE) by inserting the needle in the caudal 2/3 of the right

side of the abdomen. Pause briefly before withdrawing the needle. Return the mouse to the cage and wait 5 min.

4. When the mouse is fully anesthetized, place the mouse in dorsal recumbency. Put the pipet tip containing 30 μL of the virus inoculum in the nostril and slowly but constantly eject the solution. Be sure the mouse is inhaling the preparation by observing the inoculum drop disappearing (*see* **Note 24**).
5. Return the animal to the cage placing it in dorsal recumbency and monitor the mice for any signs of respiratory distress until it regains consciousness (30–45 min after it was anesthetized).

3.6.2 Evaluation of Viral Titers in Lungs

1. At days 2 and 4 post-infection, euthanize (*see* **Note 25**) the mice ($n = 3$/group/day) with a lethal dose (i.p.) of TBE (500 mg/kg) and collect the mouse lungs as described in [44]. Store the samples on ice (4 °C) if the samples are processed the same day or on dry ice to freeze them quickly if the samples will be processed later.
2. Place the lungs into a sterile Dounce homogenizer and add 1 mL of cold infection media. Homogenize the sample by moving the pestle up and down for approximately 1 min at room temperature until the lungs are completely disrupted. Put the homogenized sample in a sterile tube and store at 4 °C.
3. Centrifuge the samples at 300 × *g* for 5–10 min at 4 °C. Collect the supernatant in a new sterile tube. Store the supernatant at 4 °C if viral titration is performed on the same day and discard the pellet. Alternatively, freeze (−80 °C) the supernatant of the homogenized samples to evaluate viral titers later.
4. To evaluate viral titers by immunofluorescence, perform the same **steps 8–14** indicated in Subheading 3.5 for viral infection.
5. Then, remove the tissue culture medium from the 96-well plates. Fix and permeabilize the cells with fixation/permeabilization solution for 20 min at room temperature.
6. Incubate the cells with blocking solution for 1 h at room temperature.
7. Dilute the primary monoclonal (1 μg/mL of HB65) in blocking solution. Add sufficient antibody to completely cover the cell monolayer to prevent drying (~50 μL/well). Incubate for 1 h at 37 °C.
8. Carefully remove the primary antibody and wash the cells with 100 μL/well of PBS 1× three times.
9. Dilute the fluorescently labeled secondary antibody in blocking solution to a predetermined optimum working dilution. Add sufficient secondary antibody to completely cover the cell monolayer to prevent from drying (~50 μL/well). Incubate for 30 min at 37 °C.

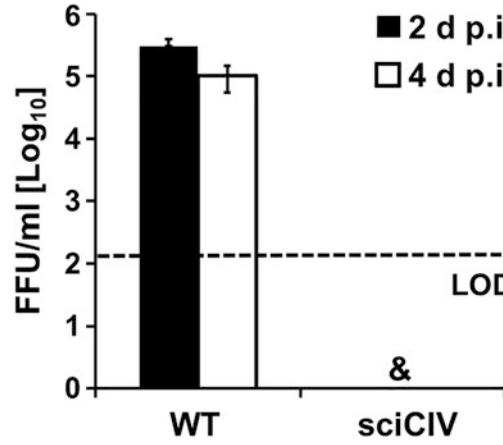

Fig. 4 Attenuation of sciCIV ΔHA/GFP in mice: Female 6- to 8-week-old C57BL/6 mice ($n = 6$) were infected (i.n.) with 1×10^5 FFU of CIV WT (WT) or sciCIV. To evaluate viral lung replication, mice were sacrificed at days 2 ($n = 3$) and 4 ($n = 3$) post-infection (d p.i.) and lungs were harvested, homogenized, and used to quantify viral titers by immunofocus assay (FFU/mL) using an anti-NP monoclonal antibody (HB-65). Dotted black lines indicate LOD of the assay (200 FFU/mL)

10. Carefully remove the secondary antibody and wash the cells with PBS 1× three times. Remove the last PBS 1× wash and add 100 μL of PBS 1×.
11. Observe the cells under a fluorescence microscope to determine the number of positive stained (green) cells. Calculate the viral titer by counting FFU/mL as indicated before (Fig. 4).

3.7 Analysis of Immunogenicity Induced by sciCIV ΔHA/GFP

3.7.1 Mouse Bleeding by Submandibular Puncture

1. Inoculate (i.n.) four groups of 6- to 8-week-old female C57BL/6 mice ($n = 6$/group) with 1×10^3 FFU of CIV WT (WT), 1×10^5 FFU of sciCIV ΔHA/GFP that was (sciCIV-UV) or was not (sciCIV) exposed to UV light (*see* Subheading 3.7.2) or mock-vaccinated with PBS 1×. Perform the i.n. mouse inoculations as described in Subheading 3.6.1.
2. Fourteen days after infection, collect the mice blood by submandibular bleeding using a 4 mm lancet [43, 44] or using another IACUC approved method.
3. Recover blood in a sterile tube (approximately 0.1–0.2 mL/mouse are recovered). Apply pressure on the puncture with a sterile napkin for a few seconds.
4. Put the tubes for 1–2 h at 37 °C and then centrifuge them at $700 \times g$ for 30 min at room temperature to separate the serum from the blood. Transfer the upper layer (that consists of the serum) to a new sterile tube. Discard the pellet. Store the serum at −20 °C to be used for the ELISA.

3.7.2 sciCIV UV Inactivation

1. sciCIV ΔHA/GFP (~500 μL) is placed in uncovered 12-well plates and exposed to shortwave UV radiation at 254 nm for 20 min on ice (4 °C) at a distance of 6 in. (approximately 15 cm) [48, 49]. Following UV irradiation, infectivity is measured in CIV H3N8 HA-expressing MDCK cells as previously described in Subheading 3.5 (Fig. 5a).
2. Seed approximately 2×10^4 CIV H3N8 HA-expressing MDCK cells/well in 96-well plates the day before the infection to reach confluence after 1 day.
3. Before infection, check the cells under a light microscope to confirm the presence of a cell monolayer.
4. Infect the CIV H3N8 HA-expressing MDCK cells with 50 μL of the tenfold serial dilutions (triplicates) of mock- or UV-treated virus. Leave rows of mock-infected CIV H3N8 HA-expressing MDCK cells as control.
5. After 1 h of viral absorption, remove the virus inoculum and add 100 μL of post-infection media containing 1 μg/mL of TPCK-treated trypsin.
6. Place the 96-well plates at 37 °C in the 5% CO_2 humidified incubator for 2–3 days.

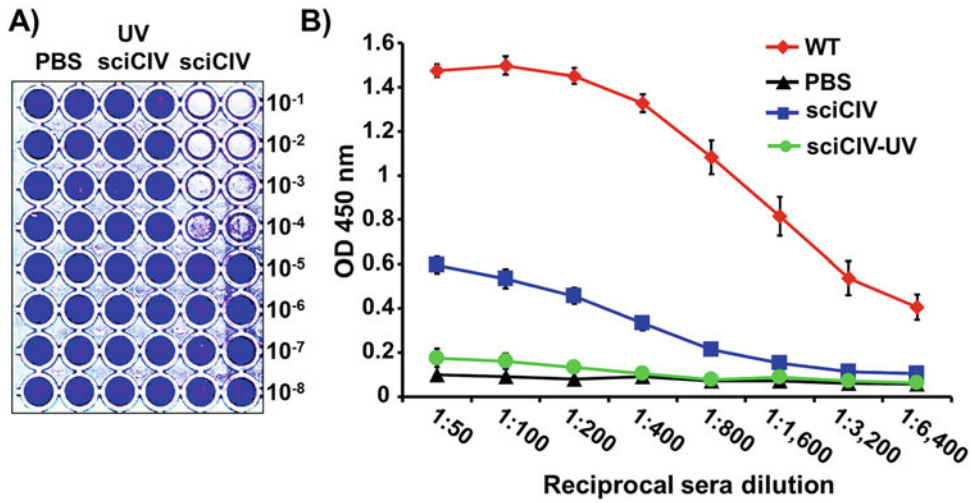

Fig. 5 Induction of humoral responses by sciCIV ΔHA/GFP vaccination. (**a**) UV inactivation of sciCIV: Tissue culture supernatants from sciCIV were UV inactivated. UV inactivation was confirmed by infecting CIV H3N8 CIV HA-expressing MDCK cells using serial dilutions of the virus. At 3 days post-infection, cells monolayers were stained with crystal violet solution. Cells mock (PBS)-infected or infected with sciCIV were used as internal controls. (**b**) ELISA: 6- to 8-week-old C57BL/6 mice were immunized (i.n.) with 1×10^3 FFU of CIV WT (WT), 1×10^5 FFU of sciCIV ΔHA/GFP that was (sciCIV-UV) or was not (sciCIV) UV inactivated. At 14 days post-infection, mice were bled and sera were collected and evaluated by ELISA for IgG antibodies against total influenza virus proteins using cell extracts of MDCK cells infected with CIV H3N8 WT. *OD* optical density. Data represent the means ± SDs of the results for four individual mice

7. Remove the tissue culture medium from the 96-well plates, wash with PBS 1× and add 100 μL/well of 0.1% crystal violet.
8. Incubate for 1 h at room temperature, remove the crystal violet solution, and wash the wells 3 times with distilled water. Dry the plates at room temperature.
9. Evaluate sciCIV ΔHA/GFP inactivation by confirming the confluent cell monolayer of CIV H3N8 HA-expressing MDCK cells after infection with UV-inactivated sciCIV ΔHA/GFP. Contrary to the wells infected with sciCIV ΔHA/GFP, wells infected with UV-inactivated virus should not result in removing of the cell monolayer, similar to mock-infected CIV H3N8 HA-expressing MDCK cells (Fig. 5a).

3.7.3 Preparation of Infected MDCK Cell Extracts

1. The day before infection, seed one 100-mm dish with 4×10^6 MDCK cells (to reach ~80–90% confluence the next day) in tissue culture media. The day of viral infection, check the cells under the microscope to confirm a monolayer before starting the viral infection.
2. Prepare a viral dilution of CIV H3N8 WT to infect cells using an MOI of 0.001 in 4 mL total final volume.
3. Aspirate the tissue culture medium from the MDCK cell plate and wash twice with 4 mL of PBS 1×. Add the viral inoculum and put the plate on a rocking platform for 1 h at room temperature to allow viral adsorption. Remove the viral inoculum and add 10 mL of post-infection media containing 1 μg/mL of TPCK-treated trypsin. Incubate infected cells for 48–72 h in a 37 °C incubator with 5% CO_2, until CPE is observed.
4. Detach the cells with the aid of a cell scraper and collect the cells and tissue culture medium with a pipet in a 15 mL tube. Centrifuge the tube at 400 × *g* for 5 min at room temperature.
5. Aspirate the supernatant and resuspend the cell pellet in 1 mL of RIPA buffer. Transfer to a centrifuge tube and incubate on ice (4 °C) for 20 min.
6. Centrifuge the tube at 1300 × *g* for 20 min at 4 °C. Carefully, recover the supernatant. Store the cell extract in 100 μL aliquots at −20 °C.
7. Titrate the cell extracts as described by enzyme-linked immunosorbent assay (ELISA, Subheading 3.7.4) before evaluating the presence of antibodies in the mouse sera samples. Include cell extracts from mock-infected MDCK cells (prepared as indicated above) as a negative control.

3.7.4 ELISA

ELISA can be performed to evaluate the presence of antibodies against total viral antigens using cell extracts from CIV H3N8 infected cells (Subheading 3.7.3) or purified virus. Alternatively, recombinant proteins can be used to determine responses against specific viral proteins (*see* **Note 26**) [15, 17, 49].

1. Coat polystyrene 96-well plates with 100 μL/well of the appropriate dilution of cell lysates from CIV H3N8-infected MDCK cells in PBS 1× (Subheading 3.7.3) (*see* **Note 27**). Coat another plate with the same dilution of cell lysates from mock-infected MDCK cells as a negative control. Incubate overnight at 4 °C.
2. Remove the supernatant and wash the plates once with 100 μL/well of PBS 1×. Block non-specific binding by adding 100 μL/well of blocking solution for 1 h at room temperature.
3. Meanwhile, make twofold serial dilutions (starting dilution 1:50) of the sera from each mouse collected in Subheading 3.7.1 in antibody dilution solution.
4. Remove the blocking solution from the 96-well plates. Add 50 μL of each mouse serum dilution in the appropriate well and incubate 1 h at 37 °C.
5. Remove the mice sera and wash the wells 3 times with distilled water.
6. Add 50 μL/well of an anti-mouse secondary antibody conjugated with HRP diluted 1:2000 in antibody dilution solution. Incubate 1 h at 37 °C.
7. Remove the secondary HRP-conjugated antibody and wash the wells 3 times with distilled water. Prepare the TMB substrate solution by mixing 1:1 solution A and B. Add 100 μL/well of substrate and incubate for 5–10 min at room temperature in the dark.
8. Stop the reaction by adding 100 μL/well of 0.2 N H_2SO_4. Read the plates at 450 nm on an ELISA plate reader.
9. Subtract the value obtained in each dilution of the MDCK mock-infected 96-well plate from the value obtained in the MDCK-infected 96-well plate. Calculate the average values for each dilution with the different sera and represent them in a graph showing standard deviations (SD) (Fig. 5b).

3.8 Analysis of Protection Efficacy

1. Fifteen days post-vaccination of mice (Subheading 3.6.1), challenge animals with 1×10^5 FFU of CIV H3N8 WT. Perform the i.n. mouse inoculations as described in Subheading 3.6.1.
2. Recover mouse lungs ($n = 3$/group/day) at days 2 and 4 post-challenge. Homogenize and analyze the lungs as described in Subheading 3.6.2 to assess viral replication of CIV H3N2 WT in challenged mice (Fig. 6).

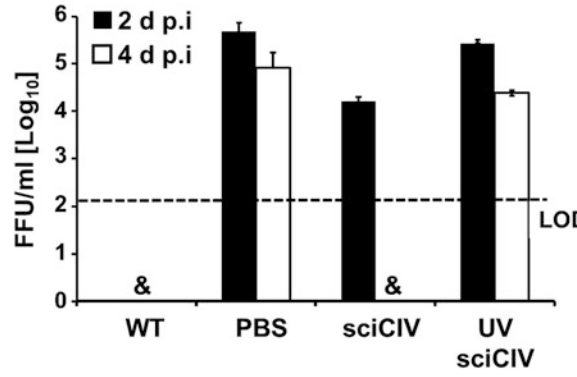

Fig. 6 Protection efficacy: Female 6- to 8-week-old C57BL/6 mice ($n = 6$) were immunized (i.n.) with 1×10^3 FFU of CIV H3N8 WT (WT), 1×10^5 FFU of sciCIV ΔHA/GFP that was (sciCIV-UV) or was not (sciCIV) UV inactivated, or mock-vaccinated with PBS 1×. At 2 weeks post-vaccination, mice were challenged (i.n.) with 1×10^5 FFU of CIV H3N8 WT. To evaluate viral lung replication, mice were sacrificed at days 2 ($n = 3$) and 4 ($n = 3$) post-infection (d p.i.) and lungs were harvested, homogenized, and used to quantify viral titers by immunofocus assay (FFU/mL) using an anti-NP monoclonal antibody (HB-65). Dotted black lines indicate LOD of the assay (200 FFU/mL). Data represent the means ± SDs and virus was not detected

4 Notes

1. Although in this chapter we describe plasmid-based reverse genetics for the generation of recombinant sciCIV ΔHA/GFP based on the backbone of influenza A/canine/NY/dog23/2009 H3N8, this protocol can be used to generate other recombinant sciIAVs subtypes.
2. It is important to keep track of the HEK293T and MDCK cell passage number, since it can affect virus rescue efficiency and virus propagation. A late cell passage (above 40) is not recommended for successful viral rescue.
3. This polyclonal antibody was originally developed against influenza A/equine/Miami/1/63 H3N8. However cross-react with A/canine/NY/dog23/2009 H3N8, as we previously described [15].
4. Other mouse strains such as BALB/c or DBA.2 could be used.
5. Other approved anesthetic and bleeding procedures can be used.
6. When using another DNA polymerase, please refer to the respective manufacturer's recommendations.
7. This protocol described the cloning of influenza A/canine/NY/dog23/2009 H3N8 HA sequence into the mammalian

expression plasmid pCAGGS. However, other mammalian influenza HA-expressing plasmids can be used.

8. CIV H3N8 HA sequence can be amplified using as template other plasmids containing the HA gene or after obtaining the cDNA from infected cells.
9. Other *E. coli* competent cells can be used for plasmid transformation.
10. pCAGGS plasmid encodes an ampicillin resistance gene. If another plasmid is used, complement with the appropriate antibiotic-containing LB liquid media and plates.
11. Plasmids containing IAV HA glycoproteins sometimes grow more efficiently at 33 °C.
12. Other miniprep purification kits can be used for DNA plasmid isolation.
13. Alternatively, selected clones can be initially screened by colony PCR to determine the presence or absence of CIV H3N8 HA.
14. When other transfection reagents are used, follow the manufacturer's recommendations for optimal plasmid DNA transfection efficiencies.
15. The days required to see individual cell colonies can vary (1–3 weeks) depending on the IAV HA glycoproteins.
16. The polyclonal cell culture can be further processed to isolate individual clones using other techniques such as limiting dilution or cell sorting after staining with appropriate antibodies.
17. Alternatively, cells can be trypsinized and washed with PBS 1× prior to lyse them with RIPA buffer. Moreover, other lysis buffers can be used.
18. The expression of GFP from the sciCIV ΔHA/GFP can be used to evaluate viral infection in vitro and in vivo. Although other foreign sequences, including alternative reporter fluorescent or luciferase genes, can be used, the sequence and length of those foreign sequences can affect viral rescue and/or foreign protein expression stability.
19. If a sciCIV ΔHA/GFP is generated before obtaining the CIV H3N8 HA-expressing stable MDCK cell line using transient transfection with the pCAGGS plasmid expressing CIV H3N8 HA, other IAV HA-expressing MDCK cell lines (e.g. PR8 HA) could be used [22, 27, 29]. The rescued sciCIV ΔHA/GFP could be later pseudotyped with CIV H3N8 HA (or other IAV HA) after generating the appropriated HA-expressing MDCK cell line.
20. We recommend performing the viral titration in triplicate and calculating viral titers using the triplicate average.

21. Longer times post-infection may lead to secondary infections and, therefore, result in an overestimation of viral titers.
22. Alternatively, cells can be fixed and plates can be kept at 4 °C to be evaluated later.
23. Proper Personal Protection Equipment (PPE) is required for working with mice.
24. Check that the mouse is breathing as TBE can depress the temperature and breathing rate.
25. Following IACUC protocol, euthanize mice with two methods of euthanasia (the second must be a physical method) to ensure that the animal is dead.
26. The optimized dilution should be tested. Generally, a dilution between 1:200 and 1:1000 is used.
27. ELISA is used to evaluate the humoral responses induced after vaccination. However, infectivity neutralization assays can be also performed to evaluate the presence of neutralizing antibodies. Alternative, the HA inhibition (HI) assay can be carried out, which measures the blockade of sialic acid binding, and for which the titers are generally similar to those from neutralizing assays.

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Chapter 14

Reverse Genetics for Influenza A and B Viruses Driven by Swine Polymerase I Promoter

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Abstract

Influenza viruses are considered prominent pathogens of humans and animals that are extensively investigated because of public health importance. Plasmid-based reverse genetics is a fundamental tool that facilitates the generation of genetically modified viruses from a cDNA copy. The ability to rescue viruses enables researchers to understand different biological characteristics including IV replication, pathogenesis, and transmission. Furthermore, understanding the biology and ability to manipulate different aspects of the virus can aid in providing a better understanding of the mechanisms of antiviral resistance and development of alternative vaccination strategies. This chapter describes the process of cloning cDNA copies of IAV and IBV RNA segments into a swine polymerase-driven reverse genetics plasmid vector, successful generation of recombinant IVs in swine cells, and propagation of virus in cells or eggs. The swine polymerase reverse genetics system was previously shown to be efficient for de novo rescue of human-, swine-, and avian-origin IAVs and IBV in swine and human origin cell lines utilizing the same protocols discussed in this chapter.

Key words Influenza, Reverse genetics, Tissue culture, Virus, Replication

1 Introduction

Influenza viruses are classified within the family orthomyxoviridae as segmented, single-stranded, negative sense RNA viruses [1]. Influenza A (IAV) and B (IBV) viruses are human and/or animal pathogens widely studied due of their importance to public health and animal production. IAV infects a broad host range of avian and mammalian species while IBV is primarily a human pathogen [2, 3]. While influenza A and B viruses differ in host range, they share many features. Both IAV and IBV possess a segmented, negative sense RNA genome composed of eight viral gene segments that encode for at least 12 viral proteins [4–6]. Each gene

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segment consists of a coding region (sometimes encoding for more than one polypeptide) flanked by short untranslated regions (UTRs). Influenza viruses contain two surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) [7], which are the main antigenic determinants that elicit neutralizing antibodies and play a key role in infection and propagation within the host [8–11]. IAVs are classified by their HA and NA into subtypes in which currently, 18 HA and 11 NA subtypes have been described [12–14]. Although IAV and IBV have similar genome structure, they diverge in the lengths of proteins and noncoding regions, the presence of accessory proteins, and antigenic properties of the internal proteins [3, 6, 15, 16]. Despite the structural and molecular differences between IAV and IBV, both can be generated by reverse genetics through similar approaches [17, 18].

Reverse genetics (RG) is an essential tool that allows for the generation of genetically modified RNA and DNA viruses from a cDNA copy [19]. This technique is highly employed in influenza virus research to understand different aspects of influenza replication, pathogenesis, transmission, and vaccine development [20–22]. Plasmid-based RG systems must produce two types of RNAs: (1) negative sense, viral genomic RNA copies of each segment for replication (vRNA), and (2) messenger RNA copies of the structural components, notably the ribonucleoprotein complex (RNP; comprised of the three RNA polymerase subunits PB1, PB2, and PA and the nucleoprotein (NP)) [23]. While many plasmid- and vector-based systems have been established, one of the more efficient systems consists of utilizing eight bidirectional plasmids containing RNA polymerase I and II transcription units in opposite orientations flanking the cloned IAV/IBV gene segment [23, 24]. The first unit contains a polymerase I promoter (*pol I*) and a polymerase I transcription terminator (TI) for the synthesis of single-stranded, negative sense, uncapped vRNAs [23, 25]. The second unit consists of an RNA polymerase II promoter (*pol II*) and a pol II poly A signal for the transcription of mRNA [6, 25, 26].

Previous reports have illustrated that the RNA *pol I* promoter has species specificity, which could potentially affect the efficiency of virus rescue [27, 28]. Recently, a bidirectional plasmid containing the swine *pol I* promoter (*spol I*) has been developed as an alternative reverse genetics bidirectional vector (referred to as pPIG2012) [29]. This plasmid vector was derived from an established bidirectional plasmid vector containing the human pol I (*hpol I*) (pDP2002) [23, 30]. The pPIG2012 RG vector was demonstrated to be efficient for de novo rescue of human-, swine- and avian-origin IAVs and IBV in swine and human origin cell lines [29]. Since swine-origin cells such as PK-15 cells have been previously used for vaccine production [31, 32], the pPIG2012 RG system expands the applicability regarding animal vaccine

production [29]. Utilizing the *spol I* RG system could be advantageous in transfection efficiency for the generation and/or expansion of influenza viruses but also limiting the potential for substrate-adapted changes, particularly to produce swine vaccines [29].

In this chapter we describe the details of reverse genetics techniques for type A and B influenza viruses using the 8-plasmid system with bidirectional promoters as described by Hoffman et al. [24] using the *spol I* system. The plasmid used in this chapter, pPIG2012, is a derivative of pDP2002 [23], in which the *hpol I* promoter was replaced with a minimal *spol I* promoter from the 45S ribosomal RNA promoter region of *Sus scrofa* [29]. The pPIG2012 plasmid (Fig. 1) contains the swine RNA *pol I* promoter (*spol I*) and an RNA polymerase II-driven cytomegalovirus (pCMV) promoter in opposite orientations. The *spol I* unit contains the promoter and TI to produce vRNAs with defined start and stop sites. The pCMV unit contains the promoter and the bovine growth hormone polyadenylation signal (aBGH). Influenza A and B cDNAs are cloned into the pPIG2012 RG vector using restriction sites artificially introduced at the 5′ and 3′ ends of each gene segment that are compatible with the restriction sites (BsmBI) on the pPIG2012 RG vector cloning region. We will provide primer specifications for cloning IAV A/turkey/Ohio/313053/2004 (H3N2) (Ty04) and IBV B/Brisbane/60/2008 (B/Bris) into pPIG2012 (Table 1).

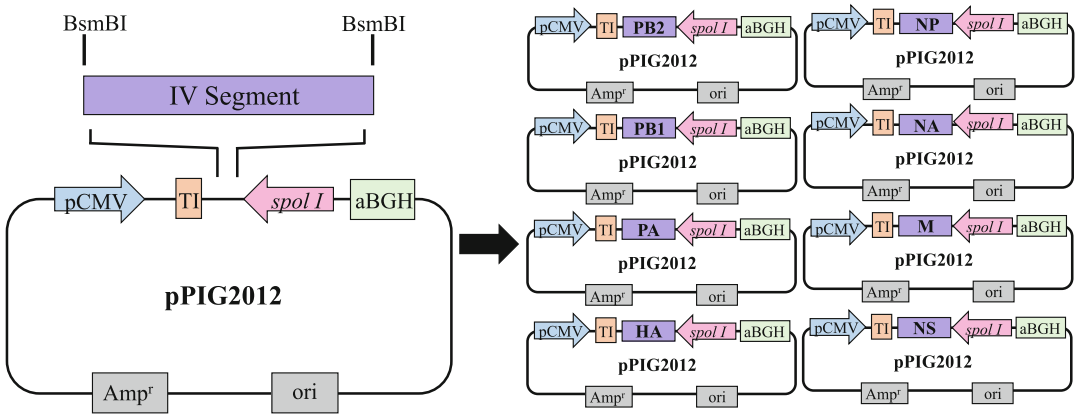

Fig. 1 Influenza vRNA cloning into pPIG2012 plasmid vector. Schematic representation of the pPIG2012 plasmid containing the swine polymerase I promoter (*spol I*, *pink arrow*) and the mouse polymerase I transcription terminator (TI, *orange box*) sequences that produce single-stranded, negative sense, uncapped IV vRNA synthesis. In the opposite direction of the *spol I* unit, a polymerase II-directed cytomegalovirus promoter (pCMV; *blue arrow*) and a bovine growth hormone polyadenylation signal (aBGH; *green box*) directs the synthesis of IV proteins from viral cDNA. The plasmid also contains an ampicillin resistant gene (Amp^r, *grey box*). Briefly, IV vRNAs are amplified by RT-PCR containing BsmBI or a compatible restriction enzyme site sequences. Following, the cDNAs are digested with the restriction enzyme and ligated into pPIG2012 BsmBI-digested plasmid vector containing the same sequence overhang. The process is to be repeated to generate eight IV plasmids for virus rescue (*right*)

Table 1
Primers used to clone IAV and IBV into pPIG2012

Gene	Forward primer		Reverse primer	
IAV—A/turkey/Ohio/313053/2004 (H3N2)				
PB2	Ba-PB2 1F	TATT*GGTCTC*AGGG**AGCGAAAGCAGGTC**	BaPIG-PB2 2341R	ATAT*GGTCTC*GAGAT**AGTAGAAACAAGG TCGTTT**
PB1	Bm-PB1 1F	TATT*CGTCTC*AGGG**AGCGAAAGCAGGCA**	BmPIG-PB1 2341R	ATAT*CGTCTC*GAGAT**AGTAGAAACAAGG CATTT**
PA	Ba-PA-1F	TATT*GGTCTC*AGGG**AGCGAAAGCAGG TAC**	B2PIG-PA 2233R	ATAT*GGTCTC*GAGAT**AGTAGAAACAAGG TACTT**
HA	Aar-HA 1F	TATT*CACCTGC*C TCAGGG**AGCAAAAGCAGGGG**	AarPIG-NS 890R	ATAT*CACCTGC*TATGAGAT**AG TAGAAACAAGGGTGTTTT**
NP	Ba-NP-1F	TATT*GGTCTC*AGGG**AGCAAAAGCAGG GTA**	BaPIG-NP 1565R	ATAT*GGTCTC*GAGAT**AGTAGAAACAAGG GTATTTTT**
NA	Bm-NA 1F	TATT*CGTCTC*AGGG**AGCAAAAGCAGG AGT**	BmPIG-NA 1413R	ATAT*CGTCTC*GAGAT**AGTAGAAACAAGG AGTTTTTT**
M	Bm-M-1F	TATT*CGTCTC*AGGG**AGCAAAAGCAGG TAG**	BmPIG-M 1027R	ATAT*CGTCTC*GAGAT**AGTAGAAACAAGG TAGTTTTT**
NS	Aar-NS 1F	TATT*CACCTGC*A TCGGGG**AGCAAAAGCAGGGTG**	AarPIG-NS 890R	ATAT*CACCTGC*TATGAGAT**AG TAGAAACAAGGGTGTTTT**
IBV—B/Brisbane/60/2008				
B-PB2	Bm-PB2b-1F_AK	TATT*CGTC TC*AGGG**AGCAGAAGCGGAGCGTTTTC**	pPig_B_Bris_PB2_R_AK	ATAT*CGTCTC*GAGAT**AG TAGAAACACGAGCATTTTTCACTC**
B-PB1	Bm-PB1b-1	TATT*CGTC TC*AGGG**AGCAGAAGCGGAGCC TTTAAGATG**	pPig_B_Bris_PB1_R_AK	ATAT*CGTCTC*GAGAT**AG TAGAAACACGAGCCTTTTTTCAT**
B-PA	Bm-Pab-1	TATT*CGTCTC*AGGG**AGCAGAAGCGG TGCGTTTGA**	pPig_B_Bris_PA_R_AK	ATAT*CGTCTC*GAGAT**AGTAGAAACACG TGCATTTTTGATTC**

B-HA	MDV-B 5′ BsmBI-HA	TATT*CGTC* *TC*AGGG**AGCAGAAGCAGAGCATTTTC TAATATC**	pPig_B_Bris_HA_R_AK	ATAT*CGTCTC*GAGAT**AGTAG TAACAAGAGCATTTTTCAAT**
B-NP	Ba-NPb-1F_AK	TATT*GGTC* *TC*AGGG**AGCAGAAGCACAGCATTTTC TTGTG**	pPig_B_Bris_NP_R_AK	ATAT*GGTCTC*GAGAT**AG TAGAAACAACAGCATTTTTTACAT**
B-NA	Bm-Nab-1	TATT*CGTC* *TC*AGGG**AGCAGAAGCAGAGCA**	pPig_B_Bris_NA_R_AK	ATAT*CGTCTC*GAGAT**AGTAG TAACAAGAGCATTTTTCAG**
B-M	Ba-Mb-1F_AK	TATT*GGTC* *TC*AGGG**AAGCAGAAGCACGCAC**	pPig_B_Bris_M_R_AK	ATAT*GGTCTC*GAGAT**AG TAGAAACAACGCACTTTTTCC**
B-NS	MDV-B 5′ BsmBI-NS F	TATT*CGTC* *TC*AGGG**AGCAGAAGCAGAGGATTTG TTTAGTC**	pPig_B_Bris_NS_R_AK	ATAT*CGTCTC*GAGAT**AGTAG TAACAAGAGGATTTTTATTTT**

Primer sequences used to amplify the eight gene segments from IAV A/turkey/Ohio/313053/2004 (H3N2) (Ty04) and IBV B/Brisbane/60/2008 (B/Bris) for cloning into pPIG2012. Shown in italics is the restriction enzyme recognition sequence and in bold is the primer sequence that encodes the 12 conserved and 13 conserved nucleotides on the 5′ and 3′ end of the cDNA. Sequences specific for individual genes are underlined. 5′ of all conserved sequences is a BsmBI (Bm), BsaI (Ba), AarI (Aar), restriction site (RE) for cloning

2 Materials

2.1 Influenza A and B Viral RNA (vRNA) Extraction

1. Viral RNA extraction kit (i.e., QiAmp, QIAGEN).
2. 200 proof ethanol (molecular grade).
3. 1.5 mL microcentrifuge tubes.
4. Nuclease-free water or DEPC-treated ddH_2O.
5. Solution for the removal of RNases (i.e., RNaseZap, Sigma).
6. Pipets to dispense from 1 to 1000 μL and corresponding filter pipet tips.
7. Laboratory equipment: pipettes and tips of appropriate sizes, vortex, microcentrifuge with a rotor capable of reaching up to 12,000 × *g*, spectrophotometer (NanoDrop or similar).

2.2 Influenza A and B cDNA Synthesis

1. Influenza A virus Universal Uni-12 primer (5′-AGCAAAAG CAGG-3′), 100 ng/μL stock.
2. Influenza B virus Universal Uni-11-A primer (5′- AGCA GAAGCGG-3′; for segments 1, 2 and 3), 100 ng/μL stock.
3. Influenza B virus Universal (Uni)-11-B primer (5′- AGCA GAAGCAG-3′; for segments 4, 6, and 8), 100 ng/μL stock.
4. Influenza B virus Universal (Uni)-11-C primer (5′- AGCA GAAGCAC-3′; for segments 5 and 7), 100 ng/μL stock.
5. Avian myeloblastosis virus (AMV) reverse transcriptase (RT) and buffer.
6. RNAse inhibitor (i.e., RNasin 40 U/μL, Promega).
7. dNTP mix, 2.5 μM each.
8. Nuclease-free water or DEPC-treated ddH_2O.
9. 1.5 mL tubes.
10. 250 μL PCR tubes.
11. Laboratory equipment: vortex, table-top minicentrifuge, and thermocycler.

2.3 Amplification of Influenza A and B Gene Segments from cDNA

1. Influenza A primer set for cloning the eight gene segments into pPig2012 bidirectional reverse genetics plasmid vector (Table 1).
2. Influenza B primer set for cloning the eight gene segments into pPig2012 bidirectional reverse genetics plasmid vector (Table 1).
3. Roche Expand high fidelity PCR system (Sigma Millipore).
4. dNTP mix, 10 μM each.
5. Nuclease-free water or DEPC-treated ddH_2O.
6. Agarose, LE analytical grade.

7. DNA gel staining solution (i.e., SYBR® Safe).
8. 1 kb ladder.
9. Gel extraction kit (i.e., QIAquick QIAGEN).
10. 1.5 mL microcentrifuge tubes.
11. 250 μL PCR tubes.
12. Ice or cooling tube racks.
13. Laboratory equipment: pipettes and tips of appropriate sizes, vortex, microcentrifuge with a rotor capable of reaching up to 12,000 × *g*, thermocycler, DNA gel electrophoresis system, transilluminator, water bath or heating block capable of reaching 50 °C, scale capable of determining weight in milligrams, NanoDrop (or similar spectrophotometer).

2.4 Bidirectional Reverse Genetics pPIG2012 Plasmid Vector

The plasmid pPIG2012 (Fig. 1) was derived from pDP2002 [23, 30], which contains a 29 nucleotides spacer sequence cloned between two BsmBI sites to easily discern the double-digested vector and to flexibly clone full length IAV or IBV cDNAs. The pPig2012 plasmid contains the RNA polymerase I and II transcription units in opposite orientations. The first unit contains the swine RNA polymerase I promoter (*spol I*, nucleotide positions 372–539 of Sus scrofa 45S ribosomal RNA promoter region) and the murine polymerase I transcription terminator (TI) for the synthesis of vRNAs. The second unit drives viral mRNA transcription from the RNA polymerase II-driven cytomegalovirus promoter (pCMV) and the bovine growth hormone polyadenylation signal (aBGH) (*see* **Note 1**). IAV and IBV cDNAs are cloned into the reverse genetics vector using restriction sites artificially engineered at the 5′ and 3′ ends of each segment in a manner compatible with the reverse genetics vector.

2.5 Cloning of Influenza A and B Virus Gene Segments into pPig2012

1. BsmBI-v2, BsaI, and AarI restriction endonucleases (New England Biolabs).
2. Alkalyne phosphatase (i.e., Antarctic phosphatase, New England Biolabs).
3. PCR purification kit (i.e., MinElute, QIAGEN).
4. DNA ligation kit.
5. Chemically competent cells (i.e., TOP10, ThermoFisher Scientific).
6. Super Optimal broth with Catabolite repression (SOC) medium.
7. BD Difco Luria Bertani (LB) broth supplemented with 50 μg/mL of ampicillin solution.
8. LB agar plates supplemented with 50 μg/mL of ampicillin solution.

9. Agarose, LE analytical grade.
10. Agarose gel staining reagent (i.e., SYBR® Safe, Invitrogen).
11. Gel extraction kit (i.e., QIAquick, QIAGEN).
12. Taq DNA polymerase reaction buffer (i.e., GoTaq® Green Master Mix Promega).
13. DNA miniprep kit (i.e., QIAprep Spin Mini).
14. Plasmid maxiprep kit (i.e., HiSpeed, Qiagen).
15. 1.5- or 2-mL microcentrifuge tubes.
16. 250 μL PCR tubes.
17. Ice or cooling tube racks.
18. Laboratory equipment: vortex, microcentrifuge with a rotor capable of reaching up to 12,000 × *g*, water bath or heating block able to reach 42 °C, thermocycler, DNA gel electrophoresis system, transilluminator, NanoDrop (or similar spectrophotometer), steady and shaker microbiological incubators set at 37 °C.

2.6 Cell Culture Growth and Maintenance

1. T-75 tissue culture flasks, canted neck.
2. Dulbecco's Modified Eagle's Medium (DMEM) high glucose (1×) liquid; with L-glutamine; without sodium pyruvate.
3. 100× Antibiotic-Antimycotic solution (100 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B.
4. L-Glutamine solution 200 mM.
5. HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) Buffer (1 M stock solution).
6. Phosphate buffered saline (1× PBS).
7. Fetal bovine serum (FBS), heat inactivated, sterile filtered.
8. Trypsin-EDTA (Trypsin-ethylenediaminetetraacetic acid, 0.05% trypsin; 0.53 mM EDTA.4Na (Sigma, cat. # T3924).
9. Serological pipets of the appropriate size as needed.
10. Sterile tubes or bottles appropriate to prepare and store media and reagents.
11. Laboratory equipment: cell culture incubator set at 37 °C with 5% CO_2, water bath set at 37 °C, pipettor for serological pipets, biosafety cabinet class II.

2.7 Generation of Influenza A and B Viruses by Reverse Genetics Using the pPig2012 Plasmid Sets

1. Madin-Darby canine kidney (MDCK) cells (ATCC, CCL-34).
2. PK-15 cells (ATCC, CCL-33) (*see* **Note 2**).
3. Influenza A and B pPIG2012 plasmid sets encoding for the eight gene segments for each virus.
4. Cell transfection reagent (i.e., Transit-LT1).

5. Opti-MEM Reduced Serum Medium (FisherScientific).
6. Trypsin L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK-trypsin) (Worthington Biochemical Corporation).
7. 100× Antibiotic-Antimycotic solution, cell culture grade (100 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B).
8. Non-coated 6-well tissue culture plates.
9. Sterile 1.5 mL screw-cap tubes.
10. Sterile conical tubes (15–50 mL).
11. Single channel pipettes capable of dispensing from 1 to 1000 μL.
12. Filtered pipet tips.
13. Sterile serological pipets (5–10 mL) as needed.
14. Laboratory equipment: cell culture incubator set at 35 °C or 37 °C with 5% CO_2, water bath set at 37 °C, hemocytometer or other cell counter, inverted microscope, pipettor for serological pipets, table-top minicentrifuge, biosafety cabinet class II, ultra-freezer set at −80 °C.

2.8 Propagation of Rescued Influenza A and B Viruses in MDCK Cells

1. Cleared infectious cell culture supernatant containing the rescued IAV or IBV.
2. Madin-Darby canine kidney (MDCK) cells (ATCC, CCL-34).
3. T-75 (or T175) tissue culture flasks, canted neck.
4. Dulbecco's Modified Eagle's Medium (DMEM) high glucose (1×) liquid; with L-glutamine; without sodium pyruvate.
5. Phosphate buffered saline (1× PBS).
6. Fetal Bovine Serum (FBS), Heat Inactivated, Sterile Filtered.
7. HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) Buffer (1 M stock solution).
8. Opti-MEM Reduced Serum Medium.
9. TPCK-trypsin (Worthington Biochemical Corporation).
10. 100× Antibiotic-Antimycotic solution (100 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B).
11. Single channel pipettes capable of dispensing from 1 to 1000 μL.
12. Filtered pipet tips.
13. Sterile serological pipets (5–10 mL) as needed.
14. Centrifuge conical tubes (15–50 mL).
15. Laboratory equipment: cell culture incubator set at 35 °C or 37 °C with 5% CO_2, water bath set at 37 °C, hemocytometer or

other cell counter, inverted microscope, pipettor for serological pipets, table-top minicentrifuge, biosafety cabinet class II, refrigerated centrifuge capable of containing 15- and 50-mL conical tubes set a t 4 °C ultra-freezer set at −80 °C.

2.9 Propagation of Rescued Influenza A and B Viruses in Embryonated Chicken Eggs

1. Cleared infectious cell culture supernatant containing the rescued IAV or IBV.
2. 9- to 11-day-old specific pathogen free (SPF) embryonated chicken eggs (ECEs).
3. 70% ethanol or Ethanol/iodine mix (70% ethanol/30% iodine).
4. Egg puncher (i.e., Dremel Multipro 7.2 V, model 770) or 16G × 1.5″ needle with robber stopper.
5. 1 mL syringes with 25G × 5/8″ needles.
6. 10 mL syringes.
7. 18G × 1″ needles.
8. Glue to seal the eggs (i.e., Elmer's glue).
9. 0.5% turkey red blood cells.
10. Phosphate buffered saline (1× PBS).
11. 96-well V-bottom plates.
12. Multichannel pipette able to dispense 50 μL with corresponding pipette tips.
13. Laboratory equipment: egg candler, egg incubator set at 33 °C or 35 °C, table-top minicentrifuge, biosafety cabinet class II, refrigerated centrifuge capable of containing 15- and 50-mL conical tubes set at 4 °C, ultra-freezer set at −80 °C.

3 Methods

3.1 vRNA Extraction from Influenza A and B Viruses

Dedicated equipment, supplies, and reagents solely for RNA work is highly recommended, *see* **Notes 3** and **4** for the precautions and recommendations to be followed during the RNA extraction procedure. We describe the RNA extraction procedure using QiAmp Viral RNA kit (QIAGEN) in the following.

1. Equilibrate Buffer AVE to room temperature.
2. Prepare Buffer AW1 and Buffer AW2 according to manufacturer's instructions.
3. Dispense 560 μL Buffer AVL into a 1.5 mL microcentrifuge tube. If sample volume is >140 μL than increase AVL buffer proportionally.
4. Add 140 μL of sample to Buffer AVL and pulse-vortex for 15 s.
5. Incubate at room temperature for 10 m.

6. Briefly centrifuge to remove droplets from the lid.
7. Add 560 μL of ethanol (96–100%) and pulse-vortex for 15 s. Briefly centrifuge to remove droplets from lid.
8. Add 630 μL of the solution from **step 5** to the column (within a 2 mL collection tube provided with the kit). Close the cap and centrifuge at 6000 × *g* for 1 m. Transfer the column into a clean 2 mL collection tube and discard previous tube.
9. Repeat **step 8** until all the lysate has been passed though the column.
10. Add 500 μL of buffer AW1 to the column. Close the cap and centrifuge at 6000 × *g* for 1 m.
11. Transfer the column into a clean 2 mL collection tube and discard previous tube.
12. Add 500 μL of buffer AW2 to the column. Close the cap and centrifuge at 6000 × *g* for 1 m.
13. Transfer the column into a clean 2 mL collection tube and centrifuge at full speed for 1 m.
14. Transfer the column into a clean 1.5 mL microcentrifuge tube and add 60 μL of room temperature buffer AVE (to elute the RNA). Close the cap and incubate at room temperature for 1 m.
15. Centrifuge at 6000 × *g* for 1 m. The RNA is in the flowthrough.
16. Measure the RNA concentration using a NanoDrop or spectrophotometer of your choice.
17. Store RNA at −80 °C if it cannot be used immediately.

3.2 IAV cDNA Synthesis (See Note 5)

1. In a 1.5 mL microcentrifuge tube, mix the following:
   (a) IAV vRNA (from Subheading 3.1): 4.0 μL
   (b) Uni-12: 0.5 μL
   (c) Nuclease-free water: 5.5 μL
   (d) Mix and heat at 70 °C for 5 m.
   (e) Immediately, incubate on ice for 2 m.
   (f) Spin down to collect all the contents at the bottom of the tube (<1 m).
   (g) Place the tube back on ice.
2. In a separate 1.5 mL microcentrifuge tube, prepare the reverse transcription cocktail by adding the following components (adjust volume based on total number of cDNA preparations to be performed):
   (a) 2.5 mM dNTPs (Thermo Fisher): 4.0 μL
   (b) 5× Reverse Transcriptase Buffer: 4.0 μL

(c) Rnasin 40 U/μL (Promega): 1.0 μL
(d) AMV Reverse transcriptase (Promega): 1.0 μL
(e) Mix well.

3. Add RT cocktail to RNA sample.
4. Incubate @ 42 °C for 1–2 h.
5. Heat inactivate @ 70 °C for 10 m.
6. Store cDNA at −20 °C until needed.

3.3 IBV cDNA Synthesis (See Note 5)

The steps below are for the preparation of IBV cDNA containing all eight gene segments in a single reaction. If interested on specific gene segments, add 0.5 μL of each desired primer and adjust the final reaction volume to 10 μL using nuclease-free water.

1. In a 1.5 mL microcentrifuge tube, mix the following:
   (a) IBV vRNA (from Subheading 3.1): 4.0 μL
   (b) Uni11-A primer: 0.5 μL
   (c) Uni11-B primer: 0.5 μL
   (d) Uni11-C primer: 0.5 μL
   (e) Nuclease-free water: 4.5 μL
   (f) Mix and heat at 70 °C for 5 m.
   (g) Immediately, incubate on ice for 2 m.
   (h) Spin down to collect all the contents at the bottom of the tube (<1 m).
   (i) Place the tube back on ice.
2. Repeat **steps 2** through **6** from Subheading 3.2.

3.4 Amplification of IAV and IBV Gene Segments from cDNA

Amplification of IAV and IBV gene segments from cDNA can be achieved with any high-fidelity PCR system; however, we recommend the use of Expand High Fidelity PCR system (Roche) as it has rendered the most robust results, and the protocol described below has been optimized for the use of this PCR system. *See* Table 1 for gene-specific primers to be used.

1. In a PCR tube, prepare reaction *mix 1* as follows and keep on ice:
   (a) cDNA: 1.0 μL
   (b) Forward Primer (100 ng/μL): 1.5 μL
   (c) Reverse Primer (100 ng/μL): 1.5 μL
2. In a 1.5 mL microcentrifuge tube, prepare reaction *mix 2* as follows (adjust volume accordingly based on number of reactions needed. 25 μL reactions can be prepared by cutting in halve the volumes for each reagent):

(a) dNTPs (10 μM each): 1.0 μL
(b) 10× Expand HF Buffer: 5.0 μL
(c) Expand HF: 0.75 μL
(d) Nuclease-free water: 39.25 μL

3. Add 45 μL of *mix 2* per each tube containing your *mix 1*.
4. Mix well and briefly spin down to collect the contents at the bottom of the tube. Keep on ice at all times until placed into the thermocycler.
5. Set the thermocycler with the following temperature conditions:

Step	Temperature	Time	No. of cycles
Initial denaturation	94 °C	4 m	1
Denaturation Annealing Extension	94 °C 56 °C 72 °C	20 s 30 s 5–7 m (*see* **Note 6**)	30
Final extension	72 °C	10 m	1
Hold	4 °C	∞	

6. Pre-heat thermocycler to the initial denaturation temperature before placing the reaction tubes.
7. Once hot, place the reaction tubes in the thermocycler and start the run.
8. Analyze PCR products by standard 1% agarose gel electrophoresis using SYBR® Safe gel staining as directed by the manufacturer.
9. Purify the PCR products from the agarose gel using QIAQuick gel extraction kit (QIAGEN) or gel extraction kit of your choice following the manufacturer's instructions.
10. Elute DNA with 27 μL of the provided elution buffer or nuclease-free water.
11. Quantify DNA using a NanoDrop or spectrophotometer of your choice.
12. Store DNA at −20 °C until needed if it cannot be used immediately.

Up to this point, eight PCR products containing full-length IAV or IBV gene segments should have been produced. Each of these segments is flanked by restriction sites compatible with the pPig2012 reverse genetics vector.

3.5 Cloning of IAV and IBV Gene Segments into pPIG2012 (See Fig. 2)

1. Using the purified PCR products from **step 10**, Subheading 3.4, set up the enzymatic digestions as follows:
   (a) Purified PCR product: 26 μL
   (b) 10× Restriction endonuclease buffer: 3 μL
   (c) Endonuclease (BsmBI, BsaI, or AarI): 1 μL
2. Perform digestions at 37 °C (BsaI and AarI) or 55 °C (BsmBI) for 4 h to overnight in a thermocycler or other device able to reach the required temperatures but some considerations need to be taken into account (*see* **Note 7**).
3. Next, purify the digested fragment using MinElute PCR Purification Kit. Digested product is eluted using 10 μL of elution buffer and measure the concentration using the spectrophotometer of your choice. Gel purification of PCR product after digestion is not necessary. DNA can be store at −20 °C if not used immediately.
4. Set digestion reaction to linearize the vector, in this case pPIG2012:
   (a) pPIG2012 (~100 ng/μL stock): 10 μL
   (b) 10× Restriction endonuclease buffer: 5 μL
   (c) Endonuclease (BsmBI): 2 μL
   (d) Nuclease-free water: 33 μL

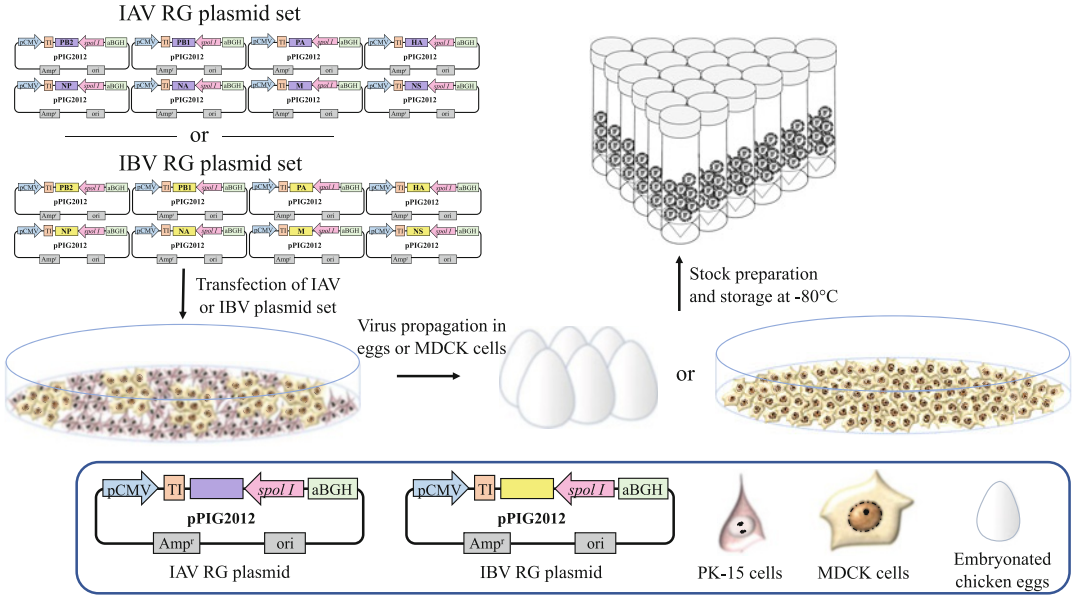

Fig. 2 Rescue of IAV and IBV viruses through reverse genetics. Eight reverse genetics plasmids carrying the corresponding IAV or IBV gene segments are transfected into PK-15/MDCK cell co-cultures. After virus recue has been confirmed, the infectious cell culture supernatants are inoculated into embryonated chicken eggs or into MDCK cell monolayers for stock preparation. Aliquots from the newly generated virus stocks are prepared and stored at −80 °C until needed

5. Set to incubate at 55 °C in the thermocycler or other device able to reach the required temperatures but some considerations need to be taken into account (*see* **Note 7**).
6. Run digested product through gel electrophoresis in a 1% agarose gel (using SYBR® Safe gel staining) at 100 V for 30 m. One band that shows an approximate size of 2,917 bp will be easily identifiable; excise the band from the gel, place into a 2 mL microcentrifuge tube, and record its weight. Use an empty 2 mL tube as blank to tare the scale.
7. Purify the DNA using the QIAquick Gel extraction kit following manufacturer's instructions and elute DNA in 30 μL of elution buffer.
8. Measure the concentration using a NanoDrop. Store DNA at −20 °C if not used immediately.
9. Proceed with ligation reactions following insert/vector ratio of 3:1 (*see* **Note 8**).
10. Calculate insert/vector ratio using the following formulas:

$$\frac{\text{Vector size in bp}}{\text{Insert size in bp}} = \text{molecular ratio}. \therefore$$

$$\frac{50 \text{ ng of vector}}{\text{Molecular ratio}} = 1 \text{ part of insert in ng} \times 3$$
$$= 3 \text{ parts of insert in ng}$$

11. Calculate the volume of insert and vector needed based on the concentrations obtained in **steps 3** and **8** and the calculations from **step 10**.
12. Set ligation reactions as follows for a total reaction volume of 21 μL (*see* **Note 9**):
    (a) Digested PCR product: *x* μL (from **step 11**)
    (b) Digested pDP2002 (50 ng total): *x* μL (from **step 11**)
    (c) 2× Quick Ligase reaction buffer: 10 μL
    (d) Nuclease-free water: Up to 20 μL
    (e) Quick Ligase: 1 μL
13. Mix well, quick spin, and set to incubate at room temperature for 5 m and then 2 m in ice. Ligation reactions can be stored at −20 °C if it cannot be used for transformation the same day.
14. Transform ligations reactions into TOP10 cells following manufacturer's instructions.
15. Spread 100 μL of transformation rxn onto LB agar plates supplemented with 50 μg of ampicillin.
16. Set to incubate overnight at 37 °C. Transformants can be screened by colony PCR (cPCR) the following day.

3.6 Screening of Transformant Colonies by cPCR

After 16 h of incubation at 37 °C, plates from **step 15**, Subheading 3.5, should have colonies with potential positive reverse genetics (RG) clones carrying the desired IAV and IBV gene segments. It is recommended to screen between 5 and 10 transformant colonies. To screen for RG clones carried into the pDP2002 vector, we recommend using the following primer set: PTI2F (5′- CTAG CAGTTAACCGGAGTACTGGT-3′) and SwinePol1Rev (5′-CAG GGCTGGGGGACAAGTG-3′).

1. Before starting, pre-warm an LB agar plate (with 50 μg/mL of ampicillin). Draw a grid and label the compartments with the corresponding colony ID numbers. This plate will serve to save a copy of the colonies selected for screening.
2. In a 1.5 mL microcentrifuge tube, prepare cPCR reactions as follows (amounts disclosed bellow are per reaction, adjust depending on the number of reactions to be performed):
   (a) 2× GoTaq Master mix: 5.0 μL
   (b) PTI2F (100 ng/μL stock): 0.5 μL
   (c) SwinePol1Rev (100 ng/μL stock): 0.5 μL
   (d) Nuclease-free water: 4.0 μL
3. Aliquot 10 μL of reaction mix into 250 μL PCR tubes.
4. With a sterile 10 μL pipette tip or a sterile toothpick, collect the selected colonies one at a time, perform this procedure under the flame to keep sterility. Then touch the pre-warmed LB plate (*see* **step 1**) to inoculate the bacteria on the surface of the agar (within the designated compartment). Set plate to incubate at 37 °C overnight.
5. Next, immerse the same bacteria containing tip and pipet up and dawn into the corresponding cPCR mix tube.
6. Mix well and briefly spin down to collect contents at the bottom of the tube. Always keep reactions on ice until they are loaded in the thermocycler.
7. Set thermocycler with the following temperature conditions:

Step	Temperature	Time	No. of cycles
Initial denaturation	94 °C	5 m	1
Denaturation Annealing Extension	94 °C 56 °C 72 °C	30 s 30 s 5 m	30
Final extension	72 °C	10 m	1
Hold	4 °C	∞	

8. Place the reaction tubes in the thermocycler and run at the specified temperature conditions.
9. Analyze cPCR products by gel electrophoresis in a 1% agarose gel stained with SYBR® Safe gel staining and select potential positive candidates for sequencing based on the cPCR results.
10. To isolate the plasmids from the selected clones, prepare 50 mL conical tubes containing 5 mL LB broth supplemented with 50 μg/mL of ampicillin.
11. Retrieve the LB plate containing grown copies of the screened clones. Using a sterile 10 μL pipette tip, pick the desired colony and inoculate the corresponding tube with LB broth.
12. Set cultures to incubate overnight (~16 h) at 37 °C/225 rpm in a shaker incubator.
13. The next day, pellet the bacterial cultures at 6800 × *g*/3 min, discard the supernatant and proceed with plasmid purification using the QIAprep Spin Mini Kit (QIAGEN). Follow manufacturer's instructions (*see* **Note 10**).

The purified plasmids are now ready for sequencing. In addition to cPCR screening, plasmids need to be sequenced to confirm the presence of unexpected mutations in either the gene segment or the plasmid vector, as well as the presence of a complete IAV or IBV gene segment and its orientation.

3.7 Cell Culture Growth and Maintenance

If cells have been kept in liquid nitrogen, perform fast thawing following source's instructions. For general growth and care, follow the recommendation listed below.

1. After extracting a vial of cells from the liquid nitrogen, thaw quickly in 37 °C water bath.
2. Dispense the contents of the vial into a T25 flask containing pre-warmed DMEM supplemented with 10% FBS and 1× Penicillin/Streptomycin solution, 2 mM L-Glutamine and 2.5% HEPES (complete media).
3. Place the cells into a cell culture incubator set at 37 °C/5% CO_2.
4. When the cells have reached confluence (about 24–48 h), remove and discard the media.
5. Wash cells twice with 2 mL of pre-warmed, sterile 1× PBS.
6. Add 1 mL of 0.25% Trypsin-EDTA solution and let incubate for 20–45 m (MDCK cells) or (PK-15 cells) at 37 °C (in the cell culture incubator) until the cells have completely detached. You may tap the sides of the flask to help with detachment.
7. Add an equal volume of complete media to stop the trypsinization and wash the bottom of the flask by pipetting up and down (*see* **Note 11**).

8. Pipette up and down a few times to resuspend the cells and transfer into a conical tube or appropriate size (15–50 mL).
9. Centrifuge at 450 × *g* for 5 min at 4 °C.
10. Discard supernatant and add 5 mL of pre-warmed media and pipette up and down until cells have been resuspended. Adjust the volume to 13 mL with complete media.
11. Transfer the cell resuspension to a T75 flask and rock to ensure even distribution of cells.
12. Place flask into the cell culture incubator under the same conditions as above.
13. Pass cells every 3 days following **steps 4–10** but using 5 mL of 1× PBS for the washes and 2–3 mL of 0.25% Trypsin-EDTA solution for trypsinization.

3.8 Rescue of IAV and IBV Using the Swine poll Reverse Genetics Plasmid Sets

Before starting a transfection, it is recommended to prepare high quality plasmid preparations (*see* **Note 10**) using the HiSpeed Plasmid Maxi kit (QIAGEN) or other similar kit of your choice.

The following procedure is very simple but also prone to cross-contamination if not careful. Use extreme care when handling cells, transfection reagents, plasmids, and media. The procedure described below is for performing transfections using a 6-well plate format and PK-15/MDCK cell co-cultures. It is also amenable for scaling down to 12- and 24-well plate formats (*see* **Note 12**).

1. Wash, treat with trypsin and pellet cells as described in Subheading 3.7.
2. Resuspend pelleted cells in 5 mL of Opti-MEM supplemented with 1× antibiotic/antimycotic solution (Opti-MEM-AB/AM) and proceed to count manually or with an automated Cellometer.
3. Based on cell count results, calculate the volume needed from each cell suspension to seed 5×10^5 PK-15 cells and 2×10^5 MDCK cells/well (6-well plate). Bring co-culture cells suspension to a volume large enough to add 2 mL of cell suspension/well.
4. Rock plate(s) to evenly distribute cells in the wells and set to incubate at 37 °C/5% CO_2 overnight (~16–24 h).
5. The next day, when the co-cultures have reached ~80–90% confluency, prepare the cocktail of 8 plasmids (one plasmid per influenza viral RNA gene segment) in a 1.5 mL micro centrifuge tube. Please note that the use of filter tips is recommended for this and subsequent steps to avoid potential contaminations. If a working stock of each plasmid has been prepared at 100 ng/μL, pipet 10 μL of each plasmid (1 μg of each plasmid, 8 μg pDNA) and set aside.

6. In a separate tube mix 104 μL of plain Opti-MEM and 16 μL of TransIT-LT1 (2 μL/μg of plasmid DNA). It is easier to calculate the total amount of Opti-MEM/ TransIT-LT1 needed for all the transfections and prepare a cocktail with the total volume (plus extra ~10% excess for both).
7. Vortex the Opti-MEM/TransIT-LT1 cocktail and add 120 μL to the plasmid DNA mixture and Mix well. It is essential to change tips before going into the next sample to avoid cross-contamination.
8. Incubate the mixture at room temperature for approximately 45 m, and then, very slowly, add 800 μL of plain Opti-MEM. DO NOT mix.
9. Remove the medium from the cells monolayers and add the transfection mixture to the corresponding wells. Washing the cells prior to addition of the transfection mixture is not necessary as no FBS was added to the seeding media. Use caution when adding transfection mixture onto the cells. It is best to place the pipet tip as close as possible to the cell monolayer and pipet down dropwise or deposit by the wall of the well to prevent aerosol formation.
10. Incubate the cells for 6 h to overnight (~16 h) at 37 °C/5% CO_2 (*35 °C/5% CO_2 for IBV*).
11. After incubation time has been completed, tilt the plate and carefully remove the transfection mixture from cells by positioning the pipette tip at the corner of the well where the liquid has accumulated. Discard in 10% bleach solution.
12. Then, add 1 mL of Opti-MEM-AB/AM to the cells and incubate at 37 °C/5% CO_2 (*35 °C/5% CO_2 for IBV*) until 30 h post-transfection (hpt) have been completed.
13. At 30 hpt, add 1 mL of Opti-MEM-AB/AM containing 2 μg/mL TPCK-trypsin to the each transfected well (1 μg/mL final concentration per well).
14. Monitor cells daily for cytopathic effect (CPE), particularly in the MDCK cells. Small foci of dead cells are indicative of active influenza virus replication may become apparent for some, but not all, successful virus rescues starting at 72 hpt.
15. If no CPE is observed at 72 hpt (or later if desired, up to 120 hpt), a blind passage in MDCK cells may be performed. Add an additional 1 mL of Opti-MEM/AB/AM + 3 μg/mL TPCK-trypsin (1 μg/mL final concentration per well) at 72 hpt if decided to prolong the incubation passing this time point.
16. To perform the blind passage, withdraw the cell culture supernatant into the appropriate centrifuge tube and centrifuge at 5000 rpm, at 4 °C, for 5 m.

17. Without disturbing the cell pellet, transfer the supernatant to a fresh tube.
18. Remove the media from the MDCK cell monolayer and add 500 μL of cleared supernatant to the corresponding well.
19. Set to incubate for 1 h at 37 °C/5% CO_2 (*35 °C/5% CO_2 for IBV*), rocking the plate every 15 m.
20. Next, discard the supernatant and add 2 mL of Opti-MEM-AB/AM+1 μg/mL TPCK-trypsin per well and monitor daily for CPE and/or HA assay at 48–72 hpi.
21. CPE and/or HA positive samples can be either stored at −70 °C for later or further propagated in either MDCK cells or 9–11-days old chicken embryonated eggs.

3.9 Propagation of IAV and IBV in MDCK Cells

1. The day before infection, wash and treat MDCK cells with trypsin as described in Subheading 3.7. Resuspend cells with 10 mL of complete growth media (DMEM supplemented with 10% FBS and 1× Penicillin/Streptomycin solution, 2 mM L-Glutamine and 2.5% HEPES).
2. Into a new T75 Flask, add 2 mL of cell suspension and 10 mL of complete media.
3. Set cells to incubate at 37 °C/5% CO_2 overnight (~16–24 h).
4. Once the cells have reached ~80–90% confluency, using plain Opti-MEM, make a 1/100 or 1/1000 from the IAV/IBV transfection or blind passage supernatant (from Subheading 3.8), based on the CPE severity and of HA titer.
5. Then wash cells 2× with 5 mL of 1× PBS.
6. Next, inoculate cells (in the T75 flask) with 2–3 mL of the IAV/IBV dilution and incubate for 1 h at 37 °C/5% CO_2 (*35 °C/5% CO_2 for IBV*), rocking the flask every 15 m.
7. Afterwards, remove the inoculum and add 10 mL of Opti-MEM-AB/AM+1 μg/mL TPCK-trypsin and monitor daily for CPE and/or HA assay at 48–72 hpi. CPE may start appearing at 48 hpi for IAV and 72 hpi for IBV. Incubation may be prolonged beyond 72 hpi (up to 120 hpi) if deemed appropriate in order to maximize the amount of virus recovered from the culture.
8. Determine the hemagglutination titer by performing HA assay as described on Subheading 3.10, **steps 12–15**.
9. Collect the cell culture supernatant into a 15 mL conical tube and clear by centrifugation at 2500 rpm for 10 m at 4 °C.
10. Transfer the cleared supernatant into a new 15 mL conical tube (set in ice), homogenize by pipetting up and down, and prepare aliquots of the desired volume.
11. Store aliquots at −80 °C.

3.10 Propagation of IAV and IBV in ECEs

1. Candle embryos and mark the limit between the air chamber and the allantoid cavity.
2. Label eggs accordingly.
3. Prepare tenfold dilutions of the virus starting by mixing 100 μL of transfection or blind passage supernatant (from Subheading 3.8) with 900 μL of 1×PBS (supplemented with 1× antibiotic/antimycotic solution) and keep in ice until needed.
4. Decontaminate eggshell with 70% ethanol or Ethanol/Iodine solution.
5. Drill/ punch a hole on the shell a few millimeters (~3 mm) from the mark between the air chamber and the allantoid cavity.
6. Using 1 mL syringes with a needle (25G × 5/8″), inoculate each dilution into 3–5 embryos (10^{-2} through 10^{-7}), with 100 μL/embryo.
7. Seal the hole with glue.
8. Set embryos to incubate at 33 °C (IBV) or 35 °C (IAV) for 48 h candling daily to remove unspecific mortality. No virus-induced mortality is expected from embryos inoculated with low pathogenic IAV or IBV.
9. Once incubation has been completed, chill embryos overnight at 4 °C.
10. Using 1 mL syringes with needle, collect 100 μL of allantoic fluid (AF) from each chilled embryo into tubes or a 96-well plate.
11. Prepare 96-well V-bottom plates (as many as needed) by adding 50 μL of 1× PBS to each well.
12. Add 50 μL of AF to individual wells of column 1.
13. Perform twofold dilutions from columns 1 through 12.
14. Next, add 50 μL of 0.5% turkey red blood cells to each well.
15. Tap or shake the plate to homogenize and set to incubate for 45 min at room temperature.
16. Select the highest dilution at which all the inoculated embryos give the highest HA titer higher to avoid viral interfering particles.
17. Into a single 50 mL conical tube (set in ice), collect as much AF as possible from each egg from the selected dilution.
18. Next, clear the AF by centrifugation at 2500 rpm for 10 m at 4 °C.
19. Transfer the cleared AF into a new 50 mL conical tube (set in ice), homogenize by pipetting up and down, and prepare aliquots of the desired volume.
20. Store aliquots at −80 °C.

4 Notes

1. Other ambisense/bidirectional plasmids, such as pDZ [33] or pHW2000 [24], can be used for reverse genetics of IAVs with appropriate amplification primers.
2. Alternatively, MDCK/human embryonic kidney (HEK293T) co-cultures could be used for the rescue of influenza viruses when using the *spol I* reverse genetics system. However, HEK293T are not an approved cell line for vaccine development. In addition, HEK293T cells are less efficient for the rescue of IAV and IBV when employing the *spol I* reverse genetics system [29].
3. Dedicated equipment, supplies, and reagents solely for RNA work is highly recommended. Disposable gloves must be worn at all times while performing the RNA extraction procedure and while handling RNA samples to avoid RNA degradation. Extracted RNA samples should be kept on ice at all times and, preferably, be used immediately for cDNA synthesis. Remaining RNA can be kept frozen at −70 °C. Typically, RNA samples stored for more than a month are not reliable and are to be discarded. Consult the Kit's manufacturer's instructions for more details about storage.
4. The vRNAs are extracted using silica-based (i.e., QiAmp Viral RNA kit) or organic-based (TRIzol reagent, Invitrogen) reagents or any other available RNA extraction method following procedures recommended by the manufacturer. RNA extraction using TRIzol reagent is preferred when extracting RNA from samples with low viral loads. We recommend the QiAmp Viral RNA kit, which has provided consistent and reliable results, with adequate vRNA purity and devoid of contaminants and ribonucleases. Viral titers of $\geq 10^6$ $TCID_{50}$ ($\geq 10^7$ EID_{50}) are typically required for efficient preparation of vRNAs and subsequent RT-PCR amplification. Virus isolation from clinical samples, tissue cultured MDCK cells or 9- to 11-days-old chicken embryonated eggs are recommended in order to obtain appropriate quantities of vRNA for downstream procedures. Extracted vRNAs are eluted and resuspended in RNase-free ddH_2O. RNA concentration is quantified using a NanoDrop or similar spectrophotometer.
5. Synthesis of cDNA can be performed using other reverse transcriptase following manufacturer's directions. The PCR amplification step is crucial; the use of other thermophilic DNA polymerase enzymes is possible but those mentioned above have provided the most robust results.
6. For optimal amplification of IAV and IBV segments, use an extension time of 5 m when amplifying the NP, NA, M, and NS

gene segments. When amplifying the PB2, PB1, PA, and HA genes, use an extension time of 7 m.

7. If using a water bath, please note the potential for excessive evaporation resulting from prolonged incubation periods.
8. The recommended insert/vector ratio for the ligation reaction depends on the size of both the insert ant the vector. When the vector is larger than the insert, a 3:1 ratio is recommended. If the insert is of equal or larger size than the vector a 1:1 ratio would be more appropriate.
9. The final volume of the ligation reaction can be adjusted if the vector and/or insert concentrations are too low that require the use of larger volumes. Just scale up the rection buffer and scale down the nuclease-free water volumes accordingly to maintain the proper concentrations in the reaction.
10. Plasmids are purified using commercially available plasmid preparation kits, following the manufacturer's recommendations, and are best stored at −20 °C at a concentration of 0.25–1.0 mg/mL. It is recommended to prepare high quality plasmids preparation for transfection experiments as impurities may interfere with the transfection efficiency. Measure DNA and RNA concentrations at an absorbance of 260 nm using a Nanodrop or similar spectrophotometer. The 260/280 ratio is used to estimate sample purity. A plasmid preparation with a 260/280 nm ratio of $\geq$1.8 is highly recommended. We also recommend that plasmid stocks are diluted into working stocks at a concentration of 100 ng/μL prior to transfection to minimize pipetting errors and to standardize pipetting volumes for each plasmid. The integrity of the plasmid DNA should be assessed by electrophoresis in agarose gels before each transfection.
11. The minimum volume of complete media to be added to stop the trypsinization has to equal the volume of trypsin that was added. One can always add more trypsin to ensure proper wash of the attachment surface and decrease bubble and foam formation.
12. Although transfection can be performed in smaller well plate formats, the efficiency of virus rescue decreases with smaller wells due to the fewer number of cells susceptible for transfection.

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Chapter 15

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 toward 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 toward a vaccine. Here, we describe staining of bovine lymphocytes with peptide (p)-MHC class I tetramers and antibodies specific toward 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, and a newly developed and simple assay to measure TCR avidity.

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

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

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[9, 10]. Intracellular staining allows for the quantification of immune 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. These spots can be counted with an ELISpot reader (Fig. 1). 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 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, e.g., radioactive chromium (^{51}Cr), washed and incubated with effector cells. Then the supernatant is collected and lysis is measured 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 to us 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

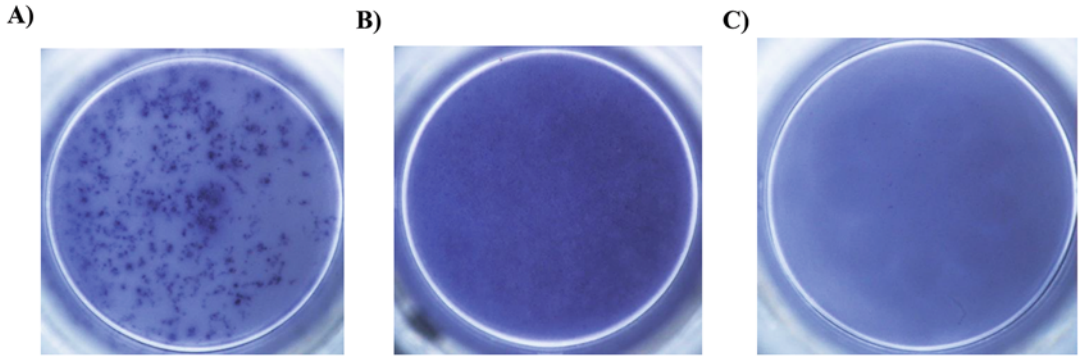

Fig. 1 ELISpot results as observed on a PVDF membrane in an individual well of a 96 well plate. (**a**) A positive well. Dark blue spots over a light blue background are indicative of the presence of positive cells secreting the cytokine of interest (in this example IFNγ is measured). Spots can be counted 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

identification [23, 24]. Furthermore, the ability to measure TCR avidity can help in selecting the best epitope to include in a vaccine. Several assays have been developed to measure the avidity of TCRs toward their epitopes, such as tetramer dilution assay [25] or the use of MHC class I-specific antibodies that compete with tetramer binding [26]. In this protocol, we describe an assay to measure both K_{OFF} and K_{ON} rates of peptide-MHC class I to TCR.

Earlier versions of some of these methods have been described in the past [27] 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, 28]. 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/mole), 51 ml of FBS. Buffer is kept at 4 °C.

4. PBS-0.5% bovine serum albumin (BSA): Dulbecco's PBS (1×), 0.5% bovine serum albumin (BSA).
5. PBS-2% formalin (or PBS-1% paraformaldehyde): Dulbecco's PBS (1×), 2% formalin or 1% paraformaldehyde.
6. Round (or V-shaped)-bottom 96-well plates and flowcytometry tubes (BD Pharmingen).
7. Ficoll-Paque (GE Healthcare).
8. 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.
9. Anti-perforin antibody (BD Pharmingen, cat #: 556577; ready-to-use therefore no dilutions are required; volume 20 μl).
10. Anti-Fas-L antibody (sc-957) (Santa Cruz Biotech).
11. Any primary antibody of interest.

2.2 TCR Avidity

1. Tetramers (can be procured through immunAware, Denmark, or any other biological supplies company).
2. Dasatinib (Selleckchem, cat # S1021). Stock solution is made in DMSO, e.g. 10 mM.
3. Anti-IgG1-FITC (SouthernBiotech cat #1070-02).
4. Fixable Viability Stain 450 (BD Horizon).
5. Graph Pad Prism software.

2.3 ELISpot

1. ELISpot plates: Millipore MAIP S45. 96 well ELISpot plate.
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. 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 sterilized. 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* under Subheading 2.1 flow cytometry.
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. Anti-rabbit IgG-alkaline phosphatase conjugated (clone R696, Sigma-Aldrich).
14. BCIP/NBT substrate tablets (cat. No. B-5655, Sigma-Aldrich). Dissolve 1 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 (Sigma, clone R696diluted 1:2000 in PBS-T/BSA).
16. Distilled (dH_2O).
17. Sterilin tubes.
18. Concanavalin A (mitogen).
19. Mitomycin or radiation source.
20. Plate shaker, CO_2 cell incubator and ELISpot reader.

2.4 CTL Cytotoxicity Assay

1. Ficoll-Paque (GE Healthcare).
2. RPMI 1640 complete: *see* under Subheading 2.3.
3. Cytotoxicity medium: as RPMI 1640 complete medium but with 5% FBS instead.
4. PBS: *see* under Subheading 2.1 flow cytometry.
5. Tissue culture plates, sterile, 24-wells, and 96-wells.
6. Recombinant human interleukin (IL)-2 (Sigma) or 15% T cell growth factor (TCGF).
7. Plastic pipettes, sterile, 5, 10, 25 ml.
8. Chromium 51 (Na_2CrO_4), aqueous sterile solution.
9. LumaPlates (PerkinElmer).
10. TopSeal film (PerkinElmer).
11. Scintillation counter for plates.
12. Mitomycin or radiation source.
13. Cell CO_2 incubator.

2.5 Immunoinformatics

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 following section, 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 polypropylene Falcon 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 × g_{AV} (g force average) 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 × 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 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 × g_{AV} for 10 min).
9. A second PBS wash is performed at 300 × g_{AV} for 10 min.
10. Resuspend in Dulbecco's PBS 1×.
11. Count cells and dispense 2–5 × 10^5 PBMC/well (*see* **Notes 3** and **4**).
12. Spin down the plate at 830 × g_{AV} 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 × $g_{AV.}$
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 × 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 30 min at room temperature (*see* **Notes 7** and **8**).
18. Spin down at 830 × g_{AV} for 3 min and remove the supernatant.
19. Add the primary antibodies of interest (20 μl of each antibody 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 5–40 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 example, for staining with our in-house primary anti-CD8 antibody (ILA51; IgG1) we use 1 μl per 2–5 × 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 20** 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 TCR Avidity Assay

This assay is designed to measure TCR avidity by using two different tetramers coupled to different fluorophores. The principle behind the assay is to measure the ability of a second tetramer, generated with the same restricting MHC class I molecule as the first tetramer used in staining but containing a different peptide, and added in excess, to outcompete the first tetramer for binding to the same TCR. Half-life and binding time can then be calculated by flow cytometry analysis. For further details, please refer to Fig. 2.

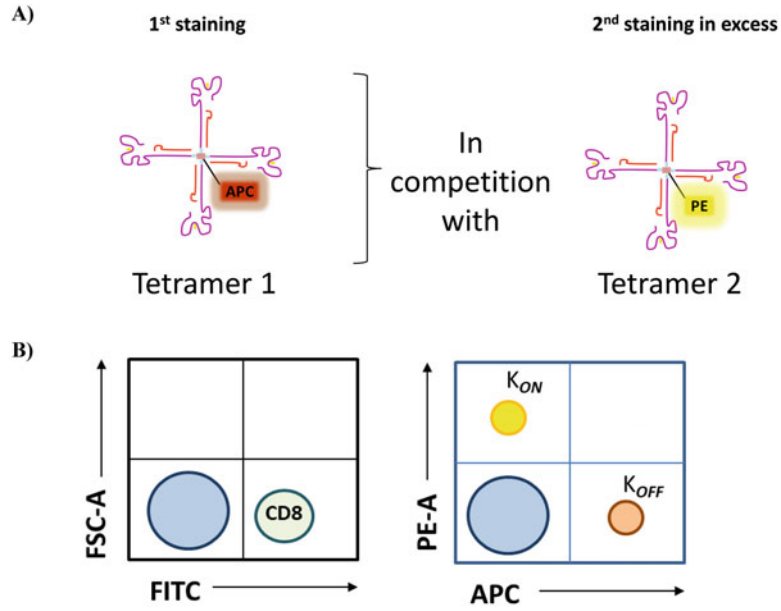

Fig. 2 TCR avidity assay by dual tetramer staining. (**a**) Schematic presentation of the principle of the TCR avidity assay using two tetramers generated with two different fluorophores. The second tetramer is added in excess after the washing step to at least five times the amount used for the first tetramer. (**b**) Schematic representation of the gating of $CD8^+$ T cells and tetramer-PE or tetramer-APC positive cells derived from the $CD8^+$ T cell quadrant. K_{ON} and K_{OFF} gates illustrates gates to measure binding time and half-life value, respectively

1. Collect cytotoxic T lymphocytes (CTL). This can be CTL lines (Subheading 3.4.1), which originates from CD8 cell that have been isolated from immune animals. They are kept growing by stimulating them with antigen in culture.
2. Wash once and resuspend in Dubelcco's PBS 1×-0.5% BSA containing 50 μM of dasatinib.
3. Plate between 2×10^5 and 5×10^5 cells per well in a round-bottom 96-well plate.
4. Incubate cells for 30 min at 37 °C to prevent TCR down-regulation and intracellular recycling.
5. Spin down the cells and remove the supernatant.
6. Add your tetramer of choice coupled to allophycocyanin (APC) (5 nM equivalent of heavy chain concentration) in 25 μl of PBS 1×-0.5% BSA containing 50 μM of dasatinib (inhibits recycling of the TCR complex from the cell surface) and anti-CD8 at dilutions of 1:250.
7. Incubate at room temperature for 20 min.
8. Wash twice with PBS 1×.

9. Resuspend cells in 25 μl of PBS 1×-0.5% BSA containing 50 μM dasatinib, anti-IgG1-FITC at a dilution of 1:500 and Fixable Viability Stain 450 diluted 1:1000.
10. Incubate at room temperature for 20 min.
11. Wash twice with PBS 1×.
12. Resuspend cells in 50 μl of PBS 1×-0.5% BSA containing 50 μM dasatinib and your competing tetramer of choice coupled to phycoerythrin (PE) at an excess concentration of at least five times the concentration of the staining tetramer (in this case 25 nM equivalent of heavy chain concentration) (Fig. 2a).
13. Incubate cells at 37 °C in a humidified atmosphere containing 5% CO_2.
14. Every 15 min: (a) collect cells for each time point, (b) wash twice with PBS 1×, (c) resuspend cells in 100 μl of PBS 1× containing 1% paraformaldehyde (PFA), (d) store at 4 °C until analysis with a flow cytometer.
15. Continue **step 14** up to 165 min (12 time points) or until your time point of choice.
16. Analyze samples on a BD FACS Canto II flow cytometer: (a) set the parameters by performing an automatic compensation using compensation controls for PE, APC, FITC, and Pacific Blue with the FACS DIVA software. (b) Select single cells, live cells, followed by cells appearing in the lymphocyte's quadrant. CD8+ cells can then be selected of which the tetramer-positives cells can be derived and analyzed (Fig. 2b). (c) Acquire at least 50,000 events in the lymphocytes gate for the analysis. For ex vivo lymphocytes, the acquired events should be between 250,000 and 500,000. (d) Calculate half-life (K_{OFF} in minutes) (Fig. 2b) as follows: Measure the percentage (%) of tetramers bound at time (*t*) Xmin in the APC positive gate only; binding at *t* 0 min is set at 100% and the % binding at (*t*) Xmin is calculated relative to the % bound at t 0 min. K_{OFF} is then determined by a non-linear curve fit (dissociation − one phase exponential decay) with GraphPad Prism. (e) Calculate binding time (K_{ON} in minutes) (Fig. 2b) as follows: Measure the % of the tetramer-PE bound at (*t*) Xmin in the PE-positive gate only, and the K_{ON}, binding time in minutes, needed to achieve a half maximum binding, which is determined by a non-linear curve fit (one site-specific binding) with GraphPad Prism.

3.3 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 manufacturer's protocol for purification of cell subsets. The way we perform this is listed below.

3.3.1 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 × 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 30 min at 4 °C.
6. Wash × 2 (6–8 min 300 × g_{AV}).
7. Pellet the cells at 300 × 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 × g_{AV}) in PBS/FBS.
10. Prepare the MiniMACS column, place on the magnet and flush through with 0.5 ml 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.

3.3.2 ELISpot

1. In a cell culture hood, coat Millipore MAIP S45 plates overnight at 4 °C with 50 μl/well of anti-bovine IFN-γ capture monoclonal antibody CC302 diluted at 1 μg/ml in sterile coating buffer. 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} M 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, at least 5000 rads) 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 dilutions starting from, e.g., 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 two minutes 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.
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 BCIP-NBT substrate solution, and incubate plates in the dark for 10 min at RT. 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.

3.4 Cytotoxicity

CTL assays are composed of a number of discrete methods, namely the generation of CTL (in this case from PBMC), which require 2–3 in vitro restimulations, labeling of target cells (here: 51-Chromium) and then the actual CTL assay.

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

1. *Primary in vitro stimulation*. 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 (TpM) using a Caesium source for 30 min at a distance of 10 cm of the Cs137 source at 265 rads per min using a top linear irradiator [Conservatome, France]) or a similar source. 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. Add TpM at a ratio of 1:5 to 1:10 (CTL:TpM), and supplement the media with 2 ng/ml of recombinant human interleukin (IL)-2 (Sigma) or 15% T cell growth factor (TCGF).
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%).
7. *Secondary* in vitro *stimulation*. *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 culture medium 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.
10. *Tertiary* in vitro restimulation. *Optional*: purification of CD8 cells. Restimulation of the bulk cultures may propagate other cell subsets than CD8 cells. Therefore, we normally purify the CD8 cells before the tertiary restimulation. 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.

3.4.2 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-labelled 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.

Infected cell lines can be used as target cells if they are MHC matched with the CTL. The labeling of infected target cells are described below.

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 repeat wash and centrifugation 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.

Peptide pulsed PBMC is another way of providing target cells for the CTL, which allow selecting specific CTL epitopes as targets. Description of the labeling of peptide pulsed PBMC is described below.

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 2–3 times during the incubation.
5. Wash 3× and adjust to 1×10^6 cells/ml in complete RPMI.

After the labeling of target cells, the cytotoxic T-cells (effectors) need to be prepared. This is described below.

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.

Finally, the cytotoxicity assay can be set up, where labeled target cells and CTL are incubated together. This is described below.

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 multi-channel 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 -C, 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 multi-channel pipette and centrifuge the plate(s) for 5 min at RT 300 × 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 (*see* **Note 21**). This will result in a very high level of counts. 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.

3.5 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- to 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 to 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 toward 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, e.g. 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 × 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, 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 × 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 auto-fluorescence 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 Iodine (PI), &-7-Aminoactinomycin (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. Singlets can be identified by plotting Forward Scatter Hight (FSC-H) versus Forward Scatter Area (FSC-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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