Chapter 14

Construction and Application of Newcastle Disease Virus-Based Vector Vaccines

Paul J. Wichgers Schreur

Abstract

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

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

1 Introduction

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

Alejandro Brun (ed.), *Vaccine Technologies for Veterinary Viral Diseases: Methods and Protocols*, Methods in Molecular Biology, vol. 1349, DOI 10.1007/978-1-4939-3008-1_14, © Springer Science+Business Media New York 2016

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

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

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

2 Materials

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

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

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

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

2.2 Components for Rescue of NDV from cDNA

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

2.3 Components for Amplification of Virus

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

2.4 Components for Evaluation of Expression of Protein of Interest

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

2.5 Evaluation of Immune Responses Against Protein of Interest in Mammals

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

3 Methods

3.1 Construction of Full-Length cDNA Clones Containing GOI

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

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

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

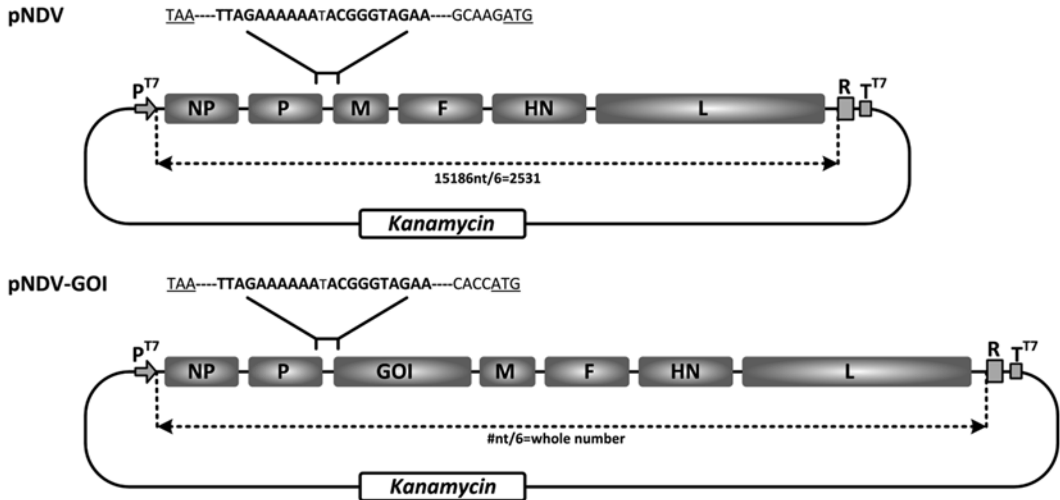

Fig. 1 Schematic presentation of pNDV and pNDV-GOI. The NDV and NDV-GOI antigenome is transcribed by a T7 polymerase recognizing the T7 promoter (P^{T7}) and the T7 terminator (T^{T7}). An encoded ribozyme (R) ensures that the resulting transcript is cleaved exactly at the end of the virus specific sequence.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

3.2 Rescue of NDV-GOI

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

1. Day −3: Subculture QM5 cells in a 75 cm² flask in complete QT-35 medium to reach confluence in 3 days.
2. Day 0: Wash the 75 cm² confluent monolayer of QM5 cells with PBS and dissociate cells using trypsin–EDTA. Subsequently, seed 500,000–700,000 cells per well of a six wells plate in 2.5 ml complete QT-35 medium (*see* **Notes 15** and **16**). N.B. It is strongly preferred to use 6-well plates.
3. Day 1: Remove medium and infect cells with FP-T7 (multiplicity of infection 1–5) in 1 ml Opti-MEM supplemented with 1 % FBS (*see* **Note 17**).
4. After 1 h incubation remove FP-T7 containing medium and add 1 ml Opti-MEM containing 1 % FBS.
5. After 30 min recovery, transfect cells with a transfection mixture containing the pCIneo-NP, pCIneo-P, and pCIneo-L expression plasmids and the pNDV-GOI transcription plasmid according to the transfection reagents manufacturer's instructions (*see* **Note 18**) (Fig. 2a). Preferably use a total of 3 μg DNA per well with a 1.5:1:1:2 ratio in μg of pCIneo-NP, pCIneo-P, pCIneo-L, and pNDV-GOI respectively. Include a positive control based on wild-type NDV and a negative control by omitting FP-T7 infection.

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

3.3 Amplification of Virus

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

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

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

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

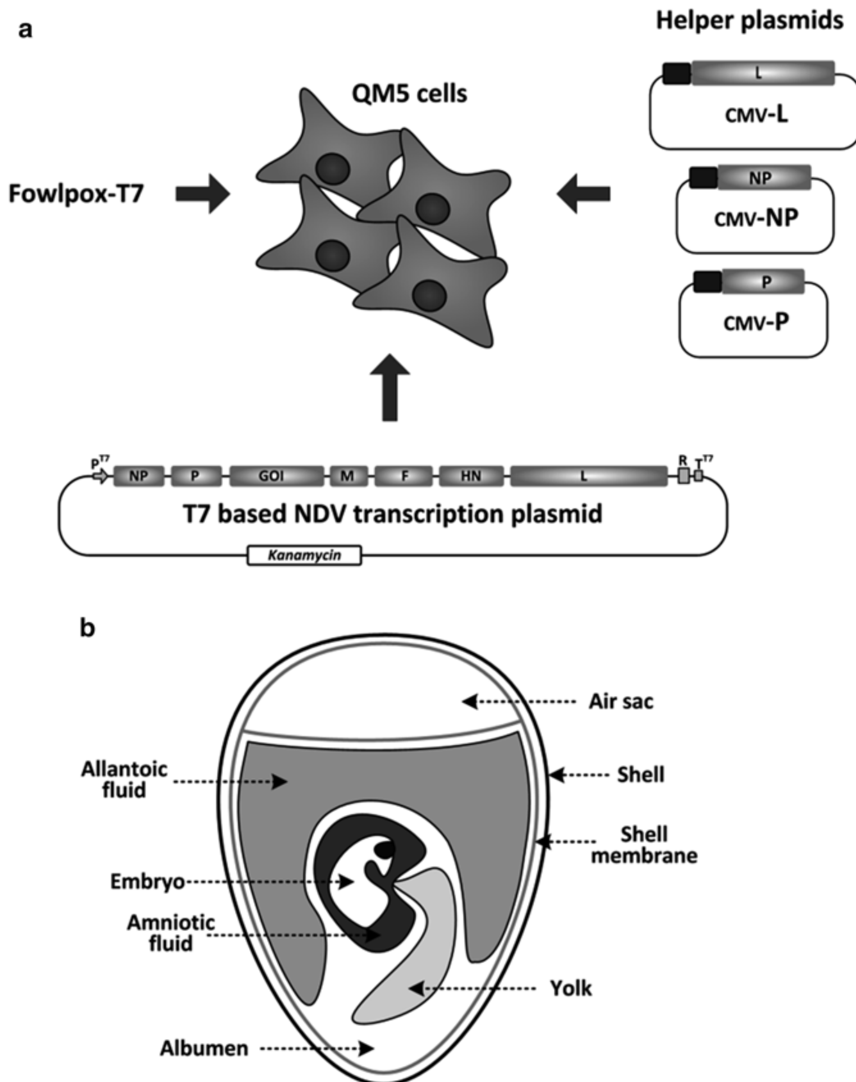

Fig. 2 Schematic presentation of NDV-GOI rescue procedure. (**a**) QM-5 cells infected with a fowlpox expressing T7 polymerase (fowlpox-T7) are transfected with three CMV promoter based expression plasmids and a T7 promoter based transcription plasmid. Approximately 3–5 days post transfection cell supernatants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

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

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

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

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

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

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

4 Notes

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

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

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

Acknowledgements

Dr. Jeroen Kortekaas is acknowledged for carefully reading of the protocol.

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