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Lentiviral vectors pseudotyped with filoviral glycoproteins

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Abstract

Pseudotyping lentivirus-based vectors is a strategy used to study conferred vector tropism and mechanisms of envelope glycoprotein function. Lentiviruses and filoviruses both assemble at the plasma membrane and have homotrimeric structural envelope glycoproteins that mediate both receptor binding and fusion. Such similarities help foster efficient pseudotyping. Importantly, filovirus glycoprotein pseudotyping of lentiviral vectors allows investigators to study virus entry at substantially less restrictive levels of biosafety containment than that required for wild-type filovirus work (biosafety level-2 vs. biosafety level-4, respectively). Standard lentiviral vector production involves transient transfection of viral component expression plasmids into producer cells, supernatant collection, and centrifuge concentration. Because the envelope glycoprotein expression plasmid is provided *in trans*, wild type or variant filoviral glycoproteins from marburgvirus or ebolavirus species may be used for pseudotyping and compared side-by-side. In this chapter we will discuss the manufacturing of pseudotyped lentiviral vector with an emphasis on small-scale laboratory grade production.

Keywords

Viral vector; Pseudotyping; Production; Purification; Titering; Lentivirus; Filovirus; Ebolavirus; Marburgvirus; Glycoprotein

1. Introduction

Several features of lentiviral vectors make them useful tools for basic science and preclinical research applications, including their large packaging capacity, efficient gene transfer capabilities, and persistent transgene expression. Lentiviral vectors also integrate into both dividing and non-dividing cells, expanding the range of cells that can be targeted [1]. Pseudotyping is the act of replacing the native envelope protein with glycoproteins from other enveloped viruses [2,3]. By pseudotyping lentiviral vectors, a wide range of cell types may be transduced at a biosafety level of containment available to most laboratories (see Note [1]). The vesicular stomatitis virus glycoprotein (VSV-G) is by far the most common envelope glycoprotein used for lentiviral pseudotyping; however, VSV-G may not be a

suitable choice for many scientific questions. A wide variety of heterologous viral envelope glycoproteins have successfully been used to pseudotype lentiviral vectors; including those from vesiculoviruses, lyssaviruses [4,5], arenaviruses [6,7], hepadnaviruses [8], flaviviruses [9], paramyxoviruses [10], orthomyxoviruses [11], baculovirus [12,13], alphaviruses [14,15] and filoviruses [16–18,11].

Pseudotyping lentivirus is a proven strategy to study filovirus receptor binding, entry, and endosomal escape outside of BSL-4 containment [19–21]. Pseudotyping lentivirus with filoviral glycoproteins effectively directs the tropism to the central nervous system or the apical surface of airway epithelial cells [22,16,23,24], providing evidence that the filovirus glycoprotein targets cells within these organs. Lentiviral vectors can be effectively pseudotyped with wild-type Ebola virus (EBOV) or Marburg virus (MARV) glycoproteins, which is of use to many filoviral-related research applications. However, if the goal is maximum pseudotyping efficiency and high titers, modifications to the glycoprotein peptide sequence have been shown to be beneficial [25,26]. Pseudotyping efficiency may be improved by either a directed evolution approach [27] or simply truncating the glycoprotein C-terminal tail [28,29]. Specifically for pseudotyping lentiviral vectors with the EBOV envelope glycoprotein, we and others observed that efficiency improved when the heavily O-glycosylated mucin domain was deleted [25,24]. Presumably, streamlined post-translational processing results in better surface display of the glycoprotein, which leads to more efficient incorporation into the budding virions.

In this chapter we outline a simple method for producing an EBOV GP pseudotyped human immunodeficiency virus (HIV)-based lentiviral vector. The approach does not require exotic equipment or materials and can easily be adapted to other lentiviral vectors such as simian or feline immunodeficiency virus- based lentiviral vectors. Furthermore, these methods will easily translate to pseudotyping with other envelope glycoproteins, such as MARV GP, VSV-G and baculovirus GP64 [30]. Many commercially available titering kits for lentiviral vectors detect VSV-G, thus they are not suitable for titering filoviral pseudotyped lentiviral vectors. Here, we outline 3 alternate methods for vector titration.

2. Materials

2.1 Transfection reagents

1. 2× HEPES buffered saline (HBS): For 1L: 11.9 g HEPES, 16.4 g NaCl, 0.21 g Na_2HPO_4. Bring up to 1 L with sterile water. Bring pH to 7.1 with 1N NaOH. Filter through a 0.22 μm bottle top filter. Aliquot and store at −20 °C.
2. 2.5M $CaCl_2$: 187.3 g $CaCl_2$ dihydrate. Bring volume up to 500 mL with sterile water. Filter through a 0.22 μm bottle top filter. Store solution at 4 °C or aliquot and store at −20 °C.

[1]All work with lentiviral vectors, should be performed inside a certified Class II, biosafety cabinet. Consult the local Institutional Biosafety Committee (IBC) for information regarding additional regulations. At most institutions, biosafety level-2 containment is sufficient.

3. Serum-free Dulbecco's Modified Eagle Medium (DMEM) with 1% penicillin/streptomycin (Pen/Strep).
4. DMEM with 2% Nu-Serum IV Culture Supplement (Corning) and 1% Pen/Strep.
5. DMEM with 10% FBS, 1% Pen/Strep for cell maintenance.
6. A filovirus glycoprotein-expressing plasmid and third generation lentiviral vector production plasmids (see Note [2]).
7. Resuspension Lactose/PBS buffer (40 mg/mL): 4 g D-lactose monohydrate. Bring up to 100 mL with 1× PBS solution (Gibco). Filter through a 0.22 μm bottle top filter. Aliquot and store at 4 °C.

2.2 Cultured cells

1. Producer Cells: HEK 293FT – Human Embryonic Kidney Cells with T antigen (Invitrogen).
2. Titering Cells: HT1080 cells (ATCC CCL-121) are derived from a human fibrosarcoma and are typically highly permissive to lentiviral transduction. Thus this cell line is useful for titration of lentiviral particles. Vero cells are commonly used for titering many viruses but should be avoided for titering HIV-based vector due to potential simian TRIM5α-mediated restriction [31].

2.3 Flow cytometry titering reagents

1. DMEM with 10% FBS, 1% Pen/Strep.
2. DMEM with 2% FBS, 1% Pen/Strep.
3. 8 mg/mL Polybrene (Hexadimethrine bromide) stock (Sigma), *optional* (long term storage at −20 °C).
4. 5 mL polystyrene tubes with caps, 12 × 75 mm (e.g. Evergreen Scientific) or BD Falcon 5 mL polystyrene round-bottom tubes, 12 × 77 mm (e.g. BD Biosciences).
5. Accumax cell detachment solution (Millipore) (storage at 4°C with a recommended shelf life of 6 weeks, or the product can be frozen in small aliquots and stored at −20°C indefinitely).
6. Falcon nylon cell strainer, 70 μm.
7. Propidium iodide (PI): 50 μg/mL in 1× PBS (store at 4 °C, protect from light).

[2]Four plasmid transfection of one 150 mm plate is performed by calcium phosphate precipitation of 7.5 μg viral glycoprotein (envelope) plasmid (i.e. EBOV GP), 22.5 μg gag/pol packaging plasmid (ie. LP1), 7.5 μg rev plasmid (ie. LP2), and 22.5 μg lentiviral vector genome plasmid containing your transgene of choice (e.g. HIV CMV eGFP). We found that a 1:3:1:3 ratio yielded optimal titers; however, results may vary. Pure, endotoxin free plasmids typically yield the best vector product. Mammalian expression vectors containing the glycoprotein of the species *Zaire ebolavirus* or the Marburg virus Musoke isolate glycoprotein are available from BEI Resources (www.beiresources.org)(Catalog #s:NR-19814 and NR-19815, respectively). Multiple HIV vectors are available through addgene.com, which carry reporter genes such as mCherry, GFP, and firefly luciferase. Empty vectors allow for construction of vectors carrying other genes of interest if desired.

2.4 Real-time PCR titering reagents

1. 12-well tissue culture plate.
2. 1.5 mL microcentrifuge tubes, sterile.
3. DMEM (e.g. Cellgro) with 2% FBS (e.g. Atlas) and 1% Pen/Strep (e.g. Cellgro).
4. Positive control - Lentivirus expressing eGFP (HIV-eGFP) with a known titer.
5. 8 mg/mL Polybrene (Hexadimethrine bromide) stock (Sigma) – *optional.*
6. 10 mg/mL RNase A stock.
7. Genomic DNA Purification Kit (Promega).
8. Isopropanol.
9. 70% Ethanol.
10. RNase/DNase Free Water.
11. MicroAmp Optical 384-well Reaction Plate with Barcode (ABI).
12. MicroAmp Optical Adhesive Film (ABI).
13. TaqMan Universal Master Mix II, No UNG (ABI).
14. RNase/DNase Free Water.
15. Primers and probe: HIVforward 5′-CGA CTG GTG AGT ACG CCA AA-3′; HIVreverse 5′-CGC ACC CAT CTC TCTCCT TCT-3′; HIVprobe 5′-/FAM/ATT TTG ACT AGC GGA GGC/Black Hole/-3′ (see Note [3]).

2.5 p24 assay

1. MagPlex Microspheres Bead Region 42 (Luminex Corporation).
2. p24 Monoclonal Antibody (ImmunoDiagnostics).
3. 0.1M Monobasic Sodium Phosphate (pH 6.2).
4. Sulfo-N-HydroxySuccinimide (Sulfo-NHS, 50mg/mL).
5. 1-Ethyl-3-[3-Dimethylaminopropyl] Carbodiimide Hydrochloride (EDC, 50mg/mL).
6. 1× PBS (pH 7.4).
7. Microsphere Resuspension Buffer: 1× PBS (pH 7.4), 0.1% BSA and 0.02% Tween-20.
8. Luminex Wash Buffer: 1× PBS with 0.01% Tween-20 and 2% 1M Tris-HCl (pH 8).
9. Normal Goat Serum (e.g. Thermo Fisher Scientific).

[3]These primer and probe sequences listed here are commonly used with standard lentiviral vector constructs. Verify that these are complementary to your lentiviral vector before use.

10. Normal Mouse Serum (e.g. Thermo Fisher Scientific).
11. Luminex Assay Buffer: (Luminex Wash buffer + 1% Normal Goat Serum + 1% Normal Mouse Serum).
12. HIV-1 p24 IIIB (Baculo) standard (NIH AIDS Reagent Program).
13. RD1-labelled anti-p24 KC57 detection antibody (Beckman Coulter).
14. 96-Well Black Flat Bottom Microplate (e.g. Greiner Bio-One).
15. Microplate Sealer (e.g. Fisher Scientific).
16. Gyrotory Shaker G2 (New Brunswick), or similar.
17. Bio-Plex Pro II Wash Station (Bio-Rad).
18. Bio-Plex 200 System (Bio-Rad).

2.6 Equipment

1. 500 mL Centrifuge bottles (e.g. Beckman Coulter).
2. Avanti J-25 centrifuge with JA-10 rotor (Beckmann Coulter), or similar.
3. 0.5 mL sterile microfuge tubes.
4. Biological Safety Cabinet (see Note [1]).
5. BioPlex Multiplex System.

3. Methods

3.1 Lentiviral vector production by four-plasmid transfection

1. Split HEK 293FT cells one to four days prior to transfecting cells in 150 mm dishes with DMEM (10% FBS) medium (see Note [4]). Seed 1.3×10^7cells per 150 mm plate the day prior to transfection, 2.5×10^6 cells per 150 mm plate three days prior to transfection, or 1.5×10^6 cells per 150 mm plate four days prior to transfection. Incubate the cells at 37 °C until ready for use.
2. Add the appropriate volume of RNase/DNase free sterile H_2O (Table 1) to a 50 mL conical tube. Note that for all values in Table 1, it is good practice to multiple by an additional 10% to account for pipetting error.
3. Add plasmids to H_2O tube (see Note [2]).
4. Add room temperature 2.5 M $CaCl_2$ to H_2O/Plasmid tube and mix by inversion.
5. Combine tubes 1 and 2 by adding the required amount of 2× HBS to Tube 2 (plasmid+H_2O+$CaCl_2$ mixture) in a dropwise manner and mix gently by inversion. After the addition of the HBS solution, the DNA should visibly precipitate (the solution will appear cloudy).

[4]For consistent results, ensure that producer cells are at a low passage number (<27) and are not over confluent.

6. Add the DNA/calcium phosphate solution to serum-free DMEM (15 mL/plate) and mix well by inversion.
7. Aspirate the culture medium from the HEK 293FT (producer) cells.
8. Gently add 15 mL of the DMEM/calcium phosphate solution to each 150 mm plate without disturbing the cell layer.
9. Incubate plates at 37°C with 5% CO_2 for a minimum of 4 hours or a maximum of 6 hours.
10. Remove the transfection medium and replace with 15 mL of DMEM with 2% Nu-Serum, 1% Pen/Strep.
11. At 24 hours post-transfection, collect the supernatant and store at 4°C. Replace 15 mL of fresh medium to each plate.
12. Repeat the collection at 40 hours post-transfection and again at 64 hours post-transfection.
13. Combine the collected supernatants and pass the entire collected volume through a 0.22 μm bottle top filter.
14. Viral supernatant can be temporarily stored at 4°C for up to 1 week or for longer periods of time at −80°C until ready to concentrate. Care should be taken to limit samples to a single freeze/thaw.

3.2 Low-speed centrifuge concentration

1. If necessary, thaw vector supernatant at room temperature or at 4°C.
2. In a biological safety cabinet, transfer vector supernatant into sterile 500 mL centrifuge bottles.
3. Spin the supernatant at 9000 × g overnight at 4°C.
4. The next day, remove the bottles from the centrifuge and visually confirm there is a pellet before removing the medium.
5. In a biological safety cabinet, aspirate medium without disturbing the pellet.
6. Invert the bottle onto a clean paper napkin and let remaining medium drain.
7. Aspirate any additional medium attached to the bottle walls and around pellet to avoid including any of the medium with your re-suspension.
8. Resuspend the pellet in 40 mg/mL of Lactose/PBS buffer (120 μL/plate).
9. Store the concentrated lentiviral vector at 4°C for a short period (0.5 h to 1 h) to allow the pellet to completely dissolve in the buffer.
10. Transfer resuspended lentiviral vector to a 0.5 mL microcentrifuge tube.
11. Invert the tube a few times to mix and then spin for 10 seconds at maximum speed in a benchtop microcentrifuge (~15,000 – 20,000 × g) to remove any protein debris.

12. Dispense 100 μL aliquots of lentiviral vector into sterile 0.5 mL tubes. Also, dispense at least one 50 μL aliquot for titering. Store for short term at 4°C, and for long term at −80°C.

13. Centrifugation also concentrates cellular debris and culture medium components such as bovine serum albumin (BSA). For potential strategies to further purify laboratory grade lentiviral vectors, see Note [5].

3.3 Vector titration using flow cytometry

This method is only suitable for the analysis of lentiviral vectors containing fluorescent transgenes (e.g. GFP, mCherry etc.).

1. Split HT1080 cells the day prior to infecting cells. Plate HT1080 cells in a 6-well plate at 7×10^5 cells/well with DMEM (10% FBS) medium. Incubate cells for ~24 hours at 37 °C. The cells should be ~80–90% confluent (or ~2.0×10^6 cells/well) before proceeding.

2. Prepare DMEM (2% FBS) with optional 1 μL/mL polybrene. Mix well.

3. Perform serial dilutions of the concentrated lentiviral vector (100-fold to 10,000-fold) in DMEM with 2% FBS/polybrene in 2.0 mL microcentrifuge tubes. Mix thoroughly between tubes.

4. Aspirate medium and plate 1 mL of lentiviral vector dilutions. You must be very gentle to avoid disrupting the cells. To negative control wells, add DMEM (2% FCS) medium only.

5. Use one or two additional control wells to trypsinize, harvest and count the number of cells. The 6-well plates have a surface area of approximately 951 mm^2 per well, although this may vary slightly depending on the supplier, and you should end up with ~2300 cells/mm^2. A visual screening and evaluation of the cell layer can be very deceptive.

6. Incubate cells for ~65 hours at 37 °C.

7. Label 5 mL polystyrene tubes and add 20 μL per tube of propidium iodide.

8. Aspirate medium from the wells and add 0.5–1.0 mL of Accumax per well.

9. Pipet dislodged cells into tubes only immediately prior to analysis. This prevents the formation of clumps that will clog the machine. Vortexing should be avoided if possible because it causes cell lysis. Cell strainers are preferable.

[5] Adverse inflammatory and immunogenic reactions resulting from *in vivo* delivery of lentiviral vectors are largely attributable to these components in the centrifuge-concentrated vector preparations [32]. Clearly, the removal of unnecessary foreign proteins from vector preparations is an important step in improving the safety and efficacy of *in vivo* gene transfer. Several methods have been proposed to partially purify pseudotyped lentiviral preparations. A strong anion exchange HPLC column was effective in purifying functional vector particles, resulting in decreased cell toxicity [33]. Weak anion exchange hollow fiber has also been used to purify pseudotyped HIV vector [34]. Mustang Q strong anion exchange capsules have been used in the purification of large-scale viral preparations [35]. These processes are based on the known electrostatic charge of the vector envelope. There are several alternative approaches for depleting stocks of contaminants. Stocks can be ultracentrifuged through a 20% sucrose (in 1× PBS) cushion (80,000 × g for 2 hours in 4°C). Alternately, size exclusion chromatography has also been used to purify large amounts of pseudotyped lentiviral particles [36]. Tangential flow filtration is also an effective and widely used technique to purify lentiviral preparations [37]; however, the equipment and materials can be cost prohibitive for small-scale laboratory grade applications.

10. Perform flow cytometry for the fluorescent reporter transgene using the appropriate filters and according to the manufacturer's instructions.

11. The titer in Transducing Units (TU)/mL is calculated as (% fluorescence positive cells/100) × (dilution factor) × total cell number/well

3.4 Vector titration using quantitative real-time PCR

This method is appropriate for the analysis of HIV-based lentiviral vectors containing any type of transgene (i.e. fluorescent, luminescent, enzymatic etc.) as long as their sequence contains the sequences corresponding to the primer and probe sequences specified in section 2.4.16.

1. Seed HT1080 (titering cells) on a 12 well plate at a density of 2.5×10^5 cells per well. Incubate at 37 °C with 5% CO_2 overnight.

2. The next day, add 1 μL of polybrene per 1 mL of DMEM, for a final concentration of 8 μg/mL. Addition of polybrene can be considered optional but may result in more reproducible titers.

3. Make serial dilutions (100- and 1000-fold) for each sample; include an HIV-eGFP control and leave one well of cells untransduced (i.e. DMEM without lentivirus) as a negative control.

4. Apply 500 μL of each dilution per well.

5. Incubate at 37°C with 5% CO_2 for 3 days.

6. Harvest cells and extract DNA using the Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions.

7. Re-suspend DNA in 100 μL of water and store at 4°C.

8. Prepare lentivirus vector samples in triplicate on a 384-well plate.

9. Prepare a linearized plasmid standard curve ranging from 10^{10} copies/μL to 10^5 copies/μL.

10. Dilute primers and probe to 100 pmol/μL (see sequences in section 2.4.16 above).

11. Make a PCR master mix using TaqMan Universal Master Mix II (Table 2) and aliquot 6.4 μL in each well. Note that for all values in Table 2, it is good practice to multiple by an additional 10% to account for pipetting error.

12. Add 3.6 μL of sample per well or 3.6 μL water for the *No Template Control*.

13. Spin plate briefly to pull down samples.

14. Run the samples using the following program: 1) 50°C 2 min., 2) 95°C 10 min., 3) 95°C 15 sec., 4) 60°C 1 min., 5) repeat steps 3 and 4 for 35 cycles.

15. Proceed with data analysis and titer calculations (see Note [6]).

3.5 Vector quantitation using a Bioplex-based p24 ELISA

This method is appropriate for the analysis of HIV-based lentiviral vectors containing any type of transgene (i.e. fluorescent, luminescent, enzymatic etc.)

1. Prior to performing a HIV p24 (capsid) detection assay, Magnetic Beads region 42 (MagPlex Carboxymethylated Microspheres) should be coupled with anti-p24 mAb.
2. Pellet Magnetic Microsphere Beads (1.25×10^7) in a low protein-binding microcentrifuge tube by spin at 8000 × g for 2 min. and resuspend in 160 µl of 0.1 M monobasic sodium phosphate (pH 6.2).
3. Activate beads by the addition of 20 µl of Sulfo-NHS and 20 µl of EDC (freshly prepared in water) for 20 min. at RT.
4. Wash the beads twice in 1 mL of 1× PBS (pH 7.4) by centrifugation at 8000 × g for 2 min. Resuspend beads in 250 µl 1× PBS (pH 7.4).
5. Add 100 µl of anti-p24 mAb (1 mg/mL) and bring to a final volume to 1 mL with 1× PBS (pH 7.4).
6. Incubate for 2h at room temperature with gentle mixing on a Gyrotory shaker G2 or thermomixer.
7. Wash the beads twice in 1 × PBS and resuspend in 1 mL of Microsphere Resuspension Buffer.
8. Determine the bead concentration by counting on an automated cell counter or a hemocytometer and store at 4°C for p24 assay.
9. Prepare Luminex Wash Buffer as described in section 2.5.8.
10. Prepare Luminex Assay Buffer as described in section 2.5.11.
11. Mix anti-p24 mAb coupled beads in Luminex assay buffer at a concentration of ~1200 beads/sample. Beads can be counted on a hemocytometer or an automated cell counter.
12. Add 50 µl of bead suspension (~1200 beads) in duplicate well for each sample in a 96-well black flat bottom microplate.

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[6]The RNA copy number (x-axis) and Cycle Threshold value (C_T; y-axis) are used to create a standard curve and the formula for a semi-logarithmic curve ($y=be^{mx}$) is generated. The sample C_T values are fitted to the curve to determine the HIV RNA copy number. Each sample is run in triplicate and averaged together. Each sample of concentrated vector was infected at 100- and 1000-fold dilutions; therefore the calculated copy number of the appropriate sample is multiplied by 100 or 1000, respectively. Because 500 µL of each dilution was added per well, the copy number is multiplied by 2 to convert it to RNA copy number per milliliter. The values obtained for RNA copy number from each dilution are averaged together to determine the real-time PCR titer. Each plate is run with a sample of genomic DNA from HT1080 cells infected with HIV-eGFP concentrated vectors. The flow cytometry titer of HIV-eGFP is determined by FACS (as described in section 3.3) and the real time PCR titers (as determined as described in section 3.5) are normalized to the flow cytometry titer using the following formula:

$$\frac{\text{control HIV}-\text{eGFP titer (flow cytometry)}}{\text{control HIV}-\text{eGFP titer (real}-\text{time)}} \times \text{Sample titer (real}-\text{time)} = \text{Normalized sample titer (TU/mL)}$$

13. Prepare p24 standards (1.5 – 10,000 pg/mL, 3 fold dilutions) and dilute samples in Luminex Buffer. Also include a negative control where all reagents are added except the antigen.
14. Add 50 μl p24 standards and samples/well in duplicates and gently mix the plate containing the magnetic beads at the bottom.
15. Seal the 96-well plate with adhesive film and cover the plates with aluminum foil.
16. Shake the plate (~50 rpm) at room temperature for 2h.
17. Wash the plate 3–4 times with Luminex wash buffer in a 96-well magnetic plate washer with 2 minutes/wash.
18. Dilute the detection Ab (RD1-labelled anti-p24 KC57) in Luminex Assay Buffer to a concentration of 0.5 μg/mL.
19. Add 100 μl of detection anti-p24 antibody to the magnetic beads and gently mix the plate.
20. Re-seal the 96-well plate with adhesive film and cover the plates with aluminum foil.
21. Shake the plate (~50 rpm) at room temperature for 1h.
22. Repeat the wash step (same as step 10 above).
23. Add 60 μl Luminex Assay Buffer to each well and gently mix without introducing air bubbles.
24. The beads are analyzed for region of difference 1 (RD1) using the Bio-Plex Multiplex Systems instrument (controlled by the Bioplex manager software 4.11). Assay results should be based on at least 50 beads/sample in order to obtain reproducible data.
25. The amount of HIV-p24 levels is calculated by the Luminex System itself which is directly proportional to the fluorescence intensity.

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Table 1

Reagent amounts for transfection of producer cells to generate psuedotyped lentiviral particles

		Amount per 150 mm plate	Amount for n 150 mm plate
TUBE 1:	**2× HBS**	2,000 µl	2,000 µl × n
TUBE 2:	**Viral glycoprotein plasmid**	7.5 µ g	7.5 µ g × n
	pLP1 (expresses gag/pol)	22.5 µg	22.5 µg × n
	pLP2 (expresses rev)	7.5 µg	7.5 µg × n
	Plasmid containing transgene-of-interest within a packagable lentiviral genome	22.5 µg	22.5 µg × n
	2.5 M $CaCl_2$	200 µl	200 µl × n
	Sterile water	Bring to 2,000 µl	Bring to 2,000 µl × n

Table 2

Preparation of quantitative real-time PCR reactions for lentiviral RNA detection

	1 Reaction	Amount for n Reactions
Water	1.16 μL	1.16 μL × n
TaqMan Universal Master Mix II	5.0 μL	5.0 μL × n
100 μM probe	0.08 μL	0.08 μL × n
100 μM Primer 1	0.08 μL	0.08 μL × n
100 μM Primer 2	0.08 μL	0.08 μL × n
Total volume	6.4 μL	6.4 μL × n