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.

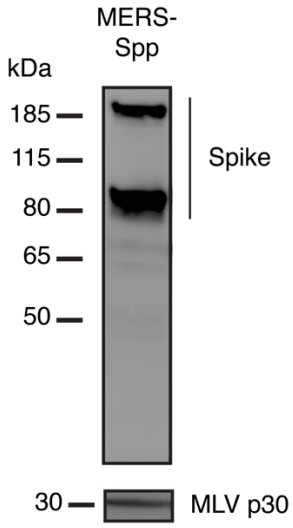

Figure A: western blot analysis of MERS-Spp incorporation of S protein. Assay performed on concentrated (centrifuged) supernatants containing MERS-Spp. Western blot performed using anti-MERS-CoV S antibodies (top) and anti-MLV p30 capsid antibodies.

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.