Murine Leukemia Virus (MLV)-based Coronavirus Spike-pseudotyped Particle Production and Infection

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[Abstract] Viral pseudotyped particles (pp) are enveloped virus particles, typically derived from retroviruses or rhabdoviruses, that harbor heterologous envelope glycoproteins on their surface and a genome lacking essential genes. These synthetic viral particles are safer surrogates of native viruses and acquire the tropism and host entry pathway characteristics governed by the heterologous envelope glycoprotein used. They have proven to be very useful tools used in research with many applications, such as enabling the study of entry pathways of enveloped viruses and to generate effective gene-delivery vectors. The basis for their generation lies in the capacity of some viruses, such as murine leukemia virus (MLV), to incorporate envelope glycoproteins of other viruses into a pseudotyped virus particle. These can be engineered to contain reporter genes such as luciferase, enabling quantification of virus entry events upon pseudotyped particle infection with susceptible cells. Here, we detail a protocol enabling generation of MLV-based pseudotyped particles, using the Middle East respiratory syndrome coronavirus (MERS-CoV) spike (S) as an example of a heterologous envelope glycoprotein to be incorporated. We also describe how these particles are used to infect susceptible cells and to perform a quantitative infectivity readout by a luciferase assay.

Keywords: Pseudotyped particle, Murine leukemia virus, Envelope glycoprotein, Coronavirus, Spike

[Background] Viral pseudotyped particles are very useful tools for studying the entry pathways that enveloped viruses use and for generating novel gene-delivery vectors. These synthetic enveloped viruses are derived from a parental virus, usually a rhabdovirus or a retrovirus, which forms the core of the particle that can incorporate in its membrane a wide range of viral envelope glycoproteins from heterologous viruses. Several model viruses such as the retroviral murine leukemia virus (MLV) and human immunodeficiency virus-1 (HIV-1) or the rhabdoviral vesicular stomatitis virus (VSV) have been successfully used to generate viral pseudotyped particles (also named pseudoviruses or pseudovirions). Virus pseudotyping is particularly useful for the following scenarios: (i) to quantify the viral entry process of enveloped viruses using reporter genes like green fluorescent protein (GFP) or luciferase, (ii) to study host cell entry of enveloped viruses that cannot be cultivated in cell culture, (iii) to study entry pathways of risk group (RG) 3 or 4 viral pathogens when biosafety level (BSL) 3 or 4 facilities are not available, (iv) to generate cells stably expressing a specific gene of interest or for specific gene silencing, (v) to produce vectors for gene delivery allowing control over cell tropism. Pseudotyped particles can be used to complement native virus infection assays, especially regarding study of virus entry events.

The protocol described here is highly adaptable both in terms of scale of production and type of envelope glycoprotein that can be incorporated. It has been extensively used in our research on viral entry of various enveloped viruses, including VSV (Sun *et al.*, 2008), influenza virus (Tse *et al.*, 2014) and coronaviruses (Belouzard *et al.*, 2009; Millet and Whittaker 2014; Millet *et al.*, 2016). We have successfully used this method to pseudotype viral envelope glycoproteins from all three classes of viral fusion proteins: influenza hemagglutinin (HA, class I), coronavirus spike (S, class I), Ebola glycoprotein (GP, class I), Semliki forest virus (SFV) E1 (class II), and vesicular stomatitis virus (VSV) G glycoprotein (class III). The technique described here is based on work performed by Bartosch and colleagues (Bartosch *et al.*, 2003), and employs the so-called ‘three-plasmid’ co-transfection strategy in which producer HEK-293T/17 cells are co-transfected with the following plasmids: a plasmid allowing expression of MLV retroviral core genes *gag* and *pol* but lacking the MLV envelope glycoprotein-encoding *env* gene, a transfer vector containing a luciferase reporter gene flanked by retroviral regulatory LTR regions and a packaging signal, along with a plasmid allowing expression of the desired envelope glycoprotein. The co-expression of these three plasmids allows synthesis of LTR-flanked reporter gene-containing RNA, MLV-derived proteins and heterologous envelope glycoprotein. During pseudotyped particle formation, which occurs at the plasma membrane, the RNAs containing the LTR-flanked luciferase gene get incorporated into nascent particles formed by assembly and budding of MLV capsid proteins that also recruit heterologous viral envelope glycoproteins. Upon infection in susceptible cells, the pseudotyped virus entry pathway is solely governed by the heterologous virus envelope glycoprotein used. As such, pseudovirions are excellent surrogates to study the entry pathway of enveloped viruses. Once virus entry has occurred, the pseudotyped virus RNAs are released in the cell and the retroviral reverse transcriptase and integrase then reverse transcribe the molecules into double stranded DNA and integrate them into the genome of target cells. Because the sequence that gets integrated only contains the gene encoding the luciferase reporter but none of the MLV genes, the pseudotyped particles are inherently safer as they only allow for one round of infection. After infection, a simple luciferase assay allows quantification of infectivity of the pseudotyped particle studied.

The following protocol can form the basis of useful experiments for the study of the heterologous envelope glycoprotein function during virus entry, for example by performing the infection assay using different infection conditions such as receptor/co-receptor expression in target cells, virus binding time and temperature, pH, endocytosis inhibitors, protease inhibitors, neutralizing antibodies, *etc.*

Materials and Reagents

1. Cell culture vessels, plates and tubes
 - a. 50 ml Falcon tubes (TrueLine, catalog number: TR2004)
 - b. Cell counting slides with grids (KOVA, catalog number: 87144)
 - c. T75 75 cm² cell culture flasks (TrueLine, catalog number: TR6002)
 - d. 6-well cell culture plates (TrueLine, catalog number: TR5000)
 - e. 24-well cell culture plates (TrueLine, catalog number: TR5002)

- f. 1.5 ml Eppendorf tubes
 - g. 0.22 µm cell culture medium filtration unit (LPS, catalog number: 1102-RLS)
 2. Pseudotyped virus solution filtration
 - a. 0.45 µm filter (Pall, catalog number: 4184)
 - b. 10 ml syringes (BD, catalog number: 309604)
 3. Pipettes and pipettors
 - a. Pipettor set:
 - P1000 (Gilson, catalog numbers: F123602)
 - P200 (Gilson, catalog numbers: F123601)
 - P20 (Gilson, catalog numbers: F123600)
 - b. Stripettor for pipetting with serological pipette (Corning, catalog number: CLS4910-1EA)
Note: This product has been discontinued.
 - c. Sterile serological pipettes:
 - 25 ml (LPS, catalog numbers: TR37129)
 - 10 ml (LPS, catalog numbers: TR37128)
 - 5 ml (LPS, catalog numbers: TR37127)
 - d. Repeater dispenser (Eppendorf, catalog numbers: 4981000.019/022260201)
Note: This product has been discontinued.
 - 10 ml sterile tips (Eppendorf, catalog numbers: 0030089677)
 - 5 ml sterile tips (Eppendorf, catalog numbers: 0030089561)
 4. Cells
 - a. Human embryonic kidney (HEK) HEK-293T/17 cells (ATCC, catalog number: CRL-11268), maintained in T75 flasks with complete DMEM (DMEM-C)
 - b. Human hepatic Huh-7 cells (National Institutes of Biomedical Innovation, Health and Nutrition, catalog number: JCRB0403), maintained in T75 flasks with DMEM-C
 5. Transfection plasmids and reagents
 - a. Plasmids pCMV-MLVgag-pol (ampicillin resistance) (Bartosch *et al.*, 2003)
 - b. pTG-Luc (ampicillin resistance) (Bartosch *et al.*, 2003)
 - c. pCAGGS-MERS-S (ampicillin resistance) (Millet and Whittaker, 2014)
 - d. pCAGGS-VSV-G (ampicillin resistance)
 - e. pCAGGS (ampicillin resistance)
 - f. Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen™, catalog number: 11668-027)
 - g. Opti-minimal essential medium (Opti-MEM) (Thermo Fisher Scientific, Gibco™, catalog number: 31985-070)
 6. Disinfection/decontamination reagents
 - a. Ethanol (95%) (VWR, BDH®, catalog number: BDH1158-4LP) diluted to 70% with water in spray bottle for surface disinfection
 - b. Paper towels to soak in 70% ethanol and wipe surfaces to disinfect (Georgia-Pacific Consumer Products, catalog number: 23304)

- c. Bleach solution for decontamination (The Clorox Company, catalog number: Germicidal Bleach)
7. Cell culture reagents
 - a. 0.25% trypsin EDTA solution (Mediatech, catalog number: 25-053-CI)
 - b. Dulbecco's phosphate buffered saline (DPBS) with Ca^{2+} and Mg^{2+} (Mediatech, catalog number: 21-030-CV)
 - c. Dulbecco's modification of Eagles medium (DMEM) with 4.5 g/L glucose, L-glutamine but without sodium pyruvate (Mediatech, catalog number: 10-017-CV)
 - d. Heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 1614071)
 - e. 100x penicillin-streptomycin (PS) solution (Mediatech, catalog number: 30-002-CI)
 - f. 1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) solution (Mediatech, catalog number: 25-060-CI)
8. Luciferase assay reagents
 - a. Luciferase assay lysis buffer (Promega, catalog number: E1531)
 - b. Luciferin substrate (Promega, catalog number: E1501)
 - c. Sterile water (VWR, catalog number: E476-1L)
9. Others
 - a. Cryovials
 - b. Complete DMEM (DMEM-C) (see Recipes)
 - c. Transfection DMEM (DMEM-T) (see Recipes)

Equipment

1. Temperature-controlled water bath (Labnet, model: W1106A)
2. Biosafety cabinet (Class II-A2) connected to a vacuum aspiration system (Labconco, model: 3440009)
3. Inverted light microscope with 10x objective (Nikon Instruments, model: TS100) for checking cell density and health
4. Plate rocker in 37 °C 5% CO₂ cell culture incubator (Thermo Fisher Scientific, Fisher Scientific™, model: 13-687-704)
5. Plate rocker, room temperature (VWR, model: 40000-300)
6. 37 °C 5% CO₂ humidified cell culture incubator – Symphony (VWR, model: 98000-368)
7. Vortex Genie (Scientific Industries, model: G560)
8. Pocket calculator (Sharp Elsimite, Sharp, model: EL-334TB)
9. Inverted light microscope with 10x objective (Carl Zeiss, model: Axiovert 200) connected to a CCD camera (PCO, model: Sensicam QE)
10. Centrifuge (Eppendorf, model: 5810R)
11. GloMax 20/20 luminometer (Promega, model: 2030-100)

12. Lab refrigerator set at 4 °C (GE Appliances, model: GMR06AAMBRWW)
13. Lab freezer set at -20 °C (SUMMIT APPLIANCE, model: FS-603)
14. Lab freezer set at -80 °C (Thermo Fisher Scientific, Thermo Scientific™, model: UXF30086A)
15. Timer (VWR, catalog number: 61161-346)

Software

1. Prism (GraphPad, [version 7](#))

Procedure

Day 1

- A. Cell culture procedures preparations (performed each time cells and viral pseudotyped particles are manipulated) – see Note 1 for basic recommendations regarding cell culture.
 1. Pre-warm cell-culture reagents required in 37 °C water bath: DMEM-C, DPBS, trypsin.
Note: This is a particularly important step since HEK-293T/17 cells are poorly adherent – see Note 2 for details.
 2. Turn on biosafety cabinet for at least 15 min before starting procedures.
 3. Disinfect all internal surfaces of biosafety cabinet (except top grills) with 70% ethanol-soaked paper towels.
- B. Cell seeding (performed in biosafety cabinet)
 1. Place an 80-90% confluent T75 flask (check by visual inspection using an inverted light microscope) containing HEK-293T/17 cells in sterilized biosafety cabinet.
 2. Wash cells twice with 10 ml of pre-warmed DPBS being careful not to dispense liquid directly on cells.
 3. Trypsinize cells with 1 ml of pre-warmed trypsin solution.
 4. Rock plate gently with hands so the trypsin solution covers the entire surface of cell monolayer.
 5. Incubate cells at 37 °C 5% CO₂ incubator for 3-5 min avoiding longer incubation times.
Note: It is crucial to avoid long trypsin incubation times because this typically leads to cells clumping – see Note 3.
 6. Gently tap sides of flask to detach cells.
 7. Place trypsinized flask back into biosafety cabinet.
 8. Add 4 ml of warmed DMEM-C to neutralize trypsin activity.
 9. Resuspend cells by performing repetitive up-down pipetting using a 5 ml serological pipette.
 10. Add 5 ml of DMEM-C to cells and resuspend cells again.
 11. Transfer 10 ml of cell suspension to a 50 ml Falcon tube.
 12. Vortex cells gently for 30 sec.

13. Transfer 10 μ l of cell suspension to a cell counting chamber using a P20 pipettor and appropriate tip.
14. Count cells using the cell counting chamber under an inverted light microscope.
15. Calculate cell density using the calculator.
16. Add DMEM-C to obtain a final cell density of 5×10^5 cells/ml.
17. Resuspend cells by up-down pipetting and gently vortex tube.
18. Seed cells to wells of a 6-well plate by adding 2 ml of cells (1 million cells) per well.
19. Gently rock plate back and forth (avoid swirling movement), sideways and diagonally.
20. Incubate plate at 37 °C 5% CO₂ incubator for 16-18 h.

Day 2

C. Transfection (performed in biosafety cabinet)

1. Check for cell confluency using a light microscope – ideally cells should be around 40-60% confluency, see Figure 1.

Figure 1. HEK-293T/17 cell confluency before transfection. For efficient transfection and pseudotyped particle production, HEK-293T/17 cell confluency should be around the 40-60% range as shown in the above microscopy picture taken with a Zeiss Axiovert 200 microscope. Scale bar represents 100 μ m.

2. Warm Opti-MEM in 37 °C incubator.
3. Calculate volumes of plasmid DNA and Opti-MEM required for mix A (the quantities given for 1 well of a 6-well plate can be scaled-up or down depending on needs).
For 1 well of a 6-well plate (1 μ g total DNA transfected):

pCMV-MLVgag-pol	300 ng
pTG-Luc	400 ng
Envelope-encoding or empty vector	300 ng
Opti-MEM	to 50 μ l

Notes:

- a. *The above DNA ratios can be optimized, if required – see Note 4.*
- b. *Also, this protocol is performed using a 6-well plate format. This can be scaled up or down according to experimental requirements – see Note 5.*

4. Include transfection conditions with co-transfection of pCMV-MLVgag-pol and pTG-Luc with a plasmid for positive control particles (VSV-Gpp, e.g., with pCAGGS-VSV-G) and for negative control particles (Δ envpp, e.g., with pCAGGS empty vector) – see Note 6 for details on pseudotyped particle controls.

5. Calculate volume of Lipofectamine 2000 transfection reagent required for mix B.

For 1 well of a 6-well plate (1:3 ratio used, 1 μ g DNA for 3 μ l Lipofectamine 2000):

Lipofectamine 2000	3 μ l
Opti-MEM	47 μ l

Note: The 1:3 ratio can be optimized, if required – see Note 4.

6. Include 2 'safety' wells in volume calculations for mixes A and B of each transfection condition to avoid running out of solutions when performing the same transfection in multiple wells.
7. Handle Lipofectamine 2000 reagent with care avoiding direct contact with plastic surfaces (tube walls) and always dispense in tubes already containing a liquid solution.
8. Perform mix A and mix B dilutions according to calculations.
9. Incubate at room temperature for 5 min.
10. Use a 1:1 ratio (i.e., 50 μ l each for one well to transfect) of mix A and B and mix solutions by performing multiple up-down pipetting.
11. Incubate at room temperature for 20 min.
12. Place plate of cells to be transfected in incubator and label wells to be transfected with name of viral enveloped used.
13. Aspirate supernatants gently and replace with 1 ml of warmed Opti-MEM.
14. Add 100 μ l of transfection mix to each well in a drop-wise manner.
15. Gently rock plates front and back and side to side.
16. Incubate in 37 °C 5% CO₂ incubator for 4-6 h.
17. Pre-warm transfection DMEM (DMEM-T, no antibiotics) in 37 °C water bath.
Note: DMEM-T is used here instead of DMEM-C because the transfection reagent increases cell membrane permeability, which leads to enhanced susceptibility to the cytotoxic effects of antibiotics in the cell culture medium – see Note 4.
18. Add gently 1 ml of DMEM-T to each well.
19. Incubate in 37 °C 5% CO₂ incubator for 48 h.

Day 4

D. Harvesting (performed in biosafety cabinet)

1. Label cryovials with name of pseudotyped virus (envelope glycoprotein used), date of harvest and initials of user.
2. Collect supernatants in 50 ml Falcon tubes (solutions from multiple wells can be pooled if they were transfected identically).
3. Spin tubes in centrifuge at 290 x g for 7 min.
4. Pass pseudotyped virus solution through a 0.45 µm filter using appropriate syringe.
5. Aliquot solution in labeled cryovials (e.g., 1 ml aliquots).
6. Store tubes at -80 °C – see Note 7 for more details on storage.
7. Decontaminate with 10% bleach solution all culture vessels, serological pipettes, syringes that have come into contact with pseudotyped virus solutions, with a minimum contact time of 15 min.

Note: This protocol describes how to produce and use pseudotyped particles for infectivity assays. To estimate quantities of particles produced as well as incorporation of specific viral envelope glycoproteins into particles, additional experiments are required such as Western blot analyses or ELISA assays – see Note 8 for details.

E. Cell seeding for infection (performed in biosafety cabinet)

1. Place an 80-90% confluent T75 flask containing Huh-7 cells in biosafety cabinet.
2. Wash cells twice with 10 ml of warmed DPBS being careful not to dispense liquid directly on cells.
3. Trypsinize cells with 1 ml of warmed trypsin solution.
4. Rock plate gently with hands so the trypsin solution covers the entire surface of cell monolayer.
5. Incubate cells at 37 °C 5% CO₂ incubator for 3-5 min avoiding longer incubation times.

Note: It is important to avoid long trypsin incubation times because this typically leads to the clumping of cells as well as cell counting and density problems – see Note 3.

6. Gently tap sides of flask to detach cells.
7. Place trypsinized flask back in biosafety cabinet.
8. Add 4 ml of warmed DMEM-C to neutralize trypsin activity.
9. Resuspend cells by performing repetitive up-down pipetting using a 5 ml serological pipettor.
10. Add 2-3 ml of DMEM-C to cells and resuspend cells again (adjust depending on cell confluency).
11. Transfer cell suspension to a 50 ml Falcon tube.
12. Vortex cells gently for around 30 sec.
13. Transfer 10 µl of cell suspension to a cell counting chamber using a P20 pipettor and appropriate tip.
14. Count cells using the cell counting chamber under a light microscope.
15. Calculate cell density using a pocket calculator.
16. Add DMEM-C to obtain a final cell density of 5 x 10⁵ cells/ml.

17. Resuspend cells by up-down pipetting and gently vortex tube.
18. Seed cells to wells of a 24-well plate by adding 500 μ l of cells (2.5×10^5 cells) per well using the repeater dispenser.
Note: The infection is performed in a 24-well plate. This can be scaled up or down depending on experimental requirements – see Note 5 for details.
19. Gently rock back and forth (avoid swirling movement), sideways and diagonally the plate.
20. Incubate plate at 37 °C 5% CO₂ incubator for 16-18 h.

Day 5

F. Infection (performed in biosafety cabinet)

1. Check for Huh-7 cell confluency using light microscope (cells should be nearly confluent as shown in Figure 2).

Figure 2. Huh-7 cell confluency before infection with pseudotyped particles. For efficient transduction by pseudotyped particles, Huh-7 cells should be almost completely confluent as shown in the above microscopy picture taken with a Zeiss Axiovert 200 microscope. Scale bar represents 100 μ m.

2. Pre-warm DPBS in 37 °C water bath.
3. Thaw pseudotyped virus solution vials at room temperature (vials can then be kept on ice).
4. Place the 24-well plate with Huh-7 monolayer in biosafety cabinet.
5. Wash three times with 200 μ l of warmed DPBS being careful not to dispense liquid directly on cells.
6. Inoculate wells with 200 μ l of pseudotyped virus solution.

7. Include non-infected (n.i.) control conditions by adding 200 μ l DMEM-C solution per well.
8. Place the plate on rocker located in 37 °C 5% CO₂ cell culture incubator.
Note: Alternatively, cells can be placed in incubator without rocker.
9. Incubate cells for 1-2 h.
10. Pre-warm DMEM-C in 37 °C water bath.
11. Add 300 μ l DMEM-C using a repeater dispenser and appropriate tip.
12. Incubate at 37 °C 5% CO₂ cell culture incubator for 72 h.
Note: This infection time can be optimized if required, see Note 9.
13. Decontaminate with 10% bleach solution all culture vessels and pipette tips, that have come into contact with pseudotyped virus solutions, with a minimum contact time of 15 min.

Day 8

G. Luciferase assay (performed in biosafety cabinet until step G5)

1. Bring luciferase assay lysis buffer (stored at -20 °C) and luciferin substrate (stored at -80 °C) at room temperature.
2. Place plate containing infected cells in biosafety cabinet and incubate until temperature has equilibrated to room temperature.
3. Dilute to 1x luciferase assay lysis buffer with sterile water.
4. Aspirate gently the supernatants of cells.
5. Add 100 μ l of 1x luciferase assay lysis buffer to each well using the repeater dispenser.
6. Incubate on rocker at room temperature for 15 min (after incubation, plate can be opened outside of biosafety cabinet).
7. Turn GloMax 20/20 luminometer on.
8. Prepare clear 1.5 ml Eppendorf tubes for each well to be analyzed by dispensing 20 μ l of luciferin substrate solution in each tube.
9. Add 10 μ l of lysed cell supernatant to each 20 μ l luciferin-containing tube, performing quick up-down pipetting and gentle tube flickering immediately followed by placing the tube in tube holder found in the luminometer.
10. Press 'Measure Luminescence' button on touch screen of luminometer.
11. Record reading of relative luciferase units (RLU).
12. Repeat measurement recording (steps G8-G11) procedure for the other wells.

Data analysis

1. Collect data for each type of pseudovirion (typical values for coronavirus spike-pseudotyped particles (CoVpp) are in the 10⁴-10⁶ RLU range, VSV-Gpp 10⁶-10⁸ RLU range and Δ envpp in the 10² baseline RLU range). If the luminometer is connected to a computer, data can be recorded on an electronic file using supplied software.
2. Include at least duplicate measurements for each infection condition analyzed.

3. Average measurements of independent experiments for statistical significance analysis.
4. Plot data using graph plotting software such as Prism 7 (an example of plotted data is shown in Figure 3).

Figure 3. MERS-CoV S-pseudotyped particle infectivity assay in human liver cells (Huh-7). Displayed are luciferase activity measurements of lysed Huh-7 cells 72 h post-infection for the following conditions: non-infected (n.i.), 'bald' (no envelope) pseudotyped particles (Δ envpp), VSV-G-pseudotyped particles (VSV-Gpp), and MERS-CoV S-pseudotyped particles (MERS-Spp). Experiments performed in triplicates and data presented is average relative luciferase units (log₁₀) of three independent experiments (n = 3). Error bars indicate standard deviation (s.d.).

Notes

1. Cell culture and transfection

HEK-293T/17 cells were chosen for transfection and production of pseudotyped particles because they are able to be very efficiently transfected. In this protocol, Huh-7 cells were chosen as target cells for infection by pseudotyped particles because they are very permissive to both native MERS-CoV and MERS-CoV S-pseudotyped particle infection. When producing other types of pseudovirions, we recommend to check what is the most suitable cell line for infectivity assays. As cell lines are the basis for production of pseudotyped particles and for infection assays, it is essential that they are maintained as well as possible by passaging them frequently to avoid having overgrown cell monolayers, using freshly made media as much as possible, and using low passage cells when transfecting or infecting them. Poor cell line health and growth often results in low transfection and infection rates, so it is important to exercise care when handling cells.

2. HEK-293T/17 cell adherence

HEK-293T/17 cells are very useful for this protocol as they typically allow for very high transfection rates. However, these cells tend to adhere poorly to cell-culture treated surfaces, and are easily detached even without trypsin. For this reason, it is important to use pre-warmed

Ca²⁺-containing DPBS when performing washes as these cations help with cell adhesion. Also, use of cell culture surfaces coated with poly-D-lysine (coating solution diluted to 40 µg/ml) are recommended to help with cell adhesion.

3. Trypsin treatment of cells

When trypsinizing cells, particularly Huh-7 cells, we found that incubations of more than 5 min at 37 °C 5% CO₂ incubator was deleterious for counting and cell seeding as cells would clump up and would no longer form evenly distributed cell monolayers. We recommend exercising care when trypsinizing cells and avoiding long incubation times with the protease.

4. Transfection recommendations

The ratio of amounts of plasmid DNA used (300 ng pCMV-MLVgag-pol:400 ng pTG-Luc:300 ng envelope-encoding or empty vector) can also be optimized if required (e.g., to increase pseudovirion infectivity), but we recommend to keep the amounts of pCMV-MLVgag-pol and pTG-Luc vectors constant and vary the amount of envelope-encoding vector. Furthermore, in this protocol we have used a 1 µg DNA:3 µl Lipofectamine 2000 ratio for the transfections, which has worked very well for our pseudotyped particle productions. However, depending on the expression plasmids and cell lines used, it may be useful to carry out testing and optimization of this ratio. Also, as the transfection procedure increases cell membrane permeability, it is important to avoid antibiotic treatment of cells during that time. Doing so ensures that the cytotoxicity associated with the transfection remains low.

5. Pseudotyped particle production and infection scaling

The following protocol is performed using 6-well plates for the transfection of HEK-293T/17 producer cells. From one well of a 6-well plate, approximately 2 ml of pseudotyped particle supernatant can be harvested. If more pseudotyped particle solution is required, the same transfection can be performed in multiple wells with the supernatants pooled at the harvesting stage. The protocol can also be scaled up or down depending on needs, by using other culture vessels (such as 10 cm dish or flasks) and by adjusting transfection conditions accordingly. The protocol also details how infection of target cells with pseudotyped particles is performed using 24-well plates. This can also be scaled to the needs of an experiment, for example for high-throughput experiments, the assay can be optimized for infections carried out in 96-well plates by adjusting volumes and using a plate-compatible luminometer.

6. Pseudotyped particle production controls

During production of pseudotyped particles, it is important to include particle production controls, an important step that is detailed in this protocol. Typically, 'bald' or 'Δenv' particles (Δenvpp) are produced by replacing the plasmid encoding the heterologous viral enveloped glycoprotein with an empty vector control. These particles bud out of producer cells, but are devoid of viral envelope glycoproteins. Also, a positive control is also useful to gauge how well the procedure was performed, and typically VSV-G-pseudotyped particles (VSV-Gpp) are used for this. These particles usually reach very high infectivity in many cell types and are thus ideal positive controls.

7. Pseudotyped particle storage

MLV-based pseudotyped particles retain their infectivity well when stored at -80 °C for several months. However, we recommend avoiding multiple freeze-thaw cycles as these can significantly reduce viral infectivity. We recommend aliquoting pseudovirions stocks into small volumes (1 ml) and thawing what is needed for each infection experiment that is being carried out.

8. Quantification of particles produced and envelope glycoprotein incorporation

This protocol details the procedures for pseudotyped particle production and infection. However, it does not describe the methodology for measuring the amount of particles for each batch produced, which is an important step, especially when comparing infectivity of pseudovirions pseudotyped with different types of viral envelope glycoproteins. Such quantifications can be done using several methodologies such as ELISA or Western blot techniques (as done in [Jaume *et al.*, 2011; Millet *et al.*, 2012; Millet and Whittaker, 2014]), allowing estimation of total capsid protein released from producer cells (p30 protein for MLV) or with a direct total particle count and analysis using devices such as Nanosight (Millet *et al.*, in press). The Western blot technique also allows to probe for the heterologous envelope protein, which is very useful for assessing efficiency of its incorporation in pseudotyped particles.

9. Incubation times

The incubation times (48 h during production and 72 h for infection assay) have been optimized for MERS-CoV S-pseudotyped particles. These incubation times can be used as guidelines for generating other types of pseudovirions, however, we recommend testing different incubation times to optimize virus production and infectivity.

10. Pseudotyping more than one envelope glycoprotein

This protocol describes the procedures to generate pseudotyped particles that harbor only one kind of envelope glycoprotein, for example the MERS-CoV S protein in the case of MERS-Spp. However, using the co-transfection strategy, it is possible to incorporate more than a single envelope protein. For example, we have successfully generated influenza hemagglutinin (HA) and neuraminidase (NA) pseudotyped viruses (Millet and Whittaker, 2014; Tse *et al.*, 2014).

11. Regulation of enveloped glycoprotein incorporation by C-terminal tail

The C-terminal tail or endodomain of class I viral fusion proteins plays important regulatory roles both for the fusion function but also for the incorporation of the protein into viral particles. It was shown that modifying the C-terminal tail of the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) S protein was associated with enhanced envelope glycoprotein incorporation into retroviral pseudotyped particles (Moore *et al.*, 2004). The modifications included truncations within the C-terminal tail and addition of a portion of the cytoplasmic tail of the HIV-1 envelope glycoprotein. This could be a useful strategy to consider if the heterologous viral envelope glycoprotein fails to efficiently incorporate into pseudotyped particles.

Recipes

1. 500 ml complete DMEM (DMEM-C)
440 ml 1x DMEM
50 ml 10% FBS (heat-Inactivated)
5 ml 1x penicillin-streptomycin (100x)
5 ml 10 mM HEPES (1 M)
Sterile-filter DMEM-C through a 0.22 µm filtration unit
2. 500 ml transfection DMEM (DMEM-T)
445 ml 1x DMEM
50 ml 10% FBS (heat-inactivated)
5 ml 10 mM HEPES (1 M)
Sterile-filter DMEM-T through a 0.22 µm filtration unit

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