Chapter 7

Development of Rabies Virus-Like Particles for Vaccine Applications: Production, Characterization, and Protection Studies

Diego Fontana, Marina Etcheverrigaray, Ricardo Kratje, and Claudio Prieto

Abstract

Rabies is a viral infection of the central nervous system for which vaccination is the only treatment possible. Besides preexposure, vaccination is highly recommended for people living in endemic areas, veterinarians, and laboratory workers. Our group has developed rabies virus-like particles (RV-VLPs) with immunogenic features expressed in mammalian cells for vaccine applications. In this chapter the methods to obtain and characterize a stable HEK293 cell line expressing RV-VLPs are detailed. Further, analytical ultracentrifugation steps to purify the obtained VLPs are developed, as well as western blot, dynamic light scattering, and immunogold electron microscopy to analyze the size, distribution, shape, and antigenic conformation of the purified particles. Finally, immunization protocols are described to study the immunogenicity of RV-VLPs.

Key words Vaccine development, Virus-like particles, Rabies, Lentiviral vectors, Stable cell line, HEK293

1 Introduction

Rabies is one of the oldest and most lethal zoonotic diseases known, with a mortality approaching 100 % when clinical symptoms occur. Once the rabies virus reaches the central nervous system, death of the infected person is almost inevitable [1, 2]. The World Health Organization (WHO) recommends preexposure prophylaxis for people living in endemic zones, travellers, veterinaries, and laboratory workers. Besides, post-exposure vaccination is the only treatment available for bitten individuals. Vaccination of pets is required as infected animals (mostly dogs) are the main and most common vector of human rabies infections [3–5]. Although cell-based vaccines for rabies are available, much effort is being made to develop novel, cheaper, and safer vaccines [6, 7].

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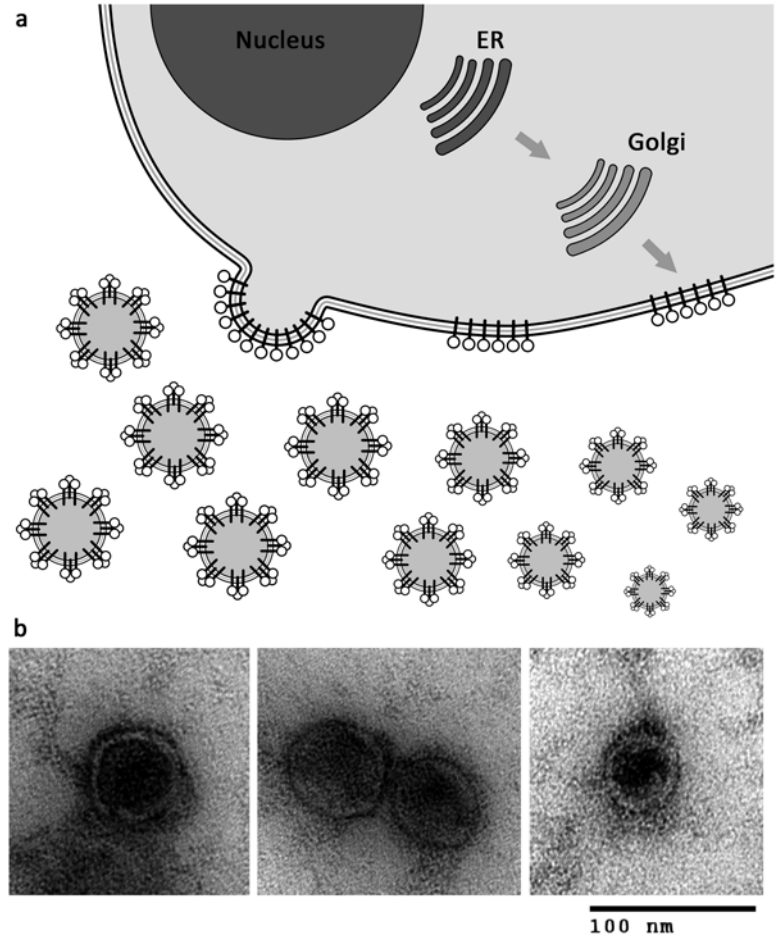

Fig. 1 (**a**) Schematic representation of enveloped VLP budding process. (**b**) Transmission electron microscopy of RV-VLPs (three different fields) found in supernatant of the stable cell line

Virus-like particles (VLPs) are great candidates for rabies vaccine development. VLPs are empty structures that mimic the native virus and are self-assembled expressing the key structural and more antigenic proteins of the virus in a chosen expression system. For enveloped viruses, the membrane glycoproteins, with or without the matrix proteins of the capsid, should be present to generate VLPs capable of inducing a protective immune response [8–10]. When these proteins are expressed adequately, they bud from the plasma membrane carrying lipids and cellular proteins present in it (Fig. 1a).

Our group has made some approaches to develop rabies virus-like particles (RV-VLPs) expressing the rabies glycoprotein (G) in mammalian cell lines [11]. When G is expressed in HEK293 cells, round-shaped VLPs have been found in the supernatant of recombinant cells (Fig. 1b). RV-VLPs proved to be immunogenic and able to induce a specific antibody response.

Here we describe the method to generate recombinant cell lines and clones that continuously express RV-VLPs. The possibility to obtain stable cell lines to produce VLPs is an interesting strategy to optimize the productivity of the production process and it has already been done [12–14]. Usually, these processes use conventional plasmids as vectors for gene transfer, the DHFR-mediated gene amplification system, or similar others. In this case, recombinant cells were constructed by lentiviral gene-mediated transfer using third-generation vectors. By using lentivirus, the introduction of genetic material to nondividing cells can be achieved and the transgene expression can be maintained stably during several passages [15–17]. The lentivirus-based gene engineering techniques have been well studied and described [18].

In this chapter, characterization of RV-VLPs is described, including density gradient analytical purification, antigenic incorporation, shape, size, and polydispersity distribution analysis. A correct particle characterization is crucial when developing novel VLPs because wrong information obtained in this stage could affect the later production process development and the downstream processing as well [10, 19]. We describe the methods to perform ultracentrifugation, western blot, dynamic light scattering (DLS), transmission electron microscopy (TEM), and immunogold electron microscopy.

Finally, the immunogenic properties of the VLPs have to be studied. As a first approach, a simple immunization protocol is recommended to analyze if the produced particles are able to trigger a specific antibody response in mice. Commercial vaccines or antigen international standards should be used to validate the experiment. In any case, in a novel vaccine candidate development, protection studies with virus challenge experiments are the proof of concept. In the case of rabies, NIH potency test is the standard method to confirm that the vaccine candidate is able to induce a protective immune response.

2 Materials

2.1 Lentivirus Production and Titration

1. Human embryonic kidney 293 cell line (HEK293T/17, ATCC® CRL-11268).
2. Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Bethesda, MD), supplemented with 10 % fetal calf serum (FCS, Gibco-BRL, Bethesda, MD), heat inactivated at 56 °C for 30 min.
3. Third-generation lentiviral packaging construct (pMDLg/pRRE), VSV-G expressing construct (pMD.G), the Rev-expressing construct (pRSV-Rev) (Addgene, USA; Plasmid numbers #12251, #12259, #12253, respectively) [15, 16].

4. Transfer lentiviral vector pLV-G (*see* **Note 1**).
5. Lipofectamine® 2000 reagent (Invitrogen™, USA).
6. Syringe filter units (0.45 μm).
7. HIV-1 p24 ELISA kit (QuickTiter™ Lentivirus Titer Kit, Cell Biolabs Inc., USA).

2.2 VLPs Production System

1. HEK293 cell line.
2. DMEM (Gibco-BRL, Bethesda, MD), supplemented with 10 % FCS (Gibco, Bethesda, MD), heat inactivated at 56 °C for 30 min.
3. T flasks for cell culture.
4. Trypsin for cell culture use.
5. Puromycin (Sigma-Aldrich, USA).

2.3 Cell Line Characterization

2.3.1 Flow Cytometry

1. GUAVA EasyCyte cytometer (Millipore, France) or other equivalent.
2. Primary antibody for rabies glycoprotein detection: mAb anti-glycoprotein (*see* **Note 2**).
3. AlexaFluor 488®-conjugated goat anti-mouse antibody (Invitrogen™, USA) or other equivalent for immunofluorescence analysis.

2.3.2 Fluorescence Microscopy

1. Polystyrene media chamber attached to a specially treated standard glass microscope slide (Nunc™ Lab-Tek™ II Chamber Slide™ System, Thermo Scientific, USA).
2. 4 % Paraformaldehyde solution.
3. Primary antibody for rabies glycoprotein detection: mAb anti-glycoprotein (*see* **Note 2**).
4. AlexaFluor 488®-conjugated goat anti-mouse antibody (Invitrogen™, USA) or other equivalent for immunofluorescence analysis.
5. Inverted fluorescence microscope (Eclipse Ti-S, Nikon Instruments Inc, USA, or other equivalent).

2.4 VLP Purification

1. Syringe filter units (0.45 μm).
2. RV-VLP stabilization buffer (50 mM Tris–HCl, 0.15 M NaCl, 1.0 mM EDTA, pH 7.4).
3. 30 % Sucrose in RV-VLP stabilization buffer.
4. Ultracentrifuge with a swinging bucket rotor.
5. Iodixanol density gradient (OptiPrep™ Density Gradient Medium, Axis-Shield, Scotland).
6. Amicon® Ultra centrifugal units (Millipore, USA) with a 100,000 MWCO.

2.5 VLP Characterization

2.5.1 Western Blot Analysis

1. Equipments and buffers for casting 10 % SDS-polyacrylamide gel electrophoresis (PAGE).
2. Equipments and buffers for western blot analysis.
3. Primary antibody for rabies glycoprotein detection: Anti-rabies rabbit polyclonal serum (*see* **Note 3**).
4. Secondary antibodies for western blot analysis: HRP-conjugated goat anti-rabbit immunoglobulin.
5. Chemiluminescent detection reagents.

2.5.2 Dynamic Light Scattering Analysis

1. Dynamic light scattering (DLS) equipment: Nano ZS particle-size analyzer (Malvern Zetasizer, Malvern Instruments Ltd, UK).
2. Specific DLS equipment software: Nanov510 (Malvern Ltd, UK).
3. Low-volume cuvette for aqueous samples.

2.5.3 Transmission Electron Microscopy and Immunogold Analysis

1. Formvar-coated 300-mesh copper grids for electron microscopy.
2. 2 % Uranyl acetate solution.
3. Tweezers, 0.22 μm filtered distilled water, and filter papers.
4. Primary antibody for rabies glycoprotein detection: mAb anti-glycoprotein (*see* **Note 2**).
5. Secondary antibody for immunogold detection: Anti-mouse 6 nm gold-conjugated antibody (Colloidal Gold-AffiniPure goat anti-mouse IgG, Jackson ImmunoResearch, USA).
6. Transmission electron microscope (TEM): Jeol JSM-100 CX II (Jeol, Japan) or other equivalent.

2.6 VLP Immunization

2.6.1 Humoral Immune Response Analysis

1. Female 4–5-week-old BALB/c mice.
2. Freund's incomplete adjuvant.
3. Syringes and needles for animal injection.
4. Inactivated rabies virus vaccines to be injected as a positive control: Human vaccine and veterinary vaccine.
5. Equipment and buffers for specific indirect ELISA.
6. Secondary antibodies for specific indirect ELISA analysis: HRP-conjugated goat anti-mouse immunoglobulin.

2.6.2 Protection Assays: Virus Challenge

1. CF-1 mice strain.
2. Sixth International Standard for Rabies vaccine (WHO International Standard, NIBSC code: 07/162).
3. Challenge Virus Standard (CVS, rabies virus) [20].
4. Syringes and needles for animal injection.

3 Methods

3.1 Lentivirus Production and Titration

1. The day before the assay, seed HEK293 cells at a concentration of 4×10^5 cells/ml in a 10 cm diameter plate and incubate ON at 37 °C with 5 % CO_2.
2. Transfer 2.5 ml of DMEM basal medium to a 15 ml sterile tube and add 50 μl of Lipofectamine® 2000 reagent. Incubate at RT for 5 min.
3. Transfer 2.5 ml of DMEM basal medium to another 15 ml sterile tube and add 2.5 μg of pRSV-REV, 3.6 μg of pMD.G, 6.5 μg of pMDLG/pRRE, and 10 μg of pLV-G constructs.
4. Mix the content of both tubes and vortex. Incubate at RT for 20 min to allow DNA–lipid complex formation.
5. During the incubation, wash gently the cells' monolayer with 5 ml of DMEM.
6. Discard the culture medium and add the DNA–lipid complex to the cells. Incubate for a minimum of 4 h.
7. Add medium supplemented with FCS to reach a final concentration of 10 % V/V. Incubate for 24–48 h.
8. Harvest and clarify the supernatant by centrifuging at $200 \times g$ for 10 min.
9. Filter using a 0.45 μm filter unit.
10. Aliquot the clarified supernatant containing the lentivirus and store at −80 °C.
11. Calculate the titer of the produced lentivirus with the HIV-1 p24 ELISA kit (QuickTiter™ Lentivirus Titer Kit, Cell Biolabs Inc., USA) or another analogue method (*see* **Note 4**).

3.2 Cell Line Development and Clone Selection

1. Seed HEK293 cells at a concentration of 3×10^4 cells/ml in a 6-well plate and incubate ON at 37 °C with 5 % CO_2.
2. Discard the supernatant and add the amount of lentivirus to achieve an MOI of 20 in a final volume of 1 ml. Incubate at 37 °C for 16 h.
3. Discard the supernatant with the rest of lentivirus and add 3 ml of DMEM 10 % FCS. Incubate for another 72 h.
4. Select recombinant cells with puromycin at 1 μg/ml using a not transduced well as a negative control (*see* **Note 5**).
5. Analyze the recombinant cell line to confirm the rabies glycoprotein expression by flow cytometry and fluorescent microscopy (Subheadings 3.3.1 and 3.3.2).
6. Clone the recombinant cell line by dilution method or FACS isolating single cells into 96-well plate.
7. Analyze the individual clones by flow cytometry.

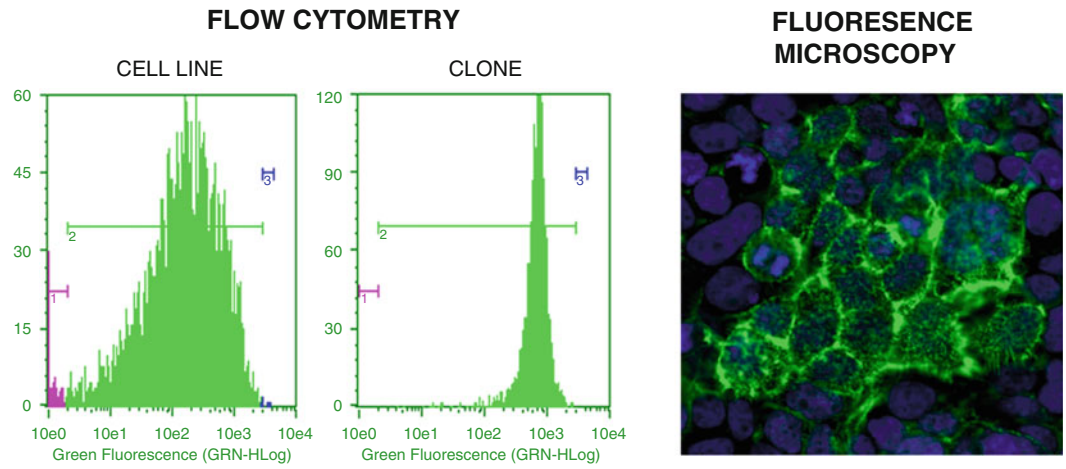

Fig. 2 Cell line and clone characterization to confirm rabies glycoprotein expression. Cells were incubated with a specific monoclonal antibody and with AlexaFluor 488®-conjugated goat anti-mouse antibody and analyzed by flow cytometry. The cell monolayer was observed by fluorescence microscopy, confirming the membrane localization of the glycoprotein

3.3 Cell Line and Clone Analysis (See Fig. 2)

3.3.1 Flow Cytometry

1. Detach the monolayer of the recombinant cell line using trypsin and count the cells. Prepare not transduced cells as a negative control for the assay.
2. Incubate a total of 1×10^5 cells in DMEM basal medium with a 1:1000 dilution of a monoclonal antibody against rabies G protein, for 30 min at RT.
3. Wash the cells with DMEM basal medium and incubate with a 1:1000 dilution of AlexaFluor 488®-conjugated goat anti-mouse antibody, for 30 min at RT.
4. Wash the cells with DMEM basal medium and analyze the cells by flow cytometry.
5. Analyze not only the percentage of fluorescent cells but also the fluorescence intensity (usually called X-*mean*) that correspond to the glycoprotein expression level.

3.3.2 Fluorescence Microscopy

1. Seed the recombinant cell line at a concentration of 1×10^5 cells/ml over a chamber slides for cell culture and incubate for 48 h.
2. Remove the supernatant and wash the monolayer gently with PBS.
3. Fix cells with a 4 % paraformaldehyde solution, during 30 min at RT.
4. Wash twice with PBS (repeat this step after each following incubation).

5. Incubate the monolayer with a G protein-specific monoclonal antibody (diluted 1:100 in PBS, 0.1 % BSA), for 30 min at RT.
6. Incubate the cells with AlexaFluor 488®-conjugated goat anti-mouse antibody for 30 min at RT (diluted 1:1000 in PBS, 0.1 % BSA).
7. Dye the nuclei with a DAPI solution (4′,6-diamidino-2-phenylindole) in a final concentration of 1 μg/ml for 5 min.
8. Analyze subcellular localization of G protein with a fluorescence microscope.

3.4 VLP Purification

1. Seed the VLPs expressing HEK293 cells in a 150 cm^2 T flask at a final concentration of 4×10^5 cells/ml with DMEM 10 % FCS and incubate ON at 37 °C with 5 % CO_2.
2. Exchange the supernatant for DMEM medium but supplemented with 1 % FCS (*see* **Note 6**). Incubate for 40–72 h at 37 °C with 5 % CO_2.
3. Harvest the medium containing the VLPs and clarify by low-speed centrifugation.
4. Centrifuge at $10{,}000 \times g$ to remove any cellular debris.
5. Filter using a 0.45 μm filter unit.
6. Layer the clarified harvest over a 30 % sucrose cushion and centrifuge for 3 h at $65{,}000 \times g$ or more.
7. Discard the supernatant and resuspend the VLP pellet with RV-VLP stabilization buffer.
8. Prepare a discontinuous iodixanol gradient (20, 30, 40, 50 %).
9. Layer the partially purified VLPs over the density gradient and centrifuge at $10{,}000 \times g$ for 6 h.
10. Analyze and aliquot the obtained band (*see* **Note 7**).
11. Exchange buffer using an Amicon® Ultra centrifugal unit with a 100,000 MWCO and RV-VLP stabilization buffer.
12. Store at 4 °C for further analysis.

3.5 VLP Characterization (See Fig. 3)

During early stages of development, VLP characterization is essential to know the main characteristics of the particles. Although there is a wide range of techniques that should be performed to fully understand the nature and structural characteristics of the particles under analysis [10, 19], simple assays, as western blot, DLS, and TEM, are recommended as the initial studies to know the size, shape, polydispersity, and antigenic content of the VLPs under development.

3.5.1 Western Blot Analysis

1. Mix 30–50 μl of the obtained band with SDS-PAGE loading buffer.
2. Load in a 10 % SDS-PAGE and run.

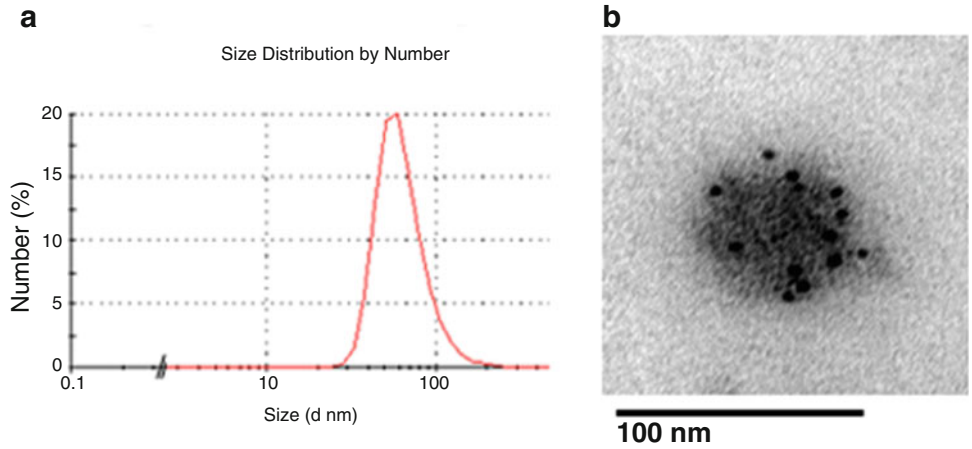

Fig. 3 Rabies virus-like particle characterization. (**a**) Dynamic light scattering analysis of purified VLPs. (**b**) Immunogold electron microscopy of purified VLPs

3. Blot gel on filter and block with 5 % skin milk in TBS buffer.
4. Incubate with a primary antibody for rabies glycoprotein detection and a secondary antibody HRP conjugated. Wash three times for 5 min with 0.05 % Tween-20/TBS buffer.
5. Reveal by chemiluminescent method.

3.5.2 Dynamic Light Scattering Analysis

1. Filter the purified VLPs through 0.45 μm and fill the cuvette for DLS.
2. Run almost ten consecutive measurement of a single sample using the specific equipment software (Nanov510, *see* **Note 8**).
3. Analyze the hydrodynamic diameter and the distribution of the purified particles (*see* **Note 9**).

3.5.3 Immunogold Electron Microscopy Analysis

1. Filter the purified VLPs, as well as all the buffers and water to be used in the assay, through 0.45 μm filter.
2. Adsorb 10 μl of purified VLPs to a formvar-coated 300-mesh copper grid for 2 min.
3. Remove the excess with filter paper and wash twice with 2 % BSA in TBS. Block with 2 % gelatine in TBS, for 30 min at RT.
4. Float the grid over a drop of monoclonal antibody anti-glycoprotein diluted in BSA/TBS and incubate for 1 h at RT (*see* **Note 10**). Wash three times with BSA/TBS.
5. Repeat the previous step but adsorbing a 1:20 dilution of secondary gold-conjugated antibody in BSA/TBS.
6. Stain the sample with 2 % uranyl acetate for 2 min.
7. Examine the grid using a transmission electron microscope.

3.6 VLP Immune Response

3.6.1 Immunization of Mice and Antibody Titration

1. Prepare the correct dilution, in RV-VLP stabilization buffer, of purified RV-VLPs containing the same glycoprotein content of a 1:20 dilution of a human rabies vaccine dose. Prepare the same dilutions of the human and veterinary rabies vaccine.
2. Mix properly the samples with Freund's incomplete adjuvant and inject 100 μl intramuscularly to five animals per group (day 0).
3. Give a booster on day 12 and collect blood samples on day 19.
4. Calculate the titer of antibodies in the obtained sera by indirect specific ELISA.

3.6.2 Protection Assays: Virus Challenge

1. Prepare 4 fivefold dilutions of the test and standard vaccine (1:5; 1:25, 1:125, 1:625) in PBS.
2. Inject intraperitoneally 16 mice per group with 0.5 ml of the dilutions of the test and reference vaccine on days 0 and 7.
3. On day 14, challenge the immunized mice injecting intracerebrally 0.03 ml of a dilution of CVS containing 25 LD_{50}. Observe mice during the following 14 days and record the number of die mice.
4. Calculate the 50 % end-point dilution ($ED_{50\%}$) of each sample and the final relative potency of the test vaccine as follows (Ref. 20):

$$RP = \frac{\text{Reciprocal of ED50 \% of TV}}{\text{Reciprocal of ED50 \% of RV}} \times \frac{\text{dose of TV}}{\text{dose of RV}}$$

where
TV = test vaccine.
RV = reference vaccine.
Dose = volume of a single vaccine dose, as stated by the producer (*see* **Note 11**).

4 Notes

1. The pLV-G [11] is a lentiviral transfer vector constructed cloning the coding sequence of the rabies glycoprotein (PV strain) into the vector pLV-PLK [21].
2. The monoclonal antibody used to analyze the rabies glycoprotein expressed by the recombinant cell lines and present in the envelope of the RV-VLPs recognizes the protein in their native form, anchored in the membrane and forming trimers. This feature is crucial when studying the antigenic properties of a protein for vaccine development, as the structural conformation is usually important to trigger the induction of neutralizing antibodies in the immune response developed.

3. For western blot analysis a polyclonal serum against rabies proteins was used. This serum was obtained by immunizing a rabbit with a commercial rabies vaccine.
4. Using this titer kit the physical titer is being calculated (the amount of p24 HIV-1 core protein associated to lentivirus particles), expressed in LP/ml. It is not the infectious titer, usually expressed as TU/ml. Generally, the infectious titer varies among different cell lines and usually 10^6 TU/ml corresponds to $10^{8–9}$ LP/ml. Other methods to quantify lentiviral particles are the reverse transcriptase (RT) activity by product-enhanced RT assay or RNA amount in viral supernatant by qPCR.
5. For recombinant cell selection a multi-step gradual selection protocol could be performed: our group has previously developed a method [21] through which cells are incubated from 1 up to 250 μg/ml of puromycin, but the selection agent is gradually changed every 7 days on the same plates. This method allows to maintain the cells in culture condition up to 200 μg/ml puromycin and achieve higher expression levels of the gen of interest.
6. It is important to reduce the total amount of FCS in the supernatant containing RV-VLPs. The FCS has a lot of bovine serum albumin (BSA) that has a similar molecular weight compared to rabies glycoprotein (62–66 kDa) and interferes in the analysis, mostly in western blot assays.
7. A simple method of determining the density of gradient fractions is to measure the absorbance (optical density) of the obtained fractions. With OptiPrep™ Density Gradient Medium it is possible to measure the absorbance at 340 nm and calculate the approximate particle density by following the manufacturer's instructions (Application Sheet C53, Axis-Shield).
8. This software could convert the intensity-based measurement to a size distribution based on the volume or number of particles. This conversion is only valid if some parameters, like particle polydispersity, are within accepted values. Besides, confirmation by other methods like TEM is recommended.
9. The hydrodynamic diameter is always higher than the diameter calculated by other methods due to the ion layer surrounding the particles in solution.
10. Several dilutions should be performed in order to achieve the best result. Try dilutions between 1:50 and 1:12,000.
11. The relative potency value obtained in the NIH test should become the minimum value recommended by WHO. The minimum potency required is 2.5 IU per human dose and 1 IU in the smallest prescribed dose for veterinary vaccine.

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