Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors

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Over the past decade, lentiviral vectors have emerged as powerful tools for transgene delivery. The use of lentiviral vectors has become commonplace and applications in the fields of neuroscience, hematology, developmental biology, stem cell biology and transgenesis are rapidly emerging. Also, lentiviral vectors are at present being explored in the context of human clinical trials. Here we describe improved protocols to generate highly concentrated lentiviral vector pseudotypes involving different envelope glycoproteins. In this protocol, vector stocks are prepared by transient transfection using standard cell culture media or serum-free media. Such stocks are then concentrated by ultracentrifugation and/or ion exchange chromatography, or by precipitation using polyethylene glycol 6000, resulting in vector titers of up to 10^{10} transducing units per milliliter and above. We also provide reliable real-time PCR protocols to titrate lentiviral vectors based on proviral DNA copies present in genomic DNA extracted from transduced cells or on vector RNA. These production/concentration methods result in high-titer vector preparations that show reduced toxicity compared with lentiviral vectors produced using standard protocols involving ultracentrifugation-based methods. The vector production and titration protocol described here can be completed within 8 d.

INTRODUCTION

The protocol described here outlines facile procedures to prepare, concentrate and titrate lentiviral vectors based on HIV-1. Lentiviral vectors are traditionally produced by transient cotransfection of human embryonic kidney 293T cells using recombinant plasmids carrying transgene sequences, sequences encoding helper (packaging) functions and sequences encoding Env glycoproteins, respectively. The vesicular stomatitis virus Env glycoprotein (VSV-G) is typically used[1–4], although a wide range of alternative glycoproteins have the ability to associate with the vector's membrane, a process that is referred to as pseudotyping[5]. Typical vector titers range from 10^6 to 10^7 transducing units per milliliter. Increased titers can be achieved by physical concentration. This has opened up the way to generate highly concentrated vector stocks for *in vivo* applications[6,7]. However, lentiviral vectors bearing VSV-G at high concentrations can be cytotoxic[8]. Fortunately, such toxicity issues can be partially overcome by using alternative pseudotypes[9,10] or improved vector concentration/purification procedures, including protocols based on anion exchange chromatography[10]. Here we outline simplified protocols for lentiviral vector concentration involving membrane-based anion exchange chromatography or precipitation using polyethylene glycol 6000 (PEG 6000). These methods were found to result in vector stocks showing reduced toxicity compared with the standard protocols involving ultracentrifugation[10].

The widespread use of VSV-G to pseudotype lentiviral vectors has made this glycoprotein the standard against which the usefulness of other viral glycoproteins to form pseudotypes are compared. Comparison of pseudotype efficiency inevitably means comparing vector titers on cell lines *in vitro*. This brings up two important issues: (i) techniques to determine lentiviral vector titers and (ii) cell lines chosen for titration of such vectors. There are different measures used to determine lentiviral vector titers. Some of them are based on the number of vector particles present in a vector stock, whereas others are derived from the number of proviral copies in transduced target cells. Vector particle numbers can be determined using real-time PCR based on strong-stop cDNA present in virions[11]. Alternatively, the amount of virus proteins present in vector cores, such as p24, are determined by ELISA to arrive at relative particle titers[12]. Unfortunately, titers based on p24 tend to be unreliable[10,13]. Functional titration assays are based on vector-encoded reporter gene expression. For example, vectors encoding green fluorescent protein (EGFP) have been titrated using fluorescence-activated cell sorting (FACS) analysis[14]. For vectors that do not contain a reporter gene, proviral DNA copy numbers determined by real-time PCR using DNA extracted from transduced cells have been used to determine vector titers[9,14–17]. As each of these methods produces different results, close attention must be paid to the titration method used. Here we present detailed real-time PCR protocols to titrate lentiviral vectors based on either vector DNA sequences present in transduced cell DNA or vector RNA present in vector particles. Advantages of vector DNA-based titration methods are that they are independent of the transgene and promoter sequences being used. Thus, they are generally applicable. Titration methods based on RNA derived from vector particles are particularly useful for adjusting lentiviral vector stocks bearing alternative Env glycoproteins. Also, they are more reliable than titration assays based on p24 (see ref. 10). Again, it must be kept in mind that assays based on vector-derived RNA or on p24 do not reflect functional vector titers.

Also significant for titration is the cell line used because receptors for a given Env glycoprotein may vary from cell line to cell line, possibly producing a falsely depressed titer. Frequently, a given cell line (such as HeLa or 293 cells) is chosen because it is considered generally permissive to transduction rather than representing the natural tropism of the virus from which the glycoprotein was derived. Additionally, results produced with a given cell line or type of cell *in vitro* do not always translate into comparable results *in vivo* due to

factors such as complement-mediated inactivation of vectors. The protocol presented here addresses some of the shortcomings of earlier protocols[7] by providing generally applicable assays for titrating lentiviral vector pseudotypes based on either proviral DNA copies present in genomic DNA extracted from transduced cells or cDNA prepared using RNA extracted from vector particles. We expect our protocols to be generally useful for the production, concentration, and titration using second or third-generation lentiviral vectors bearing alternative glycoproteins, provided that such pseudotypes are sufficiently stable to withstand concentration by ultracentrifugation or precipitation using PEG 6000 or anion exchange chromatography.

Experimental design

Production of lentiviral vectors. The production protocols outlined in this protocol were optimized in the context of our second-generation lentiviral vector system[9]. However, these protocols are compatible with other second- and third-generation lentiviral vector systems. For optimal results, the relative amounts of the transgene-containing plasmid, the packaging plasmid(s) and the Env plasmid will need to be optimized on a case-by-case basis.

Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum (FBS) is typically used for the production of lentiviral vectors that are subsequently concentrated by ultracentrifugation. If the vectors are to be used for cell transduction without previous concentration, alternative media may have to be considered. For example, unconcentrated lentiviral vector stocks produced using DMEM/10% FBS appeared to be toxic to primary cortical neuronal cultures, whereas lentiviral vectors produced in serum-free UltraCULTURE medium were not[18].

The production and handling of lentiviral vectors should be carried out using the proper biosafety containment. See Biosafety Considerations for Research with Lentiviral Vectors (http://oba.od.nih.gov/oba/rac/Guidance/LentiVirus_Containment/pdf/Lenti_Containment_Guidance.pdf).

Concentration of lentiviral vector stocks. For cell transduction *in vitro*, crude (unconcentrated) vector stocks are often sufficient, whereas concentrated vector stocks are needed for *in vivo* applications. Concentration of lentiviral vector stocks are typically performed using ultracentrifugation approaches[6]. This is a rapid and robust method for vector concentration at a small scale. Inclusion of a sucrose cushion during ultracentrifugation has been shown to be beneficial in terms of reducing vector toxicity. For example, it was shown to reduce systemic immune responses to vector proteins as well as brain inflammation exerted by VSV-G pseudotypes[19]. Anion exchange chromatography of lentiviral vector-containing cell culture supernatants using Mustang Q anion exchange membrane cartridges[10] or precipitation of lentiviral vectors using PEG 6000 (see ref. 20) are particularly useful for processing large volumes of lentiviral vector supernatants. Also, lentiviral vectors pseudotyped with VSV-G and purified on Mustang Q anion exchange membrane cartridges were found to be less toxic in the context of *in vitro* applications involving mouse mesenchymal stem cells[10].

Titration of lentiviral vectors. Flow cytometric (FACS) methods provide a convenient way to titrate lentiviral vectors expressing fluorescent proteins such as EGFP[6,9] or DsRed[14]. However, the success of this method ultimately depends on the promoter sequences being used for transgene expression. Quantitative PCR methods that determine the number of vector copies associated with genomic DNA extracted from transduced cells provide a more general method for vector titration that is independent of the transgene and promoter sequences present in the vector[9,16]. Assays that determine the number of vector particles based on virion RNA[10,15] or on p24 (see refs. 21,22) are particularly useful for adjusting lentiviral vector stocks bearing alternative Env glycoproteins.

Controls. Vector stocks that are generated using packaging constructs lacking a functional reverse transcriptase provide useful controls to rule out carryover of plasmid DNA during titration of vector stocks using quantitative PCR methods[9]. Such vector stocks should show negligible amounts of vector copies.

MATERIALS

REAGENTS

▲ CRITICAL The production and handling of lentiviral vectors should be carried out using the proper biosafety containment. See Biosafety Considerations for Research with Lentiviral Vectors (http://www4.od.nih.gov/oba/RAC/Guidance/LentiVirus_Containment/pdf/Lenti_Containment_Guidance.pdf).

Plasmids

- Second-generation lentiviral transgene plasmids: pNL-EGFP/CMV (see ref. 4); pNL-EGFP/CMV/WPREΔU3 (see ref. 10); available from Addgene
- Second-generation lentiviral packaging plasmid: pCD/NL-BH*ΔΔΔ (see ref. 9); available from Addgene
- Envelope glycoprotein-encoding plasmids: pLTR-G (see ref. 3); pLTR-RVG and pLTR-MVG (see ref. 21); pLTR-RD114A (see ref. 9); available from Addgene; the pCAGGS-LCMV-WE and pCAGGS-LCMV-Arm plasmids were provided by Dr Juan Carlos de la Torre, The Scripps Research Institute (juanct@scripps.edu) (see refs. 10,23,24)
- 293T human embryonic kidney cells (ATCC, cat. no. CRL-11268)
- Human osteosarcoma (HOS) cells (ATCC, cat. no. CRL-1543)
- H9 cells (ATCC, cat. no. HTB-176)
- DMEM high glucose (GIBCO, cat. no. 11995)
- RPMI medium 1640 (GIBCO, cat. no. 1875-093)
- UltraCULTURE Serum-free Medium (Lonza, cat. no. 12-725F)
- FBS, heat inactivated (GIBCO, cat. no. 26140-079)
- Glutamax (GIBCO, cat. no. 35050)
- Penicillin–streptomycin (GIBCO, cat. no. 15140-122)
- Trypsin-EDTA, 0.25% (GIBCO, cat. no. 3197)
- PBS without calcium chloride and magnesium chloride (without Ca/Mg) (GIBCO, cat. no. 14190)
- Hank's balanced salt solution, 1× (GIBCO, cat. no. 14025-092)
- 25 mM chloroquine dissolved in H_2O(Sigma, cat. no. C6628)
- Polybrene 8 mg ml^{-1} dissolved in H_2O (Sigma, cat. no. H9268)
- 25 mM EDTA (Invitrogen, cat. no. Y02353)
- DNaseI (Takara Mirus Bio, cat. no. 2215A)
- DNaseI Amplification grade (Invitrogen, cat. no. 18068015)
- RNase A (USB, cat. no. 21195)
- SUPERase-In (Ambion, cat. no. 2694)
- Platinum *Taq* polymerase (Invitrogen, cat. no. 10966-018)
- DNeasy tissue kit (Qiagen, cat. no. 69506)
- PureLink viral RNA/DNA kit (Invitrogen, cat. no. 12280-050)
- DNase- and RNase-free water (GIBCO, cat. no. 10977-015)
- tRNA (Sigma, cat. no. R5636)
- Fluorescent DNA Quantitation kit (Bio-Rad, cat. no. 170-3480)
- Primers and probes for real-time PCR (Integrated DNA Technologies). The sequences of the Gag-specific primers used are 5′-TTCGCAGTTAATCCTGGCCTT-3′ (forward primer), 5′-GCACACAATAGAGGACTGCTATTGTA-3′ (reverse primer) and 5′ FAM-TAGAGACATCAGAAGGCTGTAGACAAA-TAMRA 3′ (probe). The

sequences of the WPRE-specific primers are 5′-CCTTTCCGGGACTTTCGCTTT-3′ (forward primer), 5′-GCAGAATCCAGGTGGCAACA-3′ (reverse primer) and 5′-FAM-ACTCATCGCCGCCTGCCTTGCC-TAMRA-3′ (probe)
- TaqMan Universal PCR Master Mix (Applied Biosystems, cat. no. 4304437)
- TaqMan DNA Template Reagent Kit (Applied Biosystems, cat. no. 401970)
- TaqMan RNaseP control reagent (Applied Biosystems, cat. no. 4316844)
- Rodent GAPDH control reagent (Applied Biosystems, cat. no. 4308313)
- iScript cDNA synthesis kit (Bio-Rad, cat. no. 170-8890)
- Retro-Tek HIV-1 p24 antigen ELISA kit (ZeptoMetrix, cat. no. 0801111)
- HEPES (Sigma, cat. no. H4034)
- NaCl (EM Sciences, cat. no. SX0420-3)
- UltraPure sucrose (Invitrogen, cat. no. 15503-022)
- KCl (EM Sciences, cat. no. PX1405-1)
- Diethylpyrocarbonate (DEPC)-treated H_2O (Invitrogen, cat. no. 750023)
- $CaCl_2$ (Sigma, cat. no. C-2536)
- H_2O (GIBCO, cat. no. 10977-015)
- PEG 6000 (Fluka, cat. no. 81253)

EQUIPMENT
- Maxiprep kit (Qiagen, cat. no. 12263)
- 150-cm^2 dishes (Nunclone, cat. no. 168381)
- T150 tissue culture flasks
- T25 tissue culture flaks
- 6-well cell culture plates
- 12-well cell culture plates
- Optic 96-well plates (Applied Biosystems, cat. no. N801-0560)
- Optic MicroAmp Caps (Applied Biosystems, cat. no. N801-0534)
- Polystyrene round-bottomed tubes (5 ml, 12 mm × 75 mm) (Becton Dickinson, cat. no. 352005)
- PES filter units, 0.45 μm (Corning, cat. no. 430768)
- PES filter units, 0.22 μm (Corning, cat. no. 431096)
- Biosafety level 2 tissue culture facility
- Tissue culture hood
- Tissue culture incubators
- Beckman ultracentrifuge (Beckman Coulter)
- SW28 ultracentrifuge rotor (Beckman)
- Ultra-clear SW28 centrifuge tubes (Beckman, cat. no. 344058)
- Beckman Avanti J-25 I centrifuge (Beckman)
- Fixed-Angle Rotor, JLA-10.500 (Beckman)
- Polypropylene wide-mouthed bottle with cap assemblies, 250 ml (Beckman, cat. no. 356011)
- Fluorescence microscope
- Acrodisk units with Mustang Q membrane, 0.8 μm, 25 mm (Pall, cat. no. MSTG25Q6)
- ABI PRISM 7700 sequence detector or Stratagene Mx3000P real-time PCR System
- FACS machine
- ELISA microplate reader

REAGENT SETUP

2× HEPES-buffered saline (HBS) solution (50 mM HEPES, 1.5 mM Na_2HPO_4, 280 mM NaCl, 10 mM KCl, 12 mM sucrose): to prepare a 2× HBS solution, dissolve 23.8 g of HEPES, 32.0 g of NaCl, 4.32 g of UltraPure sucrose and 1.48 g of KCl in DEPC-treated H_2O and add 2 ml of a 1.5 M Na_2HPO_4 stock solution. Adjust the volume to 1,800 ml. Split the solution into four 450-ml aliquots. Adjust the pH of the first aliquot to 7.07 using 1 N NaOH. Adjust the pH of the second aliquot to 7.09, that of the third to 7.11 and that of the fourth to 7.13. For each aliquot, adjust the volume to 500 ml with H_2O and autoclave. This solution can be stored at 4 °C for at least 1 month. Test the transfection efficiency for each aliquot and store the most efficient one at −20 °C in 50-ml conical tubes containing 36 ml per tube. This solution can be stored at −20 °C for at least 1 year.
▲ CRITICAL For optimal transfection, the proper pH of the 2× HBS solution is critical.

2 M $CaCl_2$ stock solution Dissolve 14.7 g of $CaCl_2$ and adjust to 100 ml with H_2O and filter-sterilize. This solution can be stored at −20 °C in 15-ml conical tubes with 13.5 ml per tube for at least 1 year.

20% sucrose solution Dissolve 20 g of UltraPure sucrose, 100 mM NaCl, 20 mM HEPES (pH 7.4) and 1 mM EDTA. Adjust the volume to 100 ml by adding H_2O and filter-sterilize. This solution can be stored at 4 °C for at least 6 months.

Chromatography buffers Buffer A—0.15 M NaCl, 25 mM Tris-HCl, pH 8.0; buffer B—1.5 M NaCl, 25 mM Tris-HCl, pH 8.0; buffer C—1.5 M NaCl, 250 mM Tris-HCl, pH 8.0. These buffers can be stored at 4 °C for up to 1 month.

50% PEG 6000 Dissolve 250 g of PEG 6000 in 500 ml of H_2O. Autoclave and mix thoroughly. This solution can be stored at 4 °C for at least 6 months.

4.0 M NaCl stock solution Sterilize by autoclaving. This solution can be stored at 4 °C for at least 1 year.

Hank's balanced salt solution for flow cytometry Mix 20 ml of 10× Hank's balanced salt solution with 4 ml of FBS and 176 ml of distilled water. This solution can be stored at 4 °C for at least 2 months.

Real-time PCR standards for proviral DNA-copy determination To generate a standard curve, a plasmid bearing the HIV-1 Gag region, such as the pNL-EGFP/CMV plasmid[4] (9,682 bp, ~5 × 10^{12} molecules per 50 μg of purified plasmid DNA) is adjusted to 10^{10} molecules per 5 μl. Prepare serial 1–10 dilutions in the presence of 4 μg ml^{-1} tRNA. Prepare dilutions containing between 10^2 and 10^7 copies of plasmid DNA in a volume of 5 μl. The diluted plasmid DNA soutions are stable at −20 °C for up to 1 month. Multiple freeze–thaw cycles should be avoided.

Real-time PCR standards for human genomic DNA Prepare serial dilutions of human genomic DNA in the presence of 4 μg ml^{-1} tRNA resulting in 50, 10, 2, 0.4 and 0.08 ng of genomic DNA per 5 μl, respectively. A total of 1 ng of genomic DNA corresponds to 333 genomic copy equivalents. Prepare primers and probes for real-time PCR in DNase–RNase-free H_2O at a concentration of 100 pmol μl^{-1}. The diluted genomic DNA samples are stable at −20 °C for up to 1 month. Multiple freeze–thaw cycles should be avoided.

PROCEDURE

▲ CRITICAL STEP The production and handling of lentiviral vectors should be carried out using the proper biosafety containment. See Biosafety Considerations for Research with Lentiviral Vectors (http://oba.od.nih.gov/oba/rac/Guidance/LentiVirus_Containment/pdf/Lenti_Containment_Guidance.pdf).

Seeding of 293T cells for lentiviral vector production ● TIMING Day 1: early afternoon

1| Start with three semiconfluent T150 flasks containing 293T cells grown in DMEM high glucose + 10% FBS, 1% Glutamax and 1% penicillin–streptomycin.
▲ CRITICAL STEP Healthy 293T cells are important. Make sure that the cells have not been passaged more than 20 times.
? TROUBLESHOOTING

2| Split 293T cells into twelve 150-cm^2 dishes at a density of 8 × 10^6 cells per dish in 25 ml of DMEM high glucose medium supplemented with 10% FBS, 1% Glutamax and 1% penicillin–streptomycin.
? TROUBLESHOOTING

Transfection of 293T cells ● TIMING Day 2: late afternoon

3| Inspect the cells under a microscope. The cells should be around 30–40% confluent and well attached and they should show a uniform and even distribution.
? TROUBLESHOOTING

4| Add 25 µl of 25 mM chloroquine to the medium of each dish, resulting in a final concentration of 25 µM.
? TROUBLESHOOTING

5| In a sterile 50-ml conical tube, mix 31.56 ml of sterile H_2O, 4.44 ml of 2 M $CaCl_2$, 252 µg of the pNL-EGFP/CMV/WPREΔU3 lentiviral vector plasmid, 168 µg of the pCD/NL-BH*ΔΔΔ packaging plasmid and 84 µg of the VSV-G-encoding pLTR-G plasmid (see ref. 3). For plasmids encoding other Env glycoproteins, such as pLTR-RD114A, pLTR-RVG and pLTR-MVG, and pCAGGS-LCMV-WE and pCAGGS-LCMV-Arm encoding RD114, rabies virus, Mokola virus and lymphocytic choriomeningitis virus Env glycoproteins, respectively, use 252 µg.
▲ **CRITICAL STEP** Plasmid DNA prepared using Qiagen maxiprep kits (or an equivalent kit) is recommended.
? TROUBLESHOOTING

6| Mix contents of the tubes thoroughly and transfer an 18-ml aliquot of the DNA/$CaCl_2$ mixture into another 50-ml conical tube.
? TROUBLESHOOTING

7| Using a 25-ml pipette, add 18 ml of 2× HBS drop by drop while vortexing lightly (just enough to swirl the contents of the tube).
▲ **CRITICAL STEP** Proper production and storage of 2× HBS is very important.
? TROUBLESHOOTING

8| Immediately after vortexing, remove 36 ml of the mixture using a 25-ml pipette and distribute 6-ml aliquots drop by drop into each of the six 150-cm^2 dishes described in Step 4.
? TROUBLESHOOTING

9| Process another 18-ml aliquot of the DNA/$CaCl_2$ mixture from Step 5 as described in Steps 6–8.
? TROUBLESHOOTING

10| Return the 12 dishes to the tissue culture incubator. Make sure that the dishes are stacked evenly (no more than six dishes per stack) and incubate them in the presence of 5% CO_2 at 37 °C overnight (16–18 h).
? TROUBLESHOOTING

Change the medium ● TIMING Day 3: first thing in the morning

11| Observe the cells under a microscope. The cells should appear healthy and be around 60–80% confluent. A fine precipitate should be visible in areas where cells are not present.
? TROUBLESHOOTING

12| Cells can be cultured either in DMEM, 10% FBS (option A) if lentiviral vectors are to be concentrated by ultracentrifugation or UltraCULTURE serum-free medium (option B) if the vectors are to be used for cell transduction without previous concentration (see Experimental design for further discussion).

(A) DMEM, 10% FBS medium

(i) Remove the medium by aspiration and add 17 ml of fresh DMEM high glucose, 10% FBS, 1% Glutamax and 1% penicillin–streptomycin.

(B) UltraCULTURE serum-free medium

(i) Remove the medium by aspiration and add 17 ml of UltraCULTURE serum-free medium plus 0.6 mg ml^{-1} glucose, 1% Glutamax and 1% penicillin–streptomycin.
? TROUBLESHOOTING

13| Return the 12 dishes to the tissue culture incubator. Make sure that the dishes are stacked evenly (no more than six dishes per stack) and incubate them in the presence of 5% CO_2 at 37 °C for 2 d (48–50 h).
? TROUBLESHOOTING

Observe transfected cells ● TIMING Day 4

14| Observe the transfected cells under a microscope. Syncytia of fused cells should start to appear and most of the cells should still be attached. If a reporter gene encoding a fluorescent protein (such as EGFP) is used in the context of a constitutive promoter (such the cytomegalovirus immediate early promoter), more than 95% of the cells should show green fluorescence.
? TROUBLESHOOTING

Harvest the cell supernatant containing lentiviral vectors ● TIMING Day 5: first thing in the morning

15| Collect the cell supernatant in 50-ml conical tubes.

? TROUBLESHOOTING

16| Centrifuge tubes at 500*g* for 10 min at 25 °C to remove cells and large cell debris.

? TROUBLESHOOTING

17| Pour pooled supernatants (204 ml) into a 250-ml 0.45-μM PES filter flask. If the filter starts to clog up as judged by a reduced downflow rate, replace the filter flask.

■ **PAUSE POINT** The filtered cell culture supernatant can be aliquoted and frozen on crushed dry ice and then stored at −80 °C. Alternatively, it can be concentrated as outlined in Step 18. To increase vector yields, multiple vector harvests starting at 24 h after transfection can be carried out[6].

? TROUBLESHOOTING

Concentration of lentiviral vectors ● TIMING Day 5

18| Concentration of vector stocks can be performed using option A (ultracentrifugation), option B (anion exchange chromatography of lentiviral vector-containing cell culture supernatants using Mustang Q anion exchange membrane cartridges[10]) or option C (precipitation of lentiviral vectors using PEG 6000 (see ref. 20)).

(A) Lentiviral concentration by ultracentrifugation

(i) Sterilize six Ultra-clear SW28 centrifuge tubes by spraying them down with a 70% ethanol solution. Place the tubes into a laminar flow hood with the UV light on for 30 min.

(ii) Transfer ~32-ml aliquots of filtered vector-containing cell culture supernatant from Step 17 into each of the six UV-sterilized Ultra-clear SW28 tubes.

(iii) Fill a 10-ml pipette all the way up to the 12-ml mark with a 20% sucrose solution. Insert the pipette all the way to the bottom of the SW28 tube filled with the filtered vector-containing supernatant and gently underlay it by slowly expelling 4 ml of the sucrose solution. Repeat this step for two more of the SW28 tubes using the remaining 8 ml of the 20% sucrose solution from the same 10-ml pipette. For the remaining three tubes, use a new 10-ml pipette and draw up 12 ml of the 20% sucrose solution and proceed in the same way as for the first three tubes.

(iv) Adjust the weight of each tube by adding PBS until they are within 0.1 g of each other.

(v) Place all the six tubes into a Beckman SW28 ultracentrifuge rotor.

(vi) Centrifuge for 2 h at 25,000 r.p.m. (82,700*g*) and 4° C using an ultracentrifuge.

(vii) Carefully remove tubes from the rotor, pour off supernatant and leave tubes on a paper towel in an inverted position for 10 min to allow the residual liquid to drip away from the pellet. Aspirate the remaining medium droplets. There should be a miniscule pellet at the bottom of the tube.

(viii) Add 100 μl of PBS without Ca/Mg to each pellet.

(ix) Place the SW28 ultracentrifuge tubes into a 50-ml conical tube and close tube with the lid.

(x) Incubate the tubes at 4 °C for 2 h. Vortex very gently every 20 min.

(xi) Spin the tubes at 500*g* for 1 min at 25 °C to collect the vector-containing liquid.

(xii) Using a 200-μl pipette, resuspend the pellet by gently pipetting the liquid up and down. Avoid the formation of bubbles. Combine liquid from all resuspended pellets in a single SW28 ultracentrifuge tube.

■ **PAUSE POINT** If the pooled viral vector-containing suspension is to be subjected to titration at this point (proceed to Step 19), it can be aliquoted in screw-cap microfuge tubes in 25- to 100-μl portions, snap-frozen in crushed dry ice and stored at −80 °C. Alternatively, the pooled viral vector suspension can be further purified by anion exchange chromatography using Acrodisk units with Mustang Q membranes, as described in the following steps.

(xiii) For anion exchange chromatography, set up two Acrodisk units with Mustang Q membranes according to the manufacturer's instructions. Assemble the two Acrodisk units in a tandem manner.

(xiv) Add 27 ml of PBS without Ca/Mg to the sample described in Step A(xii).

(xv) Add 3 ml of buffer C.

(xvi) Pass the sample through the tandem Acrodisk units. Avoid the formation of bubbles.

(xvii) To wash the tandem Acrodisk units, attach a 5-ml syringe containing 3 ml of buffer A. Push the buffer through the two columns. Avoid the formation of bubbles.

(xviii) To elute the vectors, attach a 10-ml syringe containing 10 ml of buffer B and push the buffer through. Collect the eluate in an Ultra-clear SW28 centrifuge tube containing 25 ml of PBS.

(xix) Centrifuge the sample as described under Step A(vi).

(xx) Resuspend the pellets as described under Step A(vii–xii).

(xxi) Transfer resuspended vector sample into screw-cap microfuge tubes in 25- to 100-μl portions.

(xxii) Snap-freeze the tubes in crushed dry ice and store at −80 °C.

(B) Lentiviral concentration by anion exchange chromatography of lentiviral vector-containing cell culture supernatants using Mustang Q anion exchange membrane cartridges

(i) Sterilize six Ultra-clear SW28 centrifuge tubes by spraying them down with a 70% ethanol solution. Place tubes into a laminar flow hood with the UV light on for 30 min.
(ii) Set up two Acrodisk units with Mustang Q membranes according to the instructions provided by the manufacturer. Assemble the two Acrodisk units in a tandem manner.
(iii) Pipette 25 ml of PBS without Ca/Mg into each of the six sterilized Ultra-clear SW28 centrifuge tubes.
(iv) Add 20 ml of buffer C to 204 ml of the vector-containing cell culture supernatant (from Step 17) after passing it through a 250-ml 0.45-μM PES filter flask.
(v) To load the vector-containing sample onto the tandem Acrodisk units, draw up about 32 ml of the sample from Step B(iv) into a 30-ml syringe and pass the sample through the tandem Acrodisk units. Avoid the formation of bubbles during loading of the sample.
(vi) To wash the sample adsorbed to the Acrodisk units, attach a 5-ml syringe containing 3 ml of buffer A. Push the buffer through the two Acrodisk units. Avoid the formation of bubbles.
(vii) To elute the vectors, attach a 10-ml syringe containing 10 ml of buffer B and push the buffer through. Collect the eluate in an Ultra-clear SW28 centrifuge tube containing 25 ml of PBS.
(viii) Regenerate the Acrodisks by passing 5 ml of a 1 N NaOH solution through followed by 5 ml of buffer A.
(ix) Load another 32 ml of the vector-containing supernatant described under Step B(iv) and proceed with Step B(v–viii).
(x) Repeat this cycle four more times until all of the vector-containing supernatant has been processed. The six Ultra-clear SW28 centrifuge tubes will each contain 10 ml of vector-containing eluate and 25 ml of PBS.
(xi) Centrifuge the samples as described in Step A(iv–vi).
(xii) Resuspend the pellets as described in Step A(vii–xii).
(xiii) Aliquot resuspended sample in screw-cap microfuge tubes in 25- to 100-μl portions.
(xiv) Snap-freeze tubes in crushed dry ice and store at −80 °C.

(C) Lentiviral concentration by precipitation of lentiviral vectors using PEG 6000

(i) Take 204 ml of a vector-containing cell culture supernatant (from Step 17) after filtration using a 250-ml 0.45-μM PES filter flask.
(ii) Add 51 ml of a 50% PEG 6000 solution.
(iii) Add 21.7 ml of a 4 M NaCl stock solution.
(iv) Add 23.3 ml of PBS. This will result in a final volume of 300 ml. The final PEG 6000 concentration will be 8.5% and the final NaCl concentration will be ~0.3 M.
(v) Distribute the sample as 150-ml aliquots in two 250-ml polypropylene wide-mouthed bottles.
(vi) Store the bottles at 4 °C for 1.5 h. Mix contents every 20–30 min.
(vii) Centrifuge bottles at 7,000*g* for 10 min at 4 °C using a Beckman fixed-angel JLA-10.500 rotor.
(viii) After centrifugation, a white pellet should be visible.
(ix) Carefully decant the supernatant and add 1.2 ml of 50 mM Tris-HCl, pH 7.4, per bottle. Resuspend the pellets by vigorously pipetting liquid up and down.
(x) Vortex the bottles vigorously for 20–30 s to further resuspend the pellets.
(xi) Transfer the vector suspension into screw-cap microfuge tubes in aliquots of 100 μl.
(xii) Snap-freeze the tubes in crushed dry ice and store at −80 °C.

Titration of lentiviral vectors ● TIMING Days 5–8

19| This step can be performed using options A, B, C or D depending on the transgene and promoter sequences that are carried by the lentiviral vector or the Env glycoprotein that is used for pseudotyping; option A involves a quantitative PCR method to determine the number of vector copies associated with genomic DNA extracted from transduced HOS cells[9,16]. Option B involves a flow cytometric (FACS) method to titrate lentiviral vectors expressing fluorescent proteins such as EGFP[6,9] or DsRed[14]. Option C deals with an assay that determines relative vector particle numbers based on virion RNA[10,15]. Finally, option D describes a titration assay based on p24 (see refs. 21,22).

(A) Quantitative PCR method to determine the number of vector copies associated with genomic DNA extracted from transduced HOS cells

(i) Plate HOS cells at a density of 5×10^4 cells per well in a 6-well plate 1 d before transduction.
(ii) Twenty-four hours after seeding, count the number of cells in two of the wells using a hemacytometer. Remove the medium from other wells and replace with 0.5 ml of fresh medium containing 8 μg ml^{-1} of polybrene. Cells are transduced by adding 0.5-, 5- and 50-μl aliquots of an unconcentrated vector stock (from Step 17) per well, respectively. Tilt plates to distribute the vectors and return to the tissue culture incubator. For concentrated vector stocks (from Step 18), dilute samples 200-fold with cell culture medium (i.e., mix 1 μl of concentrated vector stock and 199 μl

of medium). Transduce HOS cells by adding 0.5-, 5- and 50-µl aliquots, respectively, of the diluted vector suspension per well.

(iii) Twenty hours after initiating the transduction, replace the medium with 0.5 ml of medium containing DNaseI (Takara Mirus Bio, final concentration 10 U ml^{-1}). Incubate for 15 min at 37 °C. Remove DNaseI and replace with 2 ml of fresh medium and continue to incubate cells for another 48 h.

(iv) Treat cell monolayer using trypsin-EDTA using 0.5 ml of a 0.25% trypsin-EDTA solution for 1 min at 37 °C and collect cells. Isolate high-molecular weight genomic DNA from transduced HOS cells using a DNeasy kit, according to the manufacturer's instructions. Elute DNA with 200 µl of elution buffer per well and determine DNA concentration using a Fluorescent DNA Quantitation kit (Bio-Rad), according to the manufacturer's instructions.

■ **PAUSE POINT** Genomic DNA can be stored in a −20 °C freezer for at least 2 months until ready for real-time PCR analysis.

(v) Set up samples for real-time PCR analysis; prepare Master mix I on ice as follows:

2× TaqMan Master Mix	25 µl × *n*
Forward primer (100 pmol $µl^{-1}$)	0.1 µl × *n*
Reverse primer (100 pmol $µl^{-1}$)	0.1 µl × *n*
Probe (100 pmol $µl^{-1}$)	0.1 µl × *n*
H_2O	19.7 µl × *n*

n = number of reactions.

An example: for 40 reactions, mix 1,000 µl of 2× TaqMan Universal PCR Master Mix, 4 µl of forward primer, 4 µl of reverse primer and 4 µl of probe plus 788 µl of H_2O. Vortex sample and store on ice.

(vi) Prepare Master Mix II as follows:

2× TaqMan Master mix	25 µl × *n*
10× RNaseP primer/probe mix	2.5 µl × *n*
H_2O	17.5 µl × *n*

n = number of reactions.

An example: for 40 reactions, mix 1,000 µl of 2× TaqMan Universal PCR Master Mix, 100 µl of RNaseP primer/probe mix and 700 µl of H_2O. Vortex sample and store on ice.

(vii) Set up real-time PCRs on a prechilled Optic 96 well plate by adding 45 µl of Master Mix I into each well of rows A–D.

(viii) Add Master Mix II into each well of rows E–G.

(ix) Add 5 µl of the diluted plasmid standard or the transduced cell DNA to be tested (in duplicate) into all wells containing Master Mix I. Add 5 µl of H_2O to one of the wells to serve as a no-template control.

(x) Add 5 µl of the diluted genomic DNA standard or the transduced cell DNA to be tested (in duplicate) into all the wells containing Master Mix II. Add 5 µl of H_2O to one of the wells to serve as a no-template control.

(xi) Real-time PCR is performed with an ABI PRISM 7700 Sequence Detector or a Stratagene Mx3000P Real-Time PCR System. Set cycling conditions as follows: 2 min at 50 °C, and 10 min at 95 °C, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

(xii) *Data analysis*: vector copy numbers in HOS cells are normalized to human RNaseP gene copies and presented as proviral copies per genome equivalent. Calculate titers (integration units per ml, IU ml^{-1}) according to the following formula: IU $ml^{-1} = (C \times N \times D \times 1{,}000)/V$, where C = proviral copies per genome, N = number of cells at time of transduction (corresponding to about 1×10^5 HOS cells per well), D = dilution of vector preparation, V = volume of diluted vector added in each well for transduction. To arrive at an accurate titer, average values obtained from at least two of the vector dilutions. A linear relationship exists between the number of proviral copies per genome and the amount of vector added at fewer than five vector copies per genome (see **Fig. 1**). To generate more accurate standards, cellular clones with known vector copy numbers determined by Southern blot analysis should be used (see ref. 25). Also, inclusion of a second 'normalization' gene may lead to more accurate measurements.

(B) Flow cytometric (FACS) method to titrate lentiviral vectors expressing fluorescent proteins, such as EGFP or DsRed

(i) Carry out transductions as described in Steps A(i) and (ii).

(ii) Twenty hours after beginning the transduction, replace the medium with 2 ml of fresh medium and continue to incubate cells.

(iii) After 2 d, remove the medium and wash cells with 1 ml of PBS. Add 0.5 ml of trypsin-EDTA per well and incubate at 37 °C for 2 min.

(iv) Add 1 ml of medium to each well, mix contents and transfer cell suspension from each well into a 5-ml round-bottomed tube and centrifuge at 1,500 r.p.m. (500*g*) for 5 min at 20 °C to pellet cells.

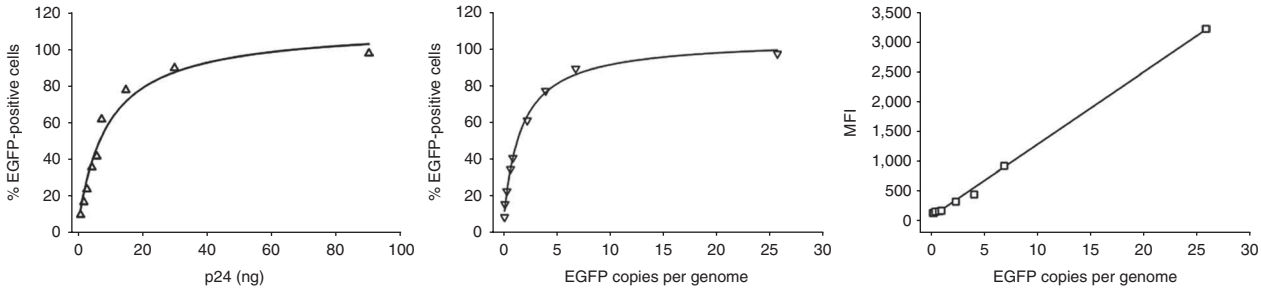

Figure 1 | Influence of vector copy numbers on EGFP transgene expression in transduced HOS cells. Cells were transduced with an EGFP-encoding lentivirus vector in DMEM-10% FBS containing 8 μg of polybrene per ml at 37 °C for 20 h. Left panel: percentage of EGFP-positive cells as a function of the amount (ng) of vector (p24) added. Centre panel: percentage of EGFP-positive cells as a function of the number of EGFP transgene copies per genome. Right panel: mean fluorescence intensity (MFI) values of the EGFP-positive cell population as a function of the number of EGFP transgene copies. The cells were analyzed by FACS 3 d after transduction. Aliquots were processed for quantitative real-time PCR with EGFP-specific primers. The MFI values and the numbers of EGFP copies per genome are shown. The data shown were obtained from two independent experiments. The vector titer was 3.6×10^4 TU ng^{-1} of p24. Reproduced with permission from ref. 9.

(v) Remove the medium by aspiration and resuspend cells pellets in 2 ml of Hank's balanced salt solution and centrifuge at 1,500 r.p.m. (500*g*) for 5 min at 20 °C to pellet cells.

(vi) Remove Hank's balanced salt solution by aspiration and resuspend the cell pellets in 300 μl of Hank's balanced salt solution.

(vii) Analyze the cells by FACS[9].

(viii) Calculate titer (transducing units (TU) ml^{-1}) according to the following formula: TU $ml^{-1} = (F \times N \times D \times 1{,}000)/V$, where F = percentage of fluorescent cells (EGFP, DsRed or other fluorescent labels), N = number of cells at the time of transduction (corresponding to about 1×10^5 HOS cells per well), D = fold dilution of vector sample used for transduction and V = volume (μl) of diluted vector sample added into each well for transduction. Combine vector titers obtained from HOS cells transduced with different amounts of vector and calculate an average titer. For accurate titer determination, the amount of vector used should fall in a range in which there is a linear relationship between the percentage of EGFP-positive cells and the amount of vector added (see **Fig. 1**). If the percentage of fluorescent cells exceeds 40%, repeat titration using additional vector dilutions.

(C) Assay to determine relative vector particle numbers based on virion RNA

(i) Into a nuclease-free Eppendorf tube, add 40 μl of 10× DNaseI reaction buffer (Invitrogen) and 353 μl of DEPC-treated H_2O.

(ii) Add 2 μl of the lentiviral vector sample to be tested.

(iii) Add 5 μl of amplification-grade DNaseI (Invitrogen).

(iv) Add 50 pg of RNase A.

▲ **CRITICAL STEP** Pipette RNase A in an area that is physically separated from that used for RNA isolation. Also, do not use pipettes that have been in contact with RNaseA for isolating RNA.

(v) Mix tubes briefly by vortexing and spin them to collect contents to the bottom of the tube.

(vi) Incubate for 10 min at room temperature (25 °C).

(vii) Add 20 μl of SUPERase-In.

(viii) Incubate at 37 °C for 10 min. Spin the tubes briefly.

(ix) Add 10 μl of 25 mM EDTA, mix the sample by pipetting and briefly spin the tubes.

(x) Incubate for 10 min at 70 °C. Spin the tubes at 2,000*g* for 10 s at 25 °C.

(xi) Proceed to RNA extraction using a PureLink kit according to the manufacturer's instructions. Elute RNA with 30 μl of elution buffer.

(xii) To RNase-free PCR tubes, add 15 μl of the eluted RNA.

(xiii) Perform a cDNA synthesis reaction using an iScript cDNA synthesis kit according to the manufacturer's instructions. In a parallel reaction, use 15 μl of the eluted RNA but omit reverse transcriptase.

(xiv) Incubate tubes at 48 °C for 1 h and then place tubes on ice.

(xv) Set up real-time PCR using 5 μl of the cDNA product; prepare Master Mix I for 40 reactions as described above for Step A(vi).

(xvi) Set up reactions in a prechilled Optic 96 well by adding 45 μl of Master Mix I in each well of rows A–D. Add 5 μl of the diluted plasmid standard or the cDNA to be tested (in duplicate) into all the wells containing Master Mix I. Add 5 μl of H_2O to one of the wells to serve as a no-template control.

(xvii) Real-time PCR is performed with an ABI PRISM 7700 sequence detector or a Stratagene Mx3000P real-time PCR system. Set cycling conditions as follows: 2 min at 50 °C and 10 min at 95 °C, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

(xviii) Calculate relative vector particles numbers per ml according to the following formula: relative vector particles ml^{-1} (VP ml^{-1}) = $(C \times D)/V$, where C = number of RNA copies, D = dilution of vector preparation (including the dilution into the PCR) and V = volume in ml.

(D) Titration assay based on p24

(i) Prepare serial dilutions of the vector samples to be tested. Use dilutions ranging from 10^{-3} to 10^{-6} for unconcentrated samples and from 10^{-5} to 10^{-8} for concentrated ones[22].

(ii) Determine p24 levels in the diluted samples using a Retro-Tek HIV-1 p24 antigen ELISA kit (ZeptoMetrix). Proceed with the assay as recommended by the manufacturer.

! CAUTION This commercial p24 ELISA kit contains inactivated wild-type HIV-1, which poses a potential safety risk. Thus, this assay should be carried out using Biosafety Level 2 (BL2) conditions.

? TROUBLESHOOTING

Monitoring the emergence of replication-competent lentivirus ● TIMING Days 5–34

20| Place 1×10^5 H9 cells in 1 ml of medium containing RPMI 1640 + 10% FBS, 1% Glutamax and 1% penicillin–streptomycin in a 12-well plate.

21| Add 0.5 ml of an unconcentrated vector preparation and 1 µl of polybrene (8 mg ml^{-1}). Mix and incubate cells at 37 °C overnight.

22| The following day, transfer cells to a 15-ml centrifuge tube and spin at 1,500 r.p.m. (500*g*) for 5 min at 25 °C.

23| Recover supernatant and filter it through a 0.45-µm filter unit. Label the filtered sample as SP0 and store it in a −80 °C freezer for up to 2 months.

24| Resuspend the cell pellets in 10 ml of fresh medium containing RPMI 1640 + 10% FBS, 1% Glutamax and 1% penicillin–streptomycin and transfer cells to a T25 flask. Incubate the flask in an upright position at 37 °C.

25| After 7 d, remove 9 ml of the cell suspension from the T25 flask to a 15-ml centrifuge tube and spin at 1,500 r.p.m. (500*g*) for 5 min at 25 °C. Remove 1 ml of the supernatant and filter it through a 0.45-µm filter unit and save the filtrate. Label it as SP1 and store it in a −80 °C freezer for up to 2 months.

26| Add 9 ml of fresh medium to the T25 flask and return the flask to the incubator.

27| After 7 d, proceed as described for Step 25. Label the supernatant as SP2 and freeze it at −80 °C.

28| After 7 d, proceed as described for Step 25. Label the supernatant as SP3 and freeze it at −80 °C.

29| After 7 d (i.e., 34 d after setting up the H9 cell transduction), proceed as described for Step 25. Label the supernatant as SP4 and store at −80 °C for up to 2 months.

30| Determine p24 levels in the SP0, SP1, SP2, SP3 and SP4 samples using a Retro-Tek HIV-1 p24 antigen ELISA kit (ZeptoMetrix). Proceed with the assay as recommended by the manufacturer.

! CAUTION The presence of replication-competent lentivirus (RCL) in vector preparations is monitored by analyzing p24 levels using a commercial p24 ELISA kit[21]. This assay kit contains inactivated wild-type HIV-1, which poses a potential safety risk. Thus, this assay should be carried out using BL2 conditions. Note that there may be recombination events occurring between sequences present in the packaging construct and sequences present in the vector backbone. These could ultimately lead to the formation of RCL. Such recombinants would not be detected using the test described here.

● TIMING

Day 1: seed 293T cells
Day 2: start transfection of 293T cells
Day 3: change the medium
Day 4: observe transfected cells
Day 5: harvest lentiviral vectors; concentrate lentiviral vectors
Days 5–8: titrate lentiviral vectors
Days 5–34: carry out RCL assay
Day 34: carry out p24 ELISA

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Steps	Problem	Possible causes	Potential solutions
1–17	Low transfection efficiency	Improper pH or storage of 2× HBS	The optimal pH of the 2× HBS solution should be determined experimentally. The 2× HBS solution should be stored at −20 °C
		Status of 293T cells	293T cells should not be used after 18–20 passages and cells should not be allowed to become overconfluent. Cells should be split twice a week at a 1:12 ratio
		FBS quality	The most useful source (and lot) of FBS should be established experimentally
		Plasmid DNA concentration	Establish optimal ratio of vector, packaging and envelope plasmid DNAs
		Cell density	Increase the cell density at the time of transfection. Cell densities up to 80% confluence have resulted in high production yields[15]
19	Low titers of lentivector preparations bearing glycoproteins other than VSV-G	Poor incorporation of Env glycoprotein	Increase the amount of Env glycoprotein-containing plasmid during transfection
			Change $CaCl_2$ to 2× HBS ratios and determine TU/particle ratios before and after concentration to rule out that glycoprotein is not lost during the concentration step
		Poor survival of certain pseudotypes upon ultracentrifugation	Carry out ultracentrifugation at reduced speed[26] or switch to alternative mode of concentration
		Poor survival of pseudotypes upon freezing	Avoid freezing of vector stocks
		Lack of receptors for particular pseudotypes	Use alternative cell lines for titration of vector stocks
	Poor lentiviral vector recovery after anion exchange chromatography	Vector preparations with titers $\leq 10^7$ TU ml^{-1} contain more competing proteins per unit volume of Mustang Q membrane	Concentrate low titer lentivirus vector preparations by ultracentrifugation before chromatography
			Carry out anion exchange chromatography using vector stocks $> 10^7$ TU ml^{-1}
	No amplification or aberrant amplification during real-time PCR	Poor DNA quality	Perform agarose gel electrophoresis to make sure that DNA is not degraded
			Make sure that DNA concentration is accurate
		Faulty design of primers and probe	Redesign primers and probe using appropriate software
	Poor standard curve	Improper amplification of DNA standards used	Avoid frequent freezing and thawing of DNA standards. Prepare new standards every 2 months
	Unexpectedly high vector copy numbers in transduced cell DNA	DNA contamination due to plasmid carryover	Make sure DNaseI treatment was included after transduction of HOS cells to prevent plasmid carryover
			Prepare control vector stocks in parallel using a package construct (pCD/NL-BH*vpu/RT$^-$) lacking a functional RT[9]. Such vector stocks should show negligible amounts of vector copies
	High backgrounds in RT-PCR control reactions lacking RT	DNA carryover	Use dedicated area that is free of plasmid DNA to set up RT-PCRs
			Make sure that DNaseI treatment is included during the preparation of vector RNA to degrade plasmid DNA that is carried over with the vectors
	Higher-than-expected relative vector particle numbers after RT-PCR	DNA carryover	Make sure DNaseI and RNaseA treatments are carried out properly. These steps will ensure that free viral RNA or plasmid DNA present in the sample will be eliminated before the extraction of the virion RNA
	Lower-than-expected functional vector titers after titration using p24 ELISA	p24 is not associated with functional vector particles	Carry out titration using concentrated vector stocks[10]
		Transgene product inhibits functional particle formation[13]	Carry out titration using quantitative PCR involving DNA from transduced cells

TABLE 2 | Anticipated pseudotype titers.

Pseudotype	Concentration method	Initial titer (TU ml^{-1})	Final titer (TU ml^{-1})	Volume change (fold)	Titer increase (fold)
VSV-G	Ultracentrifugation	5.6×10^7	2.1×10^{10}	425	375
	Mustang Q	4.3×10^7	1.5×10^{10}	450	349
	PEG	1.3×10^7	1.4×10^9	147	109
RD114A	Ultracentrifugation	2.3×10^6	5.5×10^8	286	239
	Mustang Q	2.0×10^6	1.6×10^8	82	80
Rabies PV	Ultracentrifugation	1.2×10^7	4.0×10^9	400	333
	Mustang Q	1.2×10^7	1.2×10^9	333	100

ANTICIPATED RESULTS

This protocol results in titers of at least 2×10^7 IU ml^{-1} (at least 1×10^7 TU ml^{-1}) for unconcentrated vector pseudotypes involving VSV-G using HOS cells (**Table 2**) and up to 10^5 IU per ng of p24 (see refs. 21,22). The various concentration steps will ultimately result in titers up to 10^{10} IU ml^{-1} (**Table 2**). Vector pseudotypes involving the VSV-G glycoprotein are relatively nontoxic as far as target cells *in vitro* are concerned if purified by ultracentrifugation involving a sucrose cushion[10]. Inclusion of an anion exchange chromatography step will further reduce cell toxicity. In general, pseudotypes bearing Env glycoproteins other than VSV-G appear to be less toxic to target cells *in vitro*[9,10].

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