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TITLE:

Production of Pseudotyped Particles to Study Highly Pathogenic Coronaviruses in a Biosafety Level 2 Setting

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KEYWORDS:

Pseudotyped particle, pseudovirion, coronavirus, CoV, spike protein, severe acute respiratory syndrome coronavirus, SARS-CoV, Middle East respiratory syndrome coronavirus, MERS-CoV, murine leukemia virus, MLV

SUMMARY:

Here, we present a protocol to generate pseudotyped particles in a BSL-2 setting incorporating the spike protein of the highly pathogenic viruses Middle East respiratory syndrome and severe acute respiratory syndrome coronaviruses. These pseudotyped particles contain a luciferase reporter gene allowing quantification of virus entry into target host cells.

ABSTRACT:

The protocol aims to generate coronavirus (CoV) spike (S) fusion protein pseudotyped particles with a murine leukemia virus (MLV) core and luciferase reporter, using a simple transfection procedure of the widely available HEK-293T cell line. Once formed and released from producer

cells, these pseudovirions incorporate a luciferase reporter gene. Since they only contain the heterologous coronavirus spike protein on their surface, the particles behave like their native coronavirus counterparts for entry steps. As such, they are the excellent surrogates of native virions for studying viral entry into host cells. Upon successful entry and infection into target cells, the luciferase reporter gets integrated into the host cell genome and is expressed. Using a simple luciferase assay, transduced cells can be easily quantified. An important advantage of the procedure is that it can be performed in biosafety level 2 (BSL-2) facilities instead of BSL-3 facilities required for work with highly pathogenic coronaviruses such as Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV). Another benefit comes from its versatility as it can be applied to envelope proteins belonging to all three classes of viral fusion proteins, such as the class I influenza hemagglutinin (HA) and Ebola virus glycoprotein (GP), the class II Semliki forest virus E1 protein, or the class III vesicular stomatitis virus G glycoprotein. A limitation of the methodology is that it can only recapitulate virus entry steps mediated by the envelope protein being investigated. For studying other viral life cycle steps, other methods are required. Examples of the many applications these pseudotype particles can be used in include investigation of host cell susceptibility and tropism and testing the effects of virus entry inhibitors to dissect viral entry pathways used.

INTRODUCTION:

Host cell entry constitutes the initial steps of the viral infectious life cycle. For enveloped viruses, this involves binding to a single host cell receptor or several receptors, followed by fusion of viral and cellular membranes. These essential functions are carried out by viral envelope glycoproteins[1,2]. The coronavirus envelope glycoprotein is called the spike (S) protein and is a member of the class I viral fusion proteins[2-6]. Studying viral envelope glycoproteins is critical for understanding many important characteristics of a given virus, such as: lifecycle initiation, its host and cellular tropism, interspecies transmission, viral pathogenesis, as well as host cell entry pathways. Viral pseudotyped particles, also named pseudovirions, are powerful tools that enable us to easily study the function of viral fusion proteins. Pseudotyped particles or pseudovirions are chimeric virions that consist of a surrogate viral core with a heterologous viral envelope protein at their surface. The protocol's main purpose is to show how to obtain coronavirus spike pseudotyped particles that are based on a murine leukemia virus (MLV) core and contain a luciferase reporter gene. As examples, the method to produce pseudotyped particles with the spike proteins of the highly pathogenic severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses are presented. The protocol describes the transfection procedure involved, how to infect susceptible target cells, and infectivity quantification by luciferase assay.

Since the entry steps of the pseudovirions are governed by the coronavirus S at their surface, they enter cells in a similar fashion to native counterparts. As such, they are excellent surrogates of functional infectivity assays. Pseudotyped particles are usually derived from parental model viruses such as retroviruses (MLV[7-22] and the lentivirus human immunodeficiency virus - HIV[23-35]) or rhabdoviruses (vesicular stomatitis virus - VSV[36-47]). When used in pseudotyping, the parental viruses' genomes are modified to remove essential genes, rendering them defective for accomplishing a complete replication cycle. This feature allows them to be used in intermediate

biosafety level facilities (BSL-2) and is an important advantage over using highly pathogenic native viruses that require higher biosafety facilities (BSL-3, BSL-4 which are not as readily available) when conducting virus entry studies. Here, the S proteins of risk group 3 pathogens SARS-CoV and MERS-CoV are used as examples of viral envelope proteins being incorporated into MLV pseudotyped particles, generating SARS-CoV S and MERS-CoV S pseudovirions (SARS-Spp and MERS-Spp, respectively). These pseudovirions have been successfully used in studies focusing on entry events of these viruses[48-51]. Another advantage is that the technique described here is not limited to pseudotyping coronavirus S proteins: it is very flexible and can be used to incorporate representatives of all three classes of viral fusion proteins. Examples include influenza hemagglutinin (HA, class I)[52], Ebola virus glycoprotein (GP class I), E1 protein of the alphavirus Semliki Forest virus (SFV, class II), and VSV glycoprotein (G, class III)[53]. In addition, more than one kind of viral glycoprotein can be co-incorporated into a pseudotyped particle, as in the case of influenza HA- and NA- pseudotyped particles[51].

Based on the work performed by Bartosch et al.[20], this protocol describes the generation of MLV pseudotyped particles with a three-plasmid co-transfection strategy using the widely available and highly transfection-competent HEK-293T cell line[54]. The first plasmid encodes the MLV core genes *gag* and *pol* but lacks the MLV envelope *env* gene. The second plasmid is a transfer vector that encodes a firefly luciferase reporter gene, an MLV Ψ-RNA packaging signal, along with 5'- and 3'-flanking MLV long terminal repeat (LTR) regions. The third plasmid encodes the fusion protein of interest, in this case either the SARS-CoV S or MERS-CoV S protein. Upon co-transfection of the three plasmids using a transfection reagent, viral RNA and proteins get expressed within transfected cells allowing generation of pseudotyped particles. Since MLV is used as pseudovirion backbone, this occurs at the plasma membrane: the RNAs containing the luciferase gene reporter and packaging signal get encapsulated into nascent particles that also incorporate plasma membrane-expressed coronavirus spike proteins. The particles that bud out from cells contain the coronavirus S protein at their surface and are harvested for use in infectivity assays. Because pseudotyped particles harbor the coronavirus S protein and not the MLV envelope protein, when used for infecting cells, they behave like their native coronavirus counterparts for entry steps. The viral RNA containing the luciferase reporter and flanking LTRs is then released within the cell and the retroviral polymerase activities enable its reverse transcription into DNA and integration into the host cell genome. Quantification of the infectivity of viral pseudotyped particles in infected cells is then performed with a simple luciferase activity assay. Because the DNA sequence that gets integrated into the host cell genome only contains the luciferase gene and none of the MLV or coronavirus protein-encoding genes, they are inherently safer to use than replication-competent native viruses.

In addition to being safer surrogates and highly adaptable to allow incorporation of various kinds of envelope glycoproteins, the pseudotyped particles described here are also highly versatile and can be used to study many aspects of virus entry. This includes but is not limited to: testing host cell susceptibility to virus infection, analyzing the cellular entry pathways an enveloped virus uses, studying the effects of pharmacological inhibitors and drug screenings, conducting neutralization antibody assays, characterizing host cell entry of enveloped viruses that cannot be cultured, and generating viral vectors for gene delivery, stable cellular expression of genes of

interest, or gene silencing.

PROTOCOL:

1. Cell seeding for pseudotyped particle production

NOTE: Perform this step in the biosafety cabinet.

1.1. By standard cell culture techniques, obtain an 80-90% confluent 75 cm^2 flask of HEK-293T/17 cells passaged in complete Dulbecco's Modified Eagle's Medium (DMEM-C) containing 10% (vol/vol) fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 IU/mL penicillin, and 100 µg/mL streptomycin. Prepare DMEM-T medium for transfections (its composition is the same as DMEM-C but without antibiotics).

1.2. Wash cells with 10 mL of pre-warmed (37 °C) Dulbecco's Phosphate Buffered Saline (DPBS) twice.

NOTE: Handle HEK293T/17 cells with care as they easily detach.

1.3. Aspirate the supernatant and detach cells with 1 mL of 0.25% trypsin solution pre-warmed at 37 °C. Place the flask of cells at 37 °C, 5% CO_2 incubator for 3–5 min or until cells start detaching.

NOTE: Avoid incubating cells with trypsin for more than 5 minutes as this typically leads to cell clumping.

1.4. Deactivate trypsin by adding 4 mL of DMEM-C medium and count cells using a cell counting slide and light microscope.

NOTE: To avoid having to count too many cells, an additional dilution step may be required beforehand. Remember to factor in this dilution when calculating the actual cell density of trypsinized cells.

1.5. Dilute cells to 5 x 10^5 cells/mL with DMEM-C.

1.6. Seed 6-well tissue culture plate with 2 mL of cell solution per well and gently move the plate back and forth and side to side to evenly distribute cells, avoiding circular motion.

NOTE: This is a key step. Evenly distributed cells will ensure that cells do not clump at the center of wells. In turn, this will ensure good transfection efficiencies and pseudotyped particle production.

1.7. Incubate the plate overnight (16-18 h) in a 37 °C, 5% CO_2 cell culture incubator.

2. Three-plasmid co-transfection

NOTE: Perform this step in the biosafety cabinet.

2.1. Observe cells under an inverted light microscope to check for the cell morphology and density.

NOTE: Ideally, cell density should be in the 40-60% confluency range. It is critical that cells are neither too confluent (80−90% confluent) nor too sparsely distributed (20−30% confluent) in each well. A cell density of 40−60% confluency will ensure good pseudotyped particles production.

2.2. Plasmids mix

2.2.1. Calculate the plasmid mix for each envelope glycoprotein following the quantities for one well of a 6-well plate shown in **Table 1**. Multiply quantities if transfecting several wells and include an extra well to avoid running out of mix.

NOTE: Along with the SARS-CoV S and MERS-CoV S encoding plasmids, include empty vector control for the generation of negative control particles which lack viral envelope glycoproteins (Δenv particles) along with a positive control glycoprotein such as vesicular stomatitis virus (VSV) G glycoprotein that is known to robustly infect a very wide range of cells (VSV-Gpp). Plasmids are available upon request.

2.2.2. Mix calculated volumes of plasmids and reduced serum cell culture medium (see **Table of Materials**) in a microcentrifuge tube.

2.3. Lipid-based transfection reagent mix (see **Table of Materials**)

2.3.1. Calculate the volumes for the transfection reagent mix from the quantities shown in **Table 2** for one well (1:3 transfection ratio, multiply quantities as needed). Include extra wells to avoid running out of transfection reagent mix.

2.3.2. Mix calculated volumes of lipid-based transfection reagent (3 µL per well) and reduced serum cell culture medium (47 µL per well) in a microcentrifuge tube, making sure to add the transfection reagent into the reduced serum cell culture medium and not the other way around.

2.4. Incubate both mixes (for one well: 50 µL of plasmids mix and 50 µL of lipid-based transfection reagent mix) separately for 5 min at room temperature.

2.5. Add the contents of the transfection reagent mix to the plasmids mix at a 1:1 ratio (for 1 well: 50 µL of each mix)

2.6. Perform thorough up-down pipetting of the resulting mix.

2.7. Incubate the mix for at least 20 min at room temperature.

2.8. Aspirate the spent medium of cells.

2.9. Add gently 1 mL of pre-warmed (37 °C) reduced serum cell culture medium per well.

2.10. Add dropwise 100 µL of transfection mix to each well.

NOTE: Exercise care when adding the transfection mix to wells of HEK-293T as they detach easily.

2.11. Incubate cells in a 37 °C, 5% CO_2 cell culture incubator for 4-6 h.

2.12. Add 1 mL per well of pre-warmed (37 °C) DMEM-T medium, which does not contain antibiotics

NOTE: This is a key step. Transfection reagents increase cell permeability and increase sensitivity to antibiotics. To ensure good transfection efficiency and pseudotyped particle production, it is important to avoid using cell culture medium containing antibiotics

2.13. Incubate cells in a 37 °C, 5% CO_2 cell culture incubator for 48 h.

3. Pseudotyped particles collection

NOTE: Perform this step in the biosafety cabinet.

3.1. Observe cells under inverted light microscope to check for cell morphology and general condition. Also check the color of the medium which should be light pink/slightly orange.

NOTE: This is an important step. If there is too much cell death associated with the transfection or the medium color turned orange/yellow (acidic pH), this will typically be associated with lower yields in infectious pseudotyped particles

3.2. Transfer supernatants of transfected cells to 50 mL conical centrifuge tubes.

3.3. Centrifuge tubes at 290 x *g* for 7 min to remove cell debris.

3.4. Filter clarified supernatants through a sterile 0.45 µm pore-sized filter.

3.5. Make small volume aliquots (e.g., 500 µL or 1 mL) of pseudotyped virus solution in cryovials.

3.6. Store at -80 °C.

NOTE: The protocol can be paused here. Pseudotyped particles are stable at -80 °C for many months but once thawed, avoid re-freezing them as they will lose infectivity

4. Pseudotyped particle infection of susceptible cells

NOTE: Perform this step in the biosafety cabinet.

4.1. Cell seeding of susceptible cells in 24-well plate

4.1.1. Obtain by standard cell culture techniques 80−90% confluent 75 cm^2 flask of susceptible cells: Vero-E6 cells for SARS-CoV pseudotyped particles (SARS-Spp) and Huh-7 cells for MERS-CoV S pseudotyped particles (MERS-Spp).

NOTE: To confirm whether the pseudotyped particles that have been produced are infectious, it is important to carefully choose an appropriate susceptible cell line for pseudovirion infectivity assays. Using poorly permissive cells will lead to low infectivity.

4.1.2. Wash cells twice with 10 mL of pre-warmed (37 °C) DPBS.

4.1.3. Aspirate the supernatant and detach cells with 1 mL of 0.25% trypsin solution pre-warmed at 37 °C. Place the flask of cells at 37 °C, 5% CO_2 incubator for 3−5 minutes or until cells start detaching.

NOTE: Avoid incubating cells with trypsin for more than 5 minutes as this typically leads to cell clumping. Huh-7 cells are especially sensitive to this effect.

4.1.4. Deactivate trypsin by adding DMEM-C medium and count cells using a cell counting slide and light microscope.

4.1.5. Dilute cells to 5 x 10^5 cells/mL with DMEM-C.

4.1.6. Seed wells of a 24-well plate with 0.5 mL of cell solution per well and gently move the plate back and forth and side to side to evenly distribute cells, avoiding circular motion

NOTE: This is a key step. Evenly distributed cells will ensure that cells do not clump at the center of wells. In turn, this will ensure good infectivity assays. For each pseudotyped particle (SARS-Spp, MERS-Spp) and condition, prepare three wells for three experimental replicates. Include wells for the non-infected (N.I.), empty vector Δenv particles and positive control particles such as VSV-Gpp

4.1.7. Incubate the plate overnight (16−18 h) in a 37 °C, 5% CO_2 cell culture incubator

4.2. Pseudotyped particle infection

4.2.1. Observe cells under light microscope and visually confirm that there is a confluent carpet of cells.

4.2.2. Bring cryovials of pseudotyped virus to thaw on ice.

4.2.3. Wash cells three times with 0.5 mL pre-warmed (37 °C) DPBS

NOTE: This is a key step. Cells that are not properly rinsed prior to infection typically lead to poor infectivity readouts

4.2.4. Aspirate the supernatants of cells.

4.2.5. Inoculate cells with 200 µL of thawed pseudotyped particle solution.

4.2.6. Incubate cells in a 37 °C, 5% CO_2 cell culture incubator for 1−2 h.

4.2.7. Add 300 µL of pre-warmed (37 °C) DMEM-C medium.

4.2.8. Incubate cells in a 37 °C, 5% CO_2 cell culture incubator for 72 h.

5. Infectivity quantification by luciferase assay readout

NOTE: Perform initial steps in the biosafety cabinet.

5.1. Thaw luciferin substrate (stored at −80 °C) and 5x luciferase assay lysis buffer (stored at −20 °C) until they reach room temperature.

5.2. Dilute luciferase assay lysis buffer to 1x with sterile water.

5.3. Aspirate supernatants of cells infected with pseudotyped particles.

5.4. Add 100 µL of 1x luciferase assay lysis buffer to each well.

5.5. Place the plate on a rocker and incubate for 15 min with rocking at room temperature (from this point onwards the plate can be handled outside of a biosafety cabinet).

5.6. Prepare microcentrifuge tubes for each well by adding 20 µL of luciferin substrate in each tube.

5.7. Turn on the luminometer.

5.8. Perform luciferase activity measurement one well at a time by transferring 10 µL of lysate to one tube containing 20 µL of luciferin substrate.

5.9. Flick the tube gently to mix contents, but avoid displacing the liquid on walls of tube.

5.10. Place the tube in device and close lid.

5.11. Measure the luminescence value of the tube by using the luminometer.

5.12. Record the relative light unit's measurement.

5.13. Repeat steps 5.8-5.12 until all wells are analyzed.

NOTE: With the appropriate equipment such as a plate reading luminometer, this process can be performed automatically. The assay will need to be scaled to the plate format (e.g., 96-well plate format).

6. Data analysis

6.1. Calculation and plotting of relative luciferase units' averages and standard deviations

6.1.1. Use a graph plotting software to calculate luciferase assay measurement averages and standard deviations of experimental and biological replicates.

6.1.2. Plot data as bar chart with standard deviations.

NOTE: When performing statistical analyses on data, make sure to include at least three biological replicates in data sets.

REPRESENTATIVE RESULTS:

Representative results of infectivity assays of SARS-CoV S and MERS-CoV S pseudotyped particles are shown in **Figure 1**. As expected, for both **Figure 1A and 1B**, the VSV G pseudotyped positive control particles (VSV-Gpp) gave very high average infectivity in the 10^6 to 10^7 relative luciferase units (RLU) range respectively. For SARS-CoV S pseudotyped particles (**Figure 1A**) infection of susceptible Vero-E6 cells, a strong average infectivity was measured at around 9.8 x 10^4 RLU. This value is almost 3 orders of magnitude higher than the values measured for the non-infected control (1.1 x 10^2 RLU), or the Δenv particles (1.5 x 10^2 RLU), which do not harbor any viral envelope glycoproteins at their surface. Similarly, for MERS-CoV S pseudotyped particles (**Figure 1B**) infection of Huh-7 cells, a high average infectivity was measured at around 1.0 x 10^6 RLU. This is almost 4 orders of magnitude higher than the values measured for the non-infected control (0.8 x 10^2 RLU), or the Δenv particles (2.0 x 10^2 RLU). An additional infectivity assay was performed in which the SARS-Spp and MERS-Spp were serially diluted and used to infect Vero-E6 cells (**Figure 2A**). This assay confirms that the luciferase activity measured is dependent on the concentration of the particles used to infect cells. To confirm the role of angiotensin converting enzyme 2 (ACE2) and dipeptidyl peptidase 4 (DPP4), the receptors of SARS-CoV and MERS-CoV, respectively, in mediating attachment and entry of the pseudotyped particles, we used poorly-permissive HEK-293T cells and transfected them to express either ACE2 or DPP4 (**Figure 2B**). The transfected cells were then used for an infectivity assay. This analysis demonstrates that upon overexpression of ACE2 and DPP4, there is a ~4-log and ~2-log increase for SARS-Spp and MERS-Spp infectivity,

respectively, confirming that receptor usage of pseudovirions is similar to that of native viruses.

The examples shown here demonstrate the importance of including negative (non-infected, Δenv particles) as well as positive control (VSV-Gpp) conditions when producing pseudotyped particles. Indeed, the positive control VSV-Gpp particles allow us to assess whether a particular batch of pseudotyped particles was successful in yielding functional and infective pseudovirions. Expected results of typical infection by VSV-Gpp particles in most mammalian cell lines are in the 10^6–10^7 RLU range. Problems with HEK-293T/17 producer cells (high passage number, issues with cell densities) or poor transfection efficiencies can impact overall pseudotyped particle production and infectivity. Furthermore, the negative control conditions are also important as they allow us to assess the baselines of RLU measurements in a particular cell line (non-infected condition) and non-specific internalization of particles (Δenv infection) which is not mediated by a viral envelope protein. Ideally, for a given type of particle pseudotyped with a viral envelope protein of interest, it is recommended to obtain values that are a few orders of magnitude higher than the negative control values, as shown here in **Figure 1A,B**. However, if for a given cell type a pseudotyped virus infection gives very little infectivity (i.e., close to negative controls such as in **Figure 2B** in non-transfected N.T. conditions) it does not necessarily mean that the pseudotyped particle production was faulty. It could well be that the particular cell line used is not or poorly permissive to infection. It is recommended to check whether a given cell type is expected to be susceptible to infection by the virus being investigated. Transfecting poorly permissive cells with plasmid(s) expressing the viral receptor(s) can allow more efficient viral entry and infection to occur, as shown in **Figure 2B**, where upon transfection of ACE2 and DPP4 receptors in target HEK-293T cells, there is a ~4- and ~2-log increase in infectivity, respectively.

FIGURE AND TABLE LEGENDS:

Table 1. Quantities of plasmids and reduced serum cell culture medium required to transfect one well of a 6-well plate of HEK-293T/17 cells for pseudotyped particle production. From the concentration of plasmid preparations, calculate the required volume to reach the required amount of plasmid DNA. If more than one well is transfected with the same plasmids, multiply volumes by required number of wells to transfect, and include an extra well in calculations to avoid running out of mix in later steps. The total amount of DNA being transfected is 1 µg/well. Plasmids are available upon request to authors.

Table 2. Quantities of transfection reagent and reduced serum cell culture medium required to transfect one well of a 6-well plate of HEK-293T/17 cells for pseudotyped particle production. Multiply volumes by required number of wells to transfect and include extra wells in calculations to avoid running out of mix in later steps. The transfection reagent: plasmid DNA ratio used is 3:1.

Figure 1. Coronavirus S-pseudotyped particle infectivity assays in susceptible host cells using a murine leukemia virus (MLV) backbone and luciferase reporter gene. (**A**) SARS-CoV S pseudotyped particle infectivity assay in Vero-E6 cells. (**B**) MERS-CoV S pseudotyped particle infectivity assay in Huh-7 cells. For both (**A**) and (**B**), plotted data corresponds to the average relative luciferase units from three independent experiments, with error bars corresponding to

standard deviation (s.d.). Data plotted in log_{10} scale on y-axis. N.I.: non-infected control; Δenv: infection with pseudotyped particles lacking viral envelope glycoproteins and VSV-Gpp: infection with pseudotyped particles bearing positive control VSV G envelope glycoprotein. Other abbreviation used, SARS-Spp: infection with SARS-CoV S pseudotyped particles, MERS-Spp: infection with MERS-CoV S pseudotyped particles.

Figure 2. Concentration-dependence of CoV S-pseudovirion infectivity and role of ACE2 and DPP4 receptors in SARS-Spp and MERS-Spp entry. (A) Concentration-dependence of SARS-Spp and MERS-Spp infectivity measured by luciferase activity assay. Pseudovirions were serially diluted and used to infect Vero-E6 cells. (**B**) Infectivity assay of SARS-Spp and MERS-Spp in poorly permissive HEK-293T target cells transfected to express ACE2, and DPP4 receptors, respectively. Data plotted in log_{10} scale on y-axis as in **Figure 1** from duplicate experiments, with error bars corresponding to standard deviation (s.d.). N.T.: non-transfected control HEK-293T cells; ACE2: angiotensin converting enzyme 2 (SARS-CoV receptor) and DPP4: dipeptidyl peptidase 4 (MERS-CoV receptor). Other abbreviations used are the same as in **Figure 1**.

DISCUSSION:

This protocol describes a method to efficiently produce pseudotyped particles bearing the S protein of risk group 3 coronaviruses, SARS-CoV and MERS-CoV, in a BSL-2 setting. These particles, which incorporate a luciferase reporter gene, enable us to easily quantify coronavirus S-mediated entry events by a relatively simple luciferase assay[48-51]. In infectivity assays using permissive cells, we confirm that the luciferase activity measured is dependent on the concentration of particles. In addition, ACE2 and DPP4 receptor transfection allows for more efficient entry in poorly permissive cells lines, such as HEK-293T cells. The method is highly adaptable to other viral envelope glycoproteins and has been used extensively[48-53,55-59], often to complement other assays like biochemical analyses or native virus infections.

The protocol we describe here is based on the retrovirus MLV that incorporates a luciferase reporter. However, it is important to emphasize that there is a very wide array of other pseudotyping systems that have been successfully developed for packaging coronavirus S[12,13,25,26,30-32] and other viral envelope glycoproteins[10,11,14,16,17,23,24,29,33,38,40,42,44,46]. Some of these other systems are based on the commonly used MLV retroviral core[7-20], or based on the widely used lentiviral HIV-1 pseudotyping system using different strategies[23-35], or with the rhabdovirus vesicular stomatitis virus (VSV) as core, which allows to incorporate a wide variety of envelope glycoproteins, and again with various strategies employed[37-47]. In addition, other reporters such as fluorescent proteins like GFP[11,13] and RFP[36], or enzymes other than luciferase like β-galactosidase[16,17] and secreted alkaline phosphatase (SEAP)[42] have been successfully employed for measurements. Furthermore, in the assay presented in this protocol, a transient transfection was used to express the MLV and CoV S genes and proteins. However, there are other strategies for expression, such as generation of stable cell lines for production of pseudotyped viruses[7,14]. As each of these systems have their advantages and disadvantages, it is important to consider the following important parameters when deciding which pseudotyping system best suits an investigator's needs: pseudovirion core (MLV, HIV-1, VSV or others), how selective a particular pseudotyping core is in incorporating a specific viral envelope glycoprotein, reporter for assay

readout (GFP, luciferase, SEAP or other), and the transfection strategy (number of plasmids involved in co-transfection, transient transfection or generation of stable cell lines).

There are a number of critical steps in the method that are important to emphasize. Cell density, particularly of the HEK-293T/17 producer cell line is a critical factor in ensuring successful transfection. A cell density in the range of 40-60% confluency was found to be optimal. Higher densities typically result in low transfection efficiencies and low particle production. Also, it is important to keep in mind that HEK-293T/17 cells are less adherent than other cell lines. Care should be exercised when handling them to avoid detaching them unnecessarily. One option is to treat cell culture plastic surfaces with poly-D-lysine to enhance adherence. Furthermore, higher cell passage often results in poor transfection rates. After adding the transfection reagent to HEK-293T/17 cells, it is also important to remember that cell permeability increases. This is why at this point it is best to avoid using medium containing antibiotics as they may increase cytotoxicity. Before collecting pseudotyped particles, check the color of transfected HEK-293T/17 cell supernatants. Typically, after 48 h of transfection, the cell culture medium color takes an orange-pink tinge. Yellow-colored medium usually translates to poor pseudotyped particles yields and is often a result of issues with cell seeding density or high passage number.

In this protocol, pseudotyped particle production is performed in a 6-well plate format. To increase volume of produced particles, several wells of a 6-well plate can be transfected with the same plasmids mix and the supernatants can be pooled together. The pool can then be clarified, filtered and aliquoted. Alternatively, to scale production up, other kinds of vessels (e.g., 25, or 75 cm^2 flasks) can be used. In this case, transfection conditions should be scaled up accordingly. In this protocol, the infectivity assay is performed using a 24-well plate format and a luminometer that only allows measurements one tube at a time. For high throughput screenings, other formats are also possible, such as 96-well plate format and a plate reader luminometer. Volumes and reagents for the luciferase assay need to be adapted accordingly. Storage of pseudotyped particles in cryovials at -80 °C maintains their stability for several months without noticeable decrease in infectivity. It is not recommended to subject them to freeze-thaw cycles as this will decrease their infectivity over time. Thus, it is best to store them in small aliquots such as 0.5-1 mL and thaw them before an infection.

The method presented here has several limitations. An important one is the fact that pseudotyped particles recapitulate only viral entry events. To analyze other steps in the infectious life cycle, other assays are required. Furthermore, as MLV particles bud at the plasma membrane, it is important to bear in mind that the envelope glycoprotein being studied needs to also traffic to the plasma membrane for incorporation into pseudovirions during production. As such, it is important to know where in the cell a particular viral envelope glycoprotein is expressed in transfection conditions, such as by visualizing subcellular localization with an immunofluorescence assay, and/or by checking for retention signals within the protein. Also, while the protocol describes steps to produce and test infectivity, it does not detail how to measure incorporation of viral envelope glycoproteins into pseudotyped particles. One method is to perform western blot assays on concentrated solutions of particles, as previously described[50,51] for MERS-CoV S incorporation. In these assays, the S envelope glycoprotein of

MERS-CoV is probed along with the capsid (p30) protein of MLV, which allow us to normalize incorporation of the S protein into particles. Other examples of such assays analyzing viral envelope glycoprotein incorporation into pseudovirions have been performed for SARS-CoV S incorporation in an HIV-1 lentiviral pseudovirion system[32] , Ebola glycoprotein (GP) in another MLV pseudotyped particle system[17], and influenza hemagglutinin (HA) and neuraminidase (NA) in VSV pseudovirions[38]. A recent development in characterizing pseudotyped particle production is the use of innovative imaging devices such as Nanosight: it enables us to directly visualize, quantify, and size viral particles[50]. The device provides detailed information on overall particle production; however, it is important to keep in mind that it does not provide information on envelope glycoprotein incorporation. A future direction for the application of these versatile pseudovirion particles is to analyze individual viral fusion events using single particle tracking, microfluidics and total internal reflection fluorescence microscopy[60-62]. Such approaches were successfully applied to influenza virus and feline coronavirus particles as well as influenza HA- and NA-pseudotyped VSV-based pseudovirions[63]. The deployment of such techniques applied to coronavirus S-pseudotyped MLV-based particles is currently being developed.

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DISCLOSURES:

The authors have nothing to disclose.

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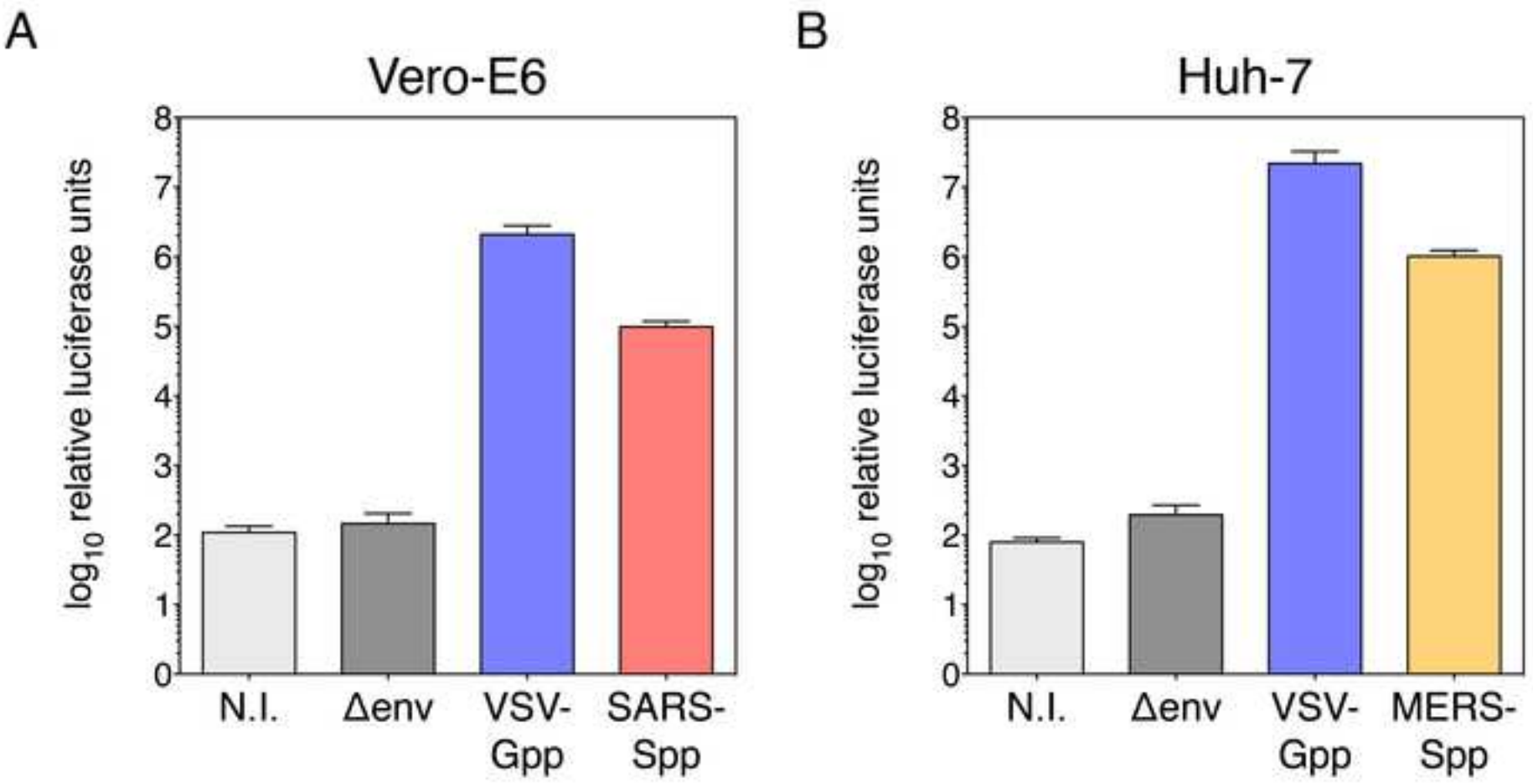
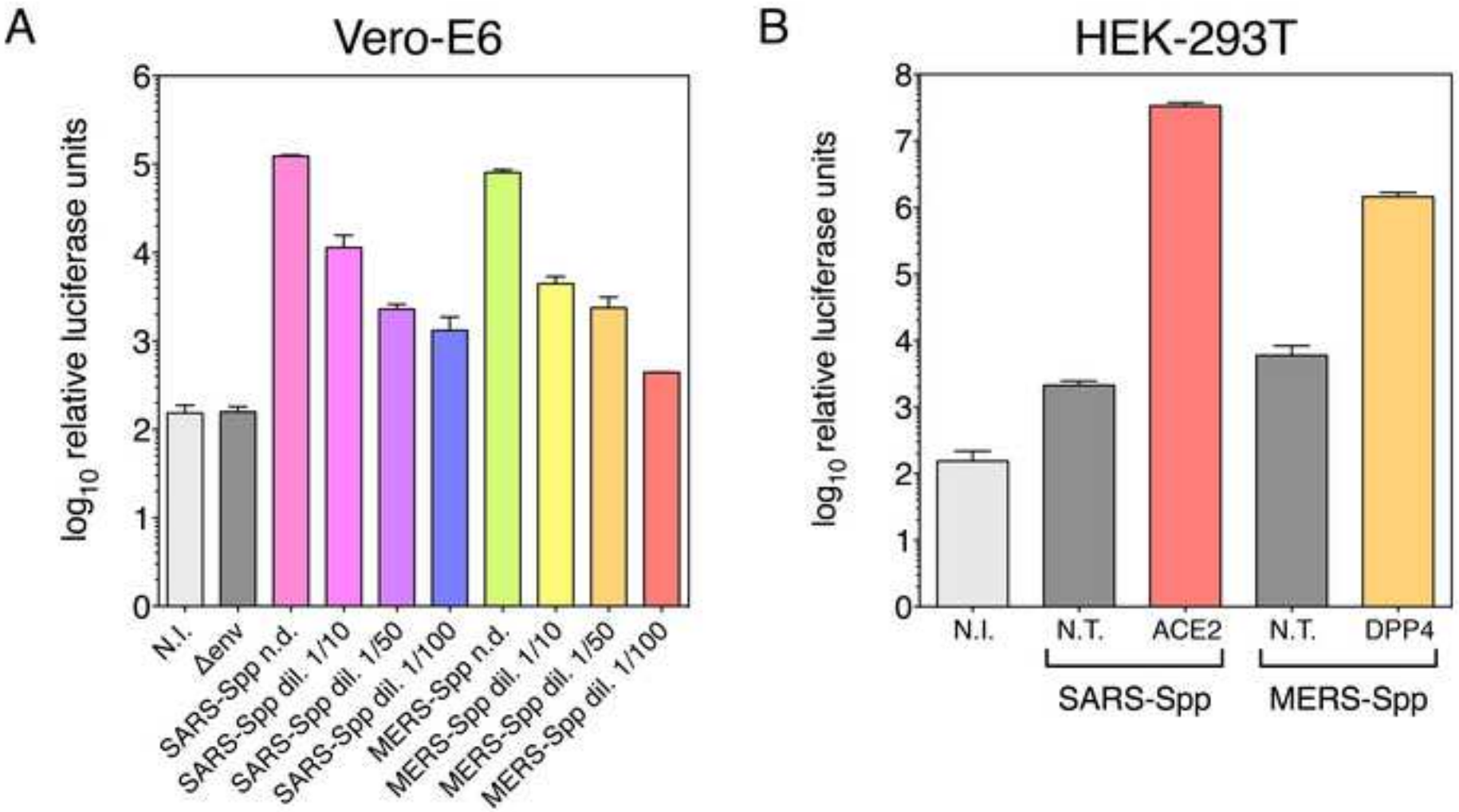
Figure



Figure



Plasmid/reagent
pCMV-MLVgagpol MLV gag and pol encoding plasmid
pTG-Luc transfer vector with luciferase reporter
pcDNA-SARS-S, pcDNA-MERS-S or empty vector
Reduced serum cell culture medium

Quantity
300 ng
400 ng
300 ng
To 50 µL

Reagent
Transfection reagent
Reduced serum cell culture medium

Quantity
3 µL
47 µL

Name of Material/ Equipment

Human embryonic kidney (HEK) HEK-293T/17 cells

African green monkey kidney epithelial Vero-E6 cells

Human hepatic Huh-7 cells

Inverted light microscope with 10 × objective

Dulbecco's modification of Eagle's medium (DMEM) with 4.5 g/L glucose, L-glutamine without sodium pyruvate

Heat-inactivated fetal bovine serum (FBS)

1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)

100 × penicillin-streptomycin (PS) solution

Dulbecco's phosphate buffered saline (DPBS) with Ca^{2+} and Mg^{2+}

0.25% trypsin, 2.21 mM ethylenediaminetetraacetic acid (EDTA) 1 × solution

Cell counting slides with grids

Opti-minimal essential medium (Opti-MEM)

Lipofectamine 2000 transfection reagent

0.45 µm pore-size sterile filter

10 mL syringes

5 × luciferase assay lysis buffer

Luciferin, substrate for luciferase assay

Sterile water

GloMax 20/20 luminometer

Company

ATCC

ATCC

Japan National Institutes of Biomedical Innovation, Health and Nutrition

Nikon

Corning Mediatech

Thermo Fisher Scientific, Gibco

Corning Mediatech

Corning Mediatech

Corning Mediatech

Corning Mediatech

Kova

Thermo Fisher Scientific, Gibco

Thermo Fisher Scientific, Invitrogen

Pall

BD

Promega

Promega

VWR

Promega

Catalog Number

CRL-11268

CRL-1586

JCRB0403

TS100

10-017-CV

1614071

25-060-Cl

30-002-Cl

21-030-CV

25-053-Cl

87144

31985-070

11668-027

4184

309604

E1531

E1501

E476-1L

2030-100

Comments/Description

Clone 17 cells are highly competent for transfection.

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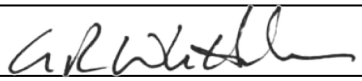
Signature:	
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Rebuttal for JoVE59010

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
The manuscript text has been proof-read for spelling mistakes and grammatical errors.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Opti-MEM, Lipofectamine, falcon, GloMax, GraphPad Prism, etc.
Commercial sounding language has been replaced with generic terms: reduced serum cell culture medium, transfection reagent, conical centrifuge tube, luminometer, graph plotting software.

3. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
More details on the protocols steps have been added, in particular 11 notes have been added and/or amended and citations of published materials have been inserted in the discussion to specify how to perform certain protocol actions. Please see our responses below for details on which steps have been amended.

4. 1.3: Please specify the trypsin concentration and the reaction time. Does "warm" mean 37 °C?
The details of temperature (37 ˚C) and incubation time (3-5 minutes) for trypsin-mediated cell detachment have been added to the manuscript text. In addition, all instances where the word "warm" is used in text has been changed to "pre-warmed (37 ˚C)".

5. 1.4: What volume of DMEM-C medium is used?
4 mL of DMEM-C is typically used to deactivate trypsin. This has been added to manuscript text. In addition, a note has been added to suggest that an additional dilution step may be required prior to the cell counting step.

6. 2.1: This step is unclear. What is observed, cell density?
The sentence was modified to explain that cell morphology and density need to be observed.

7. 2.2.1, 2.3.1: Please note that calculations are not appropriate for filming. Please consider un-highlighting this step as well as note.
Steps 2.2.1. and 2.3.1. have be un-highlighted.

8. 4.1.3: Please specify the trypsin concentration and the reaction time.
The details of temperature (37 ˚C) and incubation time (3-5 minutes) for trypsin-mediated cell detachment have been added to the manuscript text.

9. 5.5: What is the incubation temperature?
The incubation temperature (room temperature) has been added to manuscript text.

10. Tables 1 and 2: Please replace commercial language (Opti-MEM and Lipofectamine) with generic terms.
Generic terms (reduced serum cell culture medium and transfection reagent) have replaced commercial names in tables 1 and 2.

11. Figure 1: Please define error bars in the figure legend.
The error bars definition is already in the figure legend of figure 1: “with error bars corresponding to standard deviation”. However, to put more emphasis on the definition of the error bars, the abbreviation “s.d.” was added. Error bars were also defined in the new figure 2 legend.

12. Figure 1 legend: Please shorten the figure legends. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
Figure 1 legend has been shortened to remove details on the methodology and focus more on data presentation and abbreviations used. The legend of figure 2 has been similarly kept short.

13. References: Please do not abbreviate journal titles.
The references section has been modified to include full journal titles. New citations added in the revised version of the manuscript were also verified to ensure they all include full journal titles. Please note that since the modifications were performed through a reference manager application, the modified text for citations does not appear highlighted in the manuscript document even though “track changes” was turned on.

Reviewers' comments:

Reviewer #1:

In this manuscript, the authors describe a pseudotyped virus package and infection method based on a three-plasmid system, and use pseudotyped SARS-CoV and MERS-CoV as examples. Overall, the manuscript is generally well written and the assay is straightforward. Below are some comments for improving the manuscript.

1. There are a variety of applications for the pseudotyped virus system, and the authors did not specifically talk about the entry of coronaviruses, so "entry" could be removed from the title.
We thank the reviewer for the very helpful comments and suggestions. We agree to remove the word “entry” in the title to broaden the protocol’s applications.

2. In the PROTOCOL section, it would be helpful for the readers by labeling key steps for pseudotyped virus package and infection, as well as providing more notes to the critical steps to which special attentions need to be paid.
In all, 11 notes have been added or amended and important steps have been labeled as key.

3. Figure 1: The title in the Y-axis is wrong. Could be just "Relative luciferase units" (without log10), or change the labeling if the data were calculated as log10.
The data in figure 1 is presented as $\log_{10}$. As suggested by the reviewer, the mistake has been corrected by changing the y-axis labeling as log instead of powers of 10.

4. More representative results will be useful to explain this assay. For example:

1) Western blot figures could be added to show the expression of MLV antigen and SARS-CoV or MERS-CoV S protein from the generated pseudotyped viruses in order to demonstrate that the coronavirus S proteins are incorporated into the pseudotyped particles.

We agree that verifying CoV S incorporation into MLV pseudotyped particles is an important assay to perform after producing these particles. This is a technique we routinely perform, as described for MERS-CoV S MLV pseudotyped particles in the figure A shown below and in the following manuscripts: Millet and Whittaker, 2014 PMID: 25288733 and Millet et al., 2016 PMID: 2016. Similar assays have also been performed by others using MLV pseudovirions pseudotyped with other glycoproteins: Ebola Virus GP - Wool-Lewis and Bates 1998 PMID: 9525641 or SARS-CoV S pseudotyped HIV-1 lentivirus – Bertram et al., 2011 PMID: 21994442 and influenza HA and NA in VSV pseudotyped particles – Zimmer et al., 2014 PMID: 24814925. These latter examples and citations have been added to the discussion in the paragraph that specifically states that it is important to perform such analysis to obtain information on how well a particular viral envelope glycoprotein is incorporated into a pseudovirion. We believe that these statements and citations in the discussion are sufficient to guide readers to other publications that detail the procedures to perform such western blot assays.

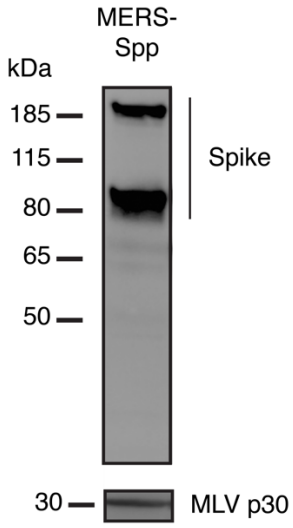

Figure A: western blot analysis of MERS-Spp incorporation of S protein. Assay performed on concentrated (centrifuged) supernatants containing MERS-Spp. Western blot performed using anti-MERS-CoV S antibodies (top) and anti-MLV p30 capsid antibodies.

2) Infectivity data from a serial dilution of pseudotyped SARS-CoV and MERS-CoV would be needed to confirm that pseudotyped virus infection is concentration-dependent, since this type of virus is single cycle and cannot replicate in infected cells.
We agree and performed an infectivity assay in Vero-E6 cells in which both SARS-Spp and MERS-Spp pseudotyped particles have been serially diluted and confirm that the luciferase assay measurements are concentration-dependent for both types of particles. This assay has been added as panel A of a new figure, fig. 2.

5. There are other assays for packaging pseudotyped SARS-CoV and MERS-CoV, such as those based on two plasmids encoding Env-defective, luciferase-expressing HIV-1 and MERS-CoV or SARS-CoV spike proteins, respectively, which are convenient and effective. The authors should cite these references and discuss differences with these assays.
We agree with the reviewer and have added many citations from other groups in the introduction and a new paragraph in the discussion to detail other pseudotyping systems that are readily available as well as comparisons with the system we present (HIV-1-, VSV- and other MLV-based pseudovirion systems).

6. In addition to infect Vero E6 cells, pseudotyped SARS-CoV can also infect other cell lines such as 293T cells expressing SARS-CoV receptor human angiotensin I converting enzyme 2 (hACE2/293T). Similarly, except for infecting Huh-7 cells, pseudotyped MERS-CoV based on the two-plasmid system can also infect 293T cells expressing MERS-CoV receptor dipeptidyl peptidase 4 (DPP4/293T), MDCK, Caco-2, HEP-G2,

and other cells. Have the authors tested infectivity of the produced pseudotyped SARS-CoV and MERS-CoV in other cell types? This should be discussed.
Yes, we have tested infectivity of pseudotyped particles in different cell types such as in a previous study with MERS-Spp (Millet and Whittaker, 2014, PMID: 25288733): Vero-E6, Huh-7, MDCK MRC-5, and WI-38 cells. As suggested by the reviewer, we have performed an experiment in which the poorly permissive (for both SARS-CoV and MERS-CoV) HEK-293T cell line was transfected to express the respective receptors of the human coronaviruses: ACE2 and DPP4. The transfected cells were then infected with SARS-Spp and MERS-Spp. The assay shows that upon expression of the respective receptor, there is a ~4- and ~2-log increase in infectivity for SARS-Spp and MERS-Spp, respectively. The data of this experiment has been added as panel B of figure 2.

Reviewer #2:

Jean Millet et al described that establishment of pseudo-type viral particle with luciferase for coronavirus by using murine leukemia virus. In this manuscript they provided reasonable methods and results. I think that this manuscript is suitable for the Journal of visualized experiments.
We thank the reviewer for the comments and remarks on our methodology manuscript.

Reviewer #3:

The authors have a strong tendency to the self-citation: 18 of 23 cited publications were done by Whittacker and/or Millet. Even if the authors are specialists in the study of protein S of coronaviruses, it is important to put the work into perspective. This article appears as not original promotion.
We thank the reviewer for the candid comments and suggestion to improve our manuscript. We agree it is important to position the method presented here into a wider perspective. To address this issue, we have added abundant citations in the introduction to inform readers of the wide array of pseudotyping systems available, based primarily on HIV-1, MLV and vesicular stomatitis virus (VSV). In addition, a new paragraph in the discussion gives elements of comparison between the wide variety of other pseudotyping systems and suggests which parameters to consider when choosing such pseudotyping systems. This should give readers some guidance to make informed decisions on which pseudotyped particle production methodology best matches their needs. In all, we have added 16 citations on MLV-based pseudotyping systems, as well as 13 citations on HIV-based pseudotyping systems and 12 article citations on VSV-based pseudotyping systems. All of these new citations were performed by other groups. In total, there are now 46 cited publications on pseudotyping systems performed by others in the manuscript, giving the reader some perspective on other pseudovirion production methods and positioning our protocol into a much larger methodological framework.

Response to Editorial comments for JoVE59010

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.
We have retained the same journal style.

2. Please address all the specific comments marked in the manuscript.
We have addressed all of the specific comments marked in the manuscript.

3. For the protocol section, Please ensure that the highlight is no more than 2.75 pages. Please combine short actions to 2-3 actions per step. Please use complete sentences throughout.
We have checked that the highlighted section is no more than 2.75 pages.

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We have not used figures from previous publications.