Chapter 13

Polycistronic Herpesvirus Amplicon Vectors for Veterinary Vaccine Development

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Abstract

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

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

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

1 Introduction

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

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

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

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

2 Materials

2.1 Transient Expression of Amplicon Vectors in Cell Cultures

2.1.1 Cell Culture

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

2.1.2 Western Analysis

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

2.1.3 Immune Fluorescence

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

2.1.4 Kinetics of Antigen Production

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

2.1.5 Luciferase Assay

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

2.1.6 Harvesting of Virus Like Particles

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

2.2 Analysis of RVLPs by Transmission Electron Microscopy

2.2.1 Infection of Cells

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

2.2.2 Preparation of Total Cell Pellet

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

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

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

2.2.3 Staining of Ultrathin Sections

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

Table 1

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

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

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

2.3 Immune Response in Laboratory Animals

2.3.1 Collecting and Preparing Serum Samples

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

2.3.2 Collecting and Preparing Fecal Samples

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

2.3.3 Collecting and Preparing Milk Samples

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

2.3.4 Antigen ELISA

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

2.3.5 IgG ELISA

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

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

2.3.6 IgA ELISA

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

3 Methods

3.1 Design and Optimization of Polycistronic Amplicon Vectors

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

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

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

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

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

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

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

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

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

3.3 Transient Expression of Amplicon Vectors in Cell Cultures

3.3.1 Selection of the Appropriate Cell Line

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

3.3.2 Maintenance of Cells and Preparation of Cells for Transduction

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

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

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

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

3.3.3 Characterization of Transgene Protein Synthesis by Western Analysis

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

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

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

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

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

3.3.4 Characterization of Transgene Protein Synthesis by Immune Fluorescence

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

3.3.5 Kinetics of Antigen Production

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

Transduction of Cells with HSV-1 Amplicon Vectors

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

Luciferase Assay

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

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

Data Analysis

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

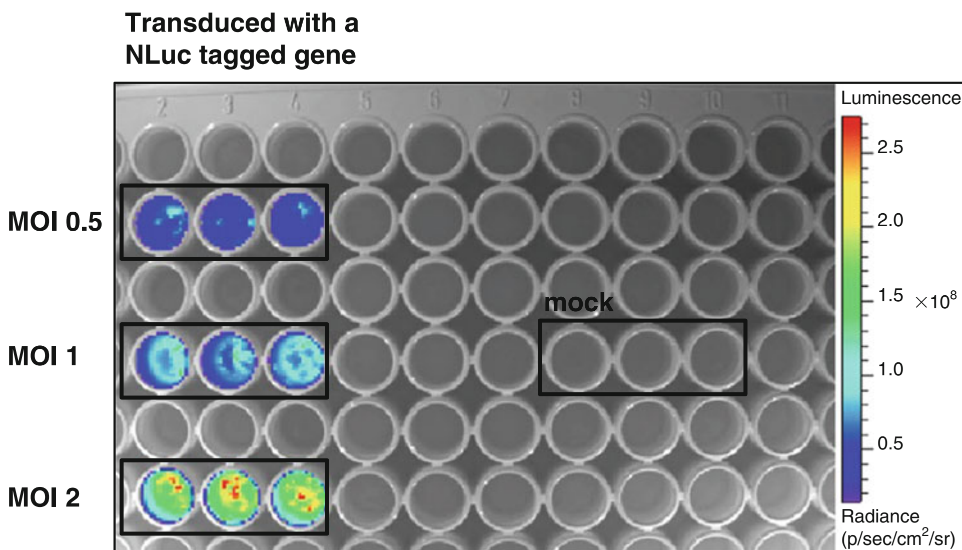

Fig. 1 Visualization of NanoLuc activity in transduced cells using an IVIS reader. Vero 2-2 cells were transduced in triplicates with HSV-1 amplicon vectors coding for the rotavirus proteins VP2, VP6, and a NLuc tagged VP7 at the indicated MOI. Substrate was added 24 hpt and light emission was recorded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

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

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

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

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

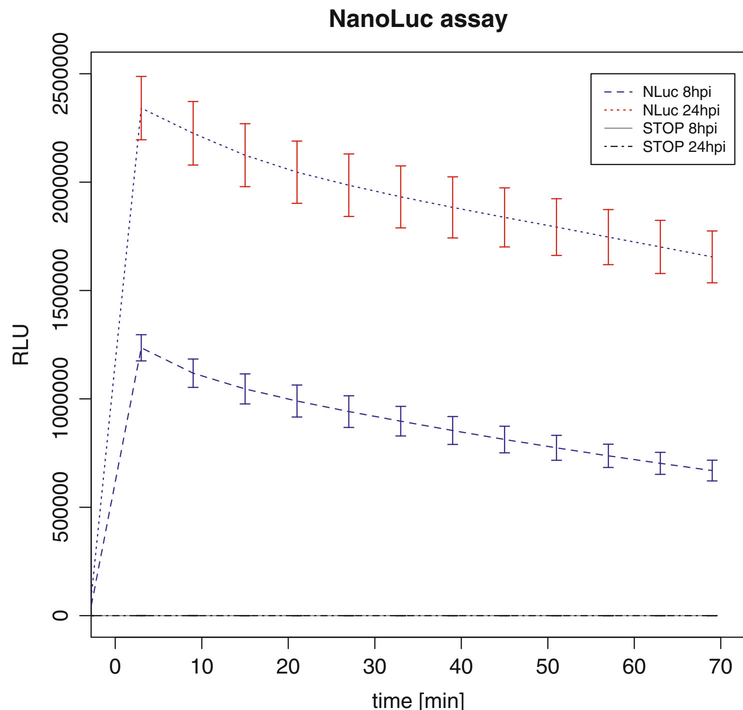

Fig. 2 NanoLuc activity over time. Vero 2-2 cells were transduced with HSV-1 amplicon vectors (MOI = 1) coding for the rotavirus proteins VP2, VP6, and either an untagged VP7 ("STOP") or a NLuc tagged VP7 ("NLuc"). Substrate was added after 8 or 24 hpt. Light emission was measured in relative light units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

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

3.4 Analysis of RVLPs by Transmission Electron Microscopy

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

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

3.4.1 Infection of Cells with HSV-1 Amplicon Vectors

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

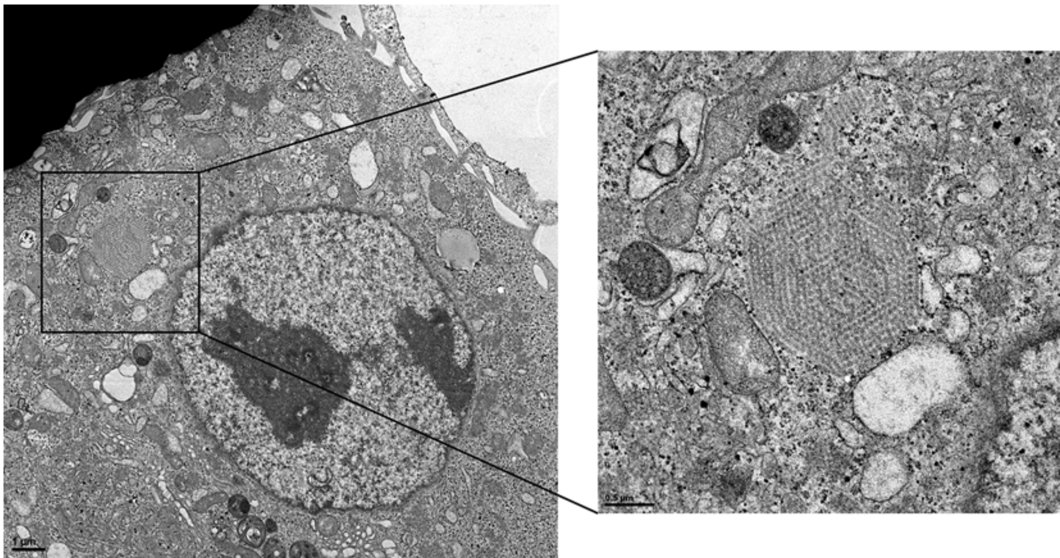

Fig. 3 Electron micrographs of ultrathin sections of amplicon vector-transduced cells. HepG2 cells were transduced (MOI = 5) with a HSV-1 amplicon vector which delivers a DNA cassette encoding a single polycistronic mRNA containing the coding sequences for the three rotavirus capsid proteins VP2, VP6,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

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

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

3.4.3 Staining Ultrathin Sections with Uranyl-Acetate and Lead Citrate

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

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

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

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

3.5 Immune Response in Laboratory Animals

3.5.1 Challenge Infection

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

3.5.2 Collecting and Preparing Samples

Serum Samples

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

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

Fecal Samples

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

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

Milk Samples

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

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

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

3.5.3 Sample Analysis Using ELISA

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

Antigen ELISA

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

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

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

IgG ELISA

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

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

IgA ELISA

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

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

3.6 Characterization of the Antibody Response in the Targeted Species

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

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

4 Notes

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

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

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

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

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

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

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

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

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

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

5. If the titer of the HSV-1 amplicon stock is too low to reach the desired MOI in the total transduction volume, transduction

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

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

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