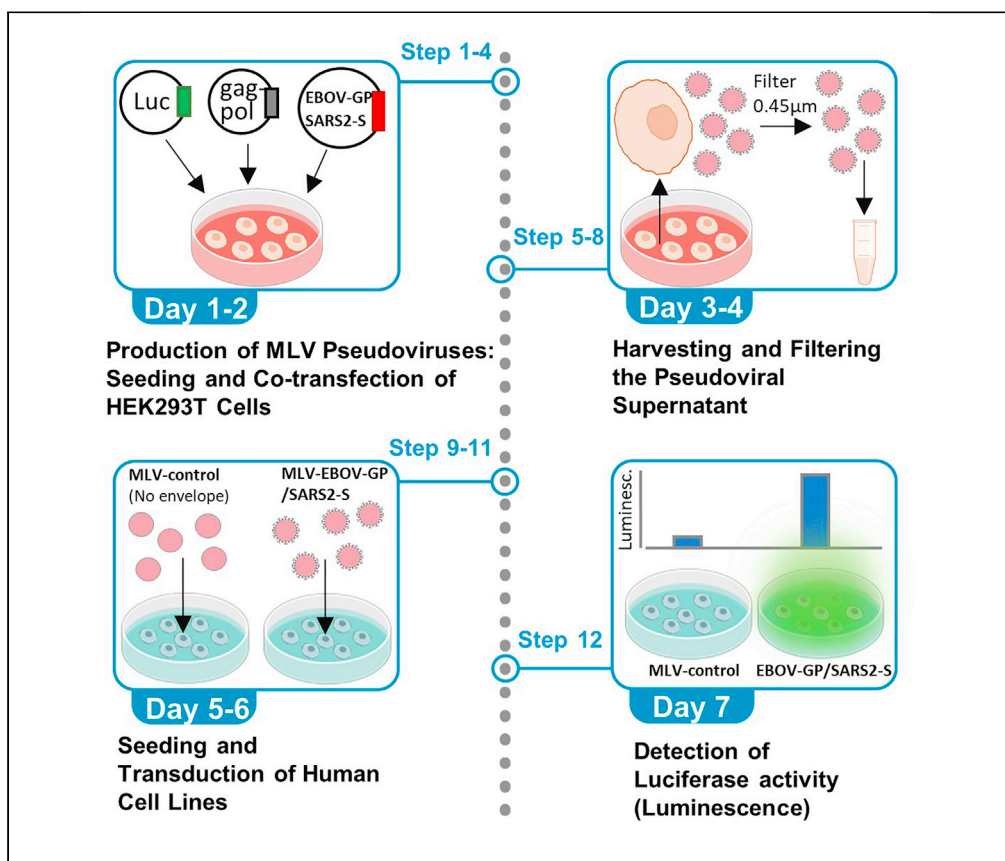


## Protocol

# Application of pseudotyped virus particles to monitor Ebola virus and SARS-CoV-2 viral entry in human cell lines



Madeleine Eichler,  
Ebru Aksi, Josef  
Pfeilschifter,  
Gergely Imre  
gimre80@gmail.com

### Highlights

MLV pseudotyped viruses are suitable for studies on viral entry in human cell lines

Replication incompetence of pseudoviruses enables bio safety level-2 conditions

Quantification of transduction efficiency by measurement of luciferase activity

Experimental work on highly pathogenic viruses such as Ebola virus and severe acute respiratory syndrome coronavirus-2 requires high-level biosafety facilities. Here we provide a detailed step-by-step protocol which details the production and application of replication-incompetent murine leukemia virus-based pseudotyped particles to monitor and quantify the viral entry efficiency in human cell lines under biosafety level-2 conditions. We describe the use of viral particles encoding *luciferase* gene and the quantification of transduction efficiency by measuring luciferase activity.

## Protocol

## Application of pseudotyped virus particles to monitor Ebola virus and SARS-CoV-2 viral entry in human cell lines

Madeleine Eichler,<sup>1,2</sup> Ebru Aksi,<sup>1</sup> Josef Pfeilschifter,<sup>1</sup> and Gergely Imre<sup>1,3,\*</sup><sup>1</sup>Institute of General Pharmacology and Toxicology, Goethe University Frankfurt, 60590 Frankfurt am Main, Germany<sup>2</sup>Technical contact<sup>3</sup>Lead contact\*Correspondence: [gimre80@gmail.com](mailto:gimre80@gmail.com)  
<https://doi.org/10.1016/j.xpro.2021.100818>

## SUMMARY

Experimental work on highly pathogenic viruses such as Ebola virus (EBOV) and severe acute respiratory syndrome coronavirus-2 requires high-level biosafety facilities. Here, we provide a detailed step-by-step protocol which details the production and application of replication-incompetent murine leukemia virus-based pseudotyped particles to monitor and quantify the viral entry efficiency in human cell lines under biosafety level-2 conditions. We describe the use of viral particles encoding *luciferase* gene and the quantification of transduction efficiency by measuring luciferase activity.

For complete details on the use and execution of this protocol, please refer to Imre et al. (2021).

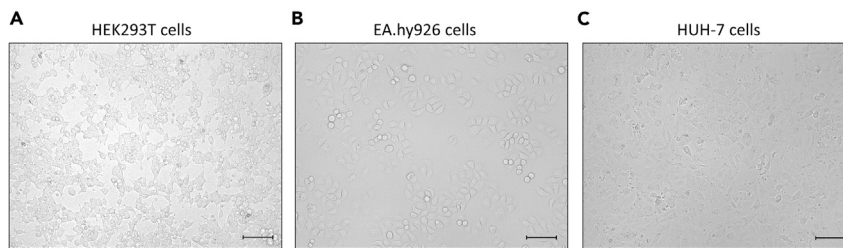
## BEFORE YOU BEGIN

Here, we describe a protocol for the production of murine leukemia virus (MLV) based pseudoviruses. To generate pseudotyped vectors of highly pathogenic viruses, lentiviral vectors such as human immunodeficiency virus (HIV) and simian immunodeficiency virus based vectors are commonly employed as a first choice. Apart from the safety concerns of HIV based particles, it has been reported that in several cases higher yield can be achieved by the application of vesicular stomatitis virus (VSV) and MLV derived systems when compared to lentiviral vectors (Temperton et al., 2007; Cosset et al., 2009). In comparison to the VSV based viral vectors MLV pseudoviruses are smaller. VSV viral particles are 200 nm in length and have a slightly elongated structure. In contrast, MLV viral vectors are 80–100 nm in size and have a spherical shape (Chen et al., 2019). The smaller size of the viral particles can result in higher yields and improved filtration efficacy of the viral particles. Similarly to replication-incompetent lentiviral and VSV pseudoviruses, MLV pseudoviruses accomplish a single round of infection, since instead of harboring viral RNA the viral particles only harbor the firefly luciferase RNA (Millet et al., 2019). Upon successful transduction of the MLV particles, the *Luciferase* gene integrates in the host genome and the light generating enzyme Luciferase is produced. This enables quantitative analysis of pseudovirus infectivity by measuring luciferase activity proportional to the detected luminescence intensity.

## Biological safety

Any work involving genetically modified virus particles should be approved by national and institutional biosafety committees. Before starting, please make sure that your lab complies all the necessary criteria and documentations required to accomplish biosafety Level-2 (BSL-2) work.





**Figure 1. Microscopy pictures of cell lines**

Microscopy pictures of (A) HEK293T, (B) EA.hy926 and (C) HuH-7 cells. The microscopy pictures were taken by Keyence BZ-8000K Microscope by using 20× 0.75 objective (200× magnification). The scale is 100 μm.

### Cell maintenance

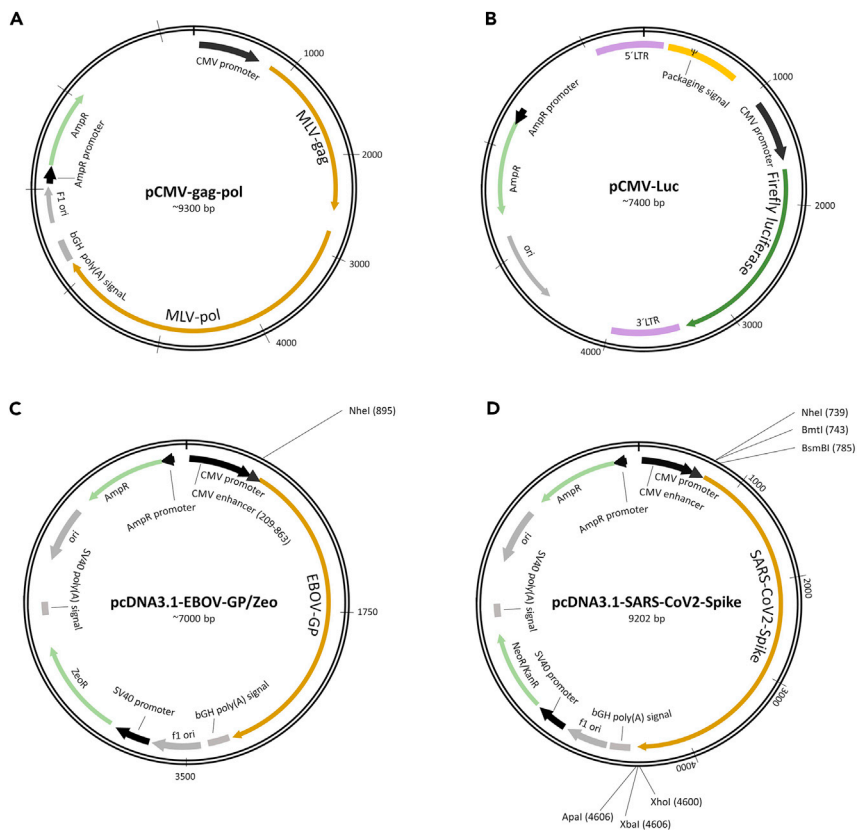
⌚ Timing: 5–7 days

1. Thaw HEK293T, EA.hy926 and HuH-7 cells (Figures 1A–1C) quickly at 37°C. Add the thawed cell suspension to 10 mL prewarmed complete medium (see step 2.) and spin at 200 × g for 4 min. Discard the supernatant and resuspend the cell pellet in fresh prewarmed complete medium. Incubate the cells in cell culture flask at 37°C with 5% CO<sub>2</sub>.
2. Maintain each cell lines in the recommended medium (see medium recipes in the [materials and equipment](#) section):
  - a. HEK293T and HuH-7 cells in complete Dulbecco's Modified Eagle Media (DMEM) (containing 4 mM glutamine, 4.5 g/l glucose) with 10% FBS and 100 μg/mL streptomycin and 100 units/mL penicillin.
  - b. EA.hy926 cells in complete Roswell Park Memorial Institute 1640 (RPMI1640) media containing 10% FBS, 100 μg/mL streptomycin and 100 units/mL penicillin.
3. Split cells:
  - a. HEK293T cells: Remove the medium. Add prewarmed fresh complete medium onto the cells. Detach the cells by repeated up and down pipetting until the cell layer is completely removed from the surface.
  - b. EA.hy926 and HuH-7 cells: Remove medium and rinse the cells twice with 5–5 mL 1 × DPBS. Add 3.5 mL Trypsin/EDTA (0.05%), rinse the cells by gently swirling the cell culture flask and remove the rest of the Trypsin/EDTA solution, leaving just a thin layer covering the whole surface. Incubate it for 5 min at 37°C. Resuspend the cells in fresh, prewarmed complete medium. The splitting ratios for the cells are the following: HEK293T: 1:5; EA.hy926: 1:10; HuH-7: 1:5.

### Prepare plasmids for transfection

⌚ Timing: 3–4 days

4. Four plasmids need to be prepared in advance (Figure 2):
  - a. Plasmid containing the Murine leukemia virus (MLV) gag and pol genes: pCMV-MLV-gag-pol (Bartosch et al., 2003; Millet and Whittaker, 2016; Millet et al., 2019).
  - b. Reporter vector encoding the firefly luciferase (Luc) gene with a viral packaging sequence: pCMV-Luc (Bartosch et al., 2003; Millet and Whittaker, 2016; Millet et al., 2019).
  - c. Plasmids encoding viral envelope proteins of interests: pcDNA3.1- Ebola virus glycoprotein (EBOV-GP) or pcDNA3.1-SARS-CoV-2-Spike (SARS-CoV-2-S).
  - d. Empty vector as control plasmid: pcDNA3-Flag-HA.
5. Transform bacterial competent cells XL-1 Blue (Agilent) by employing standard heat shock transformation procedure.
6. Grow the transformed bacteria on agar plates containing ampicillin (100 μg/mL) overnight (16 h) at 37°C.



**Figure 2. Plasmid maps**

(A–D) (A) pCMV-MLV-gag-pol, (B) pCMV-MLV-Luc, (C) pCDNA3.1-EBOV-GP, (D) pCDNA3.1-SARS-CoV-2-Spike. Amp<sup>r</sup>-Ampicillin resistance, ZeoR-Zeomycin resistance, NeoR/KanR- Neomycin/Kanamycin resistance, ori- bacterial origin of replication, CMV promoter-human Cytomegalovirus promoter, LTR-long terminal repeat, Poli (A) signal-poliadenylation signal, bGH-bovine growth hormone.

- Pick a single colony and incubate it in 100 mL LB media supplemented with 100 µg/mL ampicillin overnight at 37°C in a shaker incubator.
- Lyse the bacteria and extract the produced plasmid DNAs by employing standard midi prep columns. We use Nucleo Bond DNA extraction kit (Macherey Nagel, Cat#740573.100).
- Determine the concentration of the plasmid DNAs by nanodrop method and adjust it to 1 µg/mL.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ampicillin	Sigma-Aldrich	Cat#A5354
Dulbecco's Modified Eagle Medium (DMEM + GlutaMAX-I)	Gibco	Cat#61965-026
Dulbecco's Phosphate Buffered Saline (DPBS)	Gibco	Cat#14190-094
Fetal bovine serum (FBS)	Sigma-Aldrich	Cat#F7524-500ML
Genejuice Transfection Reagent	MERCK-Millipore	Cat#70967-1ML
Minimum Essential Medium (MEM)	Gibco	Cat#31095-029
LB-Agar	Roth	Cat#X969.1
LB-Medium	Roth	Cat#968.1

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
OPTI-MEM	Gibco	Cat#31985-062
Penicillin-streptomycin (Pen Strep)	Sigma-Aldrich	Cat#15140-122
RPMI Medium 1640 + GlutaMAX-I	Gibco	Cat#61870-010
Trypsin-EDTA 0.05%	Gibco	Cat#25300-054
D-Luciferin	Perkin Elmer	Cat#122799
<b>Critical commercial assays</b>		
Luciferase Assay System, containing Luciferase Lysis Buffer, Luciferase Assay Substrate (lyophilized) and Luciferase Assay Buffer	Promega	Cat#E1500
Luciferase Lysis Buffer	Promega	Cat#E1531
Nucleo Bond DNA extraction kit	Macherey Nagel	Cat#740573.100
<b>Experimental models: cell lines</b>		
EA.hy926	Dr. Josef Pfeilschifter	<a href="#">Edgell et al. (1983)</a>
HEK293T	ATCC	CRL-11268
HuH-7	JCRB Cell Bank	JCRB0403 RRID:CVCL0336
<b>Bacterial and virus strains</b>		
XL-1 Blue Competent Cells	Agilent	Cat#200249
<b>Recombinant DNA</b>		
Plasmid: pcDNA3-Flag-HA	Addgene #10792, gift from Dr. William Sellers	<a href="http://n2t.net/addgene:10792">http://n2t.net/addgene:10792</a> ; RRID:Addgene_10792
Plasmid: pcDNA3.1-SARS-CoV-2-Spike	Addgene #145032, gift from Dr. Fang Li ( <a href="#">Shang et al., 2020</a> )	<a href="http://n2t.net/addgene:145032">http://n2t.net/addgene:145032</a> ; RRID:Addgene_145032
Plasmid: pcDNA3.1-zEBOV-GP	Gift from Dr. Fatah Kashanchi, Laboratory of Molecular Virology, George Mason University Manassas	<a href="#">Pleat et al. (2016)</a>
Plasmid: pCMV-MLV-gag-pol	Dr. Stefan Pöhlmann	<a href="#">Bartosch et al. (2003)</a>
Plasmid: pCMV-Luc	Dr. Stefan Pöhlmann	<a href="#">Bartosch et al. (2003)</a> ; <a href="#">Millet and Whittaker (2016)</a> ; <a href="#">Millet et al. (2019)</a>
<b>Software and algorithms</b>		
SoftMax Pro Software	Molecular Devices	Version 5.4.6.005
<b>Others</b>		
6-Well cell culture plate	Greiner	Cat#657160
12-Well cell culture plate	Greiner	Cat#665180
96-Well white microplate	Corning-Costar	Cat#CLS3922
Cell culture flask with filter cap 75 cm <sup>2</sup>	Greiner	Cat#658175
Centrifuge tube 50 mL	Greiner	Cat#227261
Filtertube 0.45 µm PVDF	Merck-Millipore	Cat#SE2M230104
Luminometer	Molecular Devices	SpectraMax M5E
Nanodrop ND-1000 Spectrophotometer	Thermo Scientific	#2353-30-0010
Pipettes 200 µL-1 mL, 20-200 µL and 0.5 µL-10 µL	Eppendorf Research Plus	3123000063; 3123000055; 3123000020
Pipette tips 1 mL, 200 µL, 10 µL	Sarstedt	70.3050; 70.3030; 70.1130

## MATERIALS AND EQUIPMENT

### Complete DMEM medium

Add the following ingredients to DMEM (containing 4 mM glutamine, 4.5 g/l glucose):

Reagent	Final concentration
FBS	10%
Streptomycin	100 µg/mL
Penicillin	100 units/mL
Storage at 4°C (max. till date of expire)	

### Complete RPMI1640 medium

Add the following ingredients to RPMI1640 (containing 2 mM glutamine, 2 g/l glucose):

Reagent	Final concentration
FBS	10%
Streptomycin	100 µg/mL
Penicillin	100 units/mL
Storage at 4°C (max. till date of expire)	

### Luciferase Lysis Buffer

**Alternatives:** We have employed Luciferase Lysis Buffer (Cat#E1531) provided by the company Promega, however lysis buffer can be also self-prepared by using the following recipe:

Reagent	Final concentration
Tris-phosphate (pH 7.8)	25 mM
Dithiothreitol (DTT)	2 mM
1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (DCTA)	2 mM
Glycerol	10% (w/v)
Triton X-100	1% (w/v)

**Note:** Store Luciferase Lysis Buffer at  $-20^{\circ}\text{C}$  (max. 1 month) or at  $-80^{\circ}\text{C}$  (max. 1 year).

### Luciferase Substrate

**Alternatives:** We have used Luciferase Assay System, including Luciferase Assay Substrate (luciferin, lyophilized) and Luciferase Assay Buffer for Luciferase activity detection (Cat#E1500, Promega), however alternatively the following Luciferase Substrate Protocol adapted from [Ratnapriya et al., \(2020\)](#) can also be applied:

Solution A: Firefly Luciferase Assay Buffer	
Reagent	Final concentration
MgSO <sub>4</sub>	15 mM
Phosphate Buffer (KH <sub>2</sub> PO <sub>4</sub> /K <sub>2</sub> HPO <sub>4</sub> ) pH 7.8	15 mM
ATP	1 mM
DTT	1 mM

**Note:** Store Firefly Luciferase Assay Buffer in the dark at  $-20^{\circ}\text{C}$  (max. 1 month) or at  $-80^{\circ}\text{C}$  (max. 1 year).

### Solution B: Luciferin substrate solution

Dissolve lyophilized D-Luciferin (#122799, Perkin Elmer) in ddH<sub>2</sub>O adjusted to pH 6.3. Prepare aliquots with 225 µg/mL concentration and store them at  $-20^{\circ}\text{C}$  (max. 1 month) or at  $-80^{\circ}\text{C}$  (max. 1 year).

## STEP-BY-STEP METHOD DETAILS

### Production of recombinant MLV-EBOV-GP/MLV-SARS-CoV-2-S replication-incompetent pseudoviruses

⌚ Timing: 4 days

**Table 1. Transfection complex solution**

Reagent	Final amount
OPTI-MEM	100 $\mu$ L
Genejuice	7.2 $\mu$ L
pCMV-MLV-gag-pol	0.6 $\mu$ g
pCMV-Luc	1.2 $\mu$ g
Viral envelope protein vector/control vector	0.6 $\mu$ g

In these steps, the MLV-EBOV-GP or MLV-SARS-CoV-2-S pseudovirus particles will be produced by employing co-transfection of three plasmids in HEK293T cells.

- Day 1. Keep HEK293T cells in cell culture for at least 2 passages (Ratnapriya et al., 2020) and maximum 20 passages before starting the experiment. The cells should grow exponentially and exhibit healthy morphology (Figure 1A). Wash HEK293T cells gently with 1  $\times$  DPBS and resuspend them in fresh complete DMEM medium.
- Detach the cells with repeated up and down pipetting.
- Count the cells by using a hemocytometer and adjust the cell concentration to  $5 \times 10^5$  cells/well in a 6-well plate. Ideally, the cells should reach 50–60% confluency at the day of transfection.
- Day 2. Transfection of the cells.

**△ CRITICAL:** At this point, continue the work in a BSL-2 facility. Before starting, make sure that all the required equipment for BSL-2 work, waste management and decontamination are available and properly function. Prepare all the necessary solutions for decontamination in a sufficient amount.

- Replace the medium with 1 mL fresh complete DMEM medium/well.
- Prepare the transfection complex solution in a 1.5 mL tube. Table 1 shows the amount of reagents needed for 1 well of a 6-well plate. After mixing the OPTI-MEM and Genejuice, briefly vortex and spin ( $200 \times g$ , 30 s) the solution, and incubate it for 5 min at room temperature (RT; 20°C–23°C).

Components of the transfection mixture. The final amounts for the transfection complex solution are sufficient for 1 well of a 6-well plate. The ratio of total plasmid to Genejuice is 1:3.

**Alternatives:** Other commercially available or self-prepared transfection reagents can also be employed. We describe the protocol by employing Genejuice transfection reagent.

- Add pCMV-MLV-gag-pol, pCMV-Luc and pcDNA3.1-EBOV-GP or pcDNA3.1-SARS-CoV-2-S to the transfection reagent mix and briefly vortex/spin ( $200 \times g$ , 30 s) the sample. Table 1 demonstrates the amounts of plasmids necessary for efficient pseudovirus production.
- To produce MLV pseudovirus particles without viral surface glycoprotein (MLV-control) add pCMV-MLV-gag-pol, pCMV-Luc and pcDNA3.1 empty vector to the transfection complex solution and briefly vortex/spin ( $200 \times g$ , 30 s) the sample.
- After 15 min incubation, the transfection complex solution is gently pipetted drop-wise on the cells. The transfection complex solutions are mixed by gentle swirling of the plates to ensure equal distribution. The transfected cells are incubated in a cell culture incubator at 37°C and with 5% CO<sub>2</sub>.
- 6 h later, replace the medium with fresh complete medium by gentle pipetting.

**△ CRITICAL:** It is important to remove the medium with extra caution in order to avoid cell loss, because HEK293T cells tend to detach easily from plastic surfaces.

- Day 3. Collect the pseudovirus supernatants at 24 h after medium change in 50 mL centrifuge tubes, store them at  $-80^{\circ}\text{C}$  and add 1 mL fresh complete DMEM on the cells.



6. Day 4. Collect the pseudoviral supernatants of the cells in 50 mL centrifuge tubes at 48 h after medium change, store them at  $-80^{\circ}\text{C}$ . The two batches are stored separately.

**Note:** Cells tend to round up and detach at this stage.

**Note:** In general, the medium does not show significant pH changes at this stage. The color of medium does not turn to yellow or purple.

**▮▮ Pause point:** At this point, pseudoviral supernatants can be stored at  $-80^{\circ}\text{C}$  until further usage. Alternatively, according to our experiments viral supernatants can be stored in  $-20^{\circ}\text{C}$  for a shorter period of time ( $\sim 1$  week) without losing efficiency.

7. Thaw the pseudoviral supernatants and pool the two batches. Next, filter them by employing a  $0.45\ \mu\text{m}$  PVDF filter unit to remove cell debris. Store the filtered pseudoviral supernatants at  $-80^{\circ}\text{C}$ .

**Optional:** Centrifugation can be employed prior to filtration to remove cell debris ( $600\text{--}900 \times g$  for 8 minutes at  $4^{\circ}\text{C}$ ). This might help to avoid clotting of the cells in the filter.

**Note:** Prepare aliquots of the pseudoviral supernatants (e.g. 1-1 mL), which can be completely used up for a single experiment in order to avoid freeze-thaw cycles.

**▮▮ Pause point:** At this point, viral supernatants can be kept at  $-80^{\circ}\text{C}$  until further usage.

8. In order to confirm efficient transfection, wash and lyse the remaining cells (1 well/6-well) in plate employing  $55\ \mu\text{L}$  luciferase lysis buffer. Transfer  $50\ \mu\text{L}$  cell lysate to a 96-well white microtiter plate and incubate it for 15 min at RT. Add  $90\ \mu\text{L}$  luciferin substrate to the lysates and measure the luciferase activity immediately by a luminometer with 500 ms integration. We typically measure  $\sim 8\text{--}10 \times 10^7$  Relative Luminescence Unit (RLU) in 1 well of a 6-well plate of HEK293T cells.

### Transduction of EA.hy926 cells with MLV-EBOV-GP pseudoviruses

We employed EA.hy926 endothelial hybridoma cells because they are susceptible to Ebola virus infection (Imre et al., 2021). In these steps, human EA.hy926 cells are transduced by MLV-EBOV-GP pseudoviral particles. The pseudovirus entry efficiency is quantified by measuring luciferase activity.

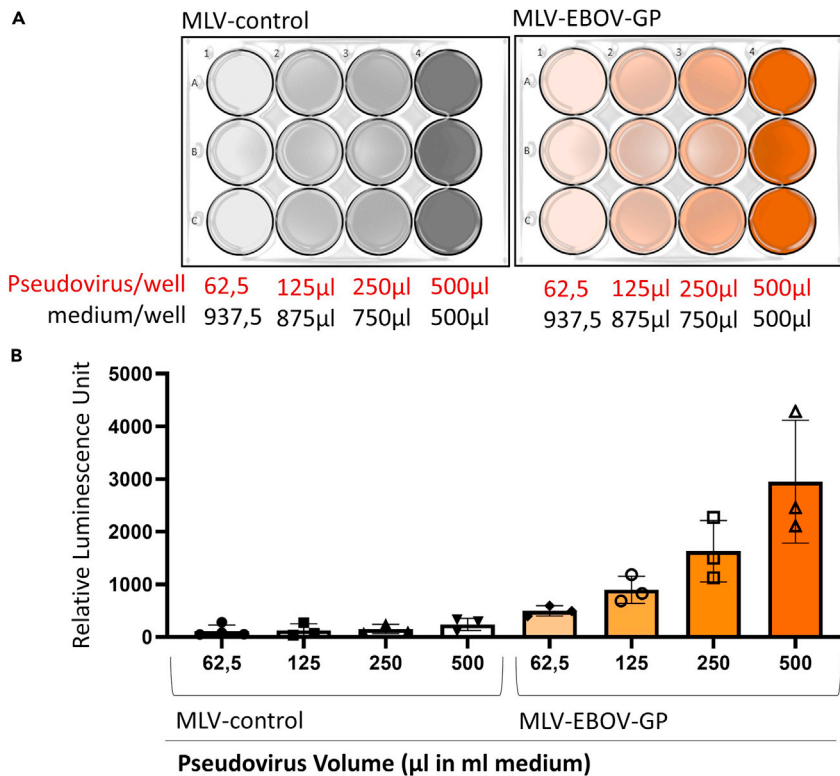
⌚ Timing: 3 days

9. Day 1. Keep EA.hy926 cells in cell culture. In our experiments, we leave cells in cell culture for at least 2 passages before starting the transduction. The cells should grow exponentially and exhibit healthy morphology (Figure 1B). Wash EA.hy926 cells with  $1 \times$  DPBS and detach the cells by employing Trypsin/EDTA (0.05%) typically incubating less than 5 min at  $37^{\circ}\text{C}$ . Resuspend the cells in 10 mL prewarmed fresh complete RPMI1640 medium and count the cells.
10. Seed  $2 \times 10^5$  cells in 1 mL/well in a 12-well plate to reach 70–80% confluency on the next day.
11. Day 2. Thaw MLV-control/MLV-EBOV-GP viral supernatants and prewarm them at  $37^{\circ}\text{C}$  (not longer than 15 min prior to experiment). Completely remove medium on the cells and replace it with 1-1 mL pseudoviral supernatant. Incubate the cells at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ .

**Note:** In our experiments, the optimal titer to infect the cells is the undiluted pseudoviral supernatant.

**Optional:** Prior to first measurement, it is recommended to titrate the pseudoviruses. Make 1:2 serial dilution of the supernatants as shown in Figure 3A. Measure luciferase activity as





**Figure 3. Two-fold serial dilution of MLV-control and MLV-EBOV-GP pseudoviral supernatants**

(A) Dilution scheme on a 12-well plate using three technical replicates. The increasing darkness of the color represents the increasing concentration of the pseudovirus particles in the actual well. MLV-control is depicted with gray and MLV-EBOV-GP is depicted with orange color. The red numbers represent the volume of the pseudovirus supernatants and the black numbers show the volume of complete medium in one well (total volume is 1 mL/well).

(B) The detected light intensity by employing a 1:2 serial dilution of the viral supernatants measured by luminometer. EA.hy926 cells were transduced with indicated volumes of MLV-control and MLV-EBOV-GP pseudoviral supernatants. The samples were harvested at 24 h and luciferase activity was measured as described in the protocol (n=3). The data represent means +/- sd.

described at step 12 and plot a Titer/Relative Luminescence Unit (RLU) curve to determine the most efficient volume for transduction (Figure 3B).

## 12. Day 3. Detection of the luciferase activity

- Thaw the Luciferase Lysis Buffer and prepare sufficient amount of 1× solution by adding ddH<sub>2</sub>O to the concentrate.
- Prepare the Luciferase substrate by dissolving Luciferase Assay Substrate (lyophilized) with Luciferase Assay Buffer. Prepare aliquots to avoid freeze-thaw cycles and store them at -80°C.
- Remove the supernatant of the cells. Wash the cells by using 300 μL 1× DPBS solution and add 200 μL Trypsin/EDTA (0.05%) solution to the cells. Incubate at 37°C for 5 min.

**Note:** Some proportion of the infected cells tend to float, therefore it is recommended to keep the removed supernatant and the 1× DPBS that was used for washing the cells, and pool them together with the trypsinized cells (step d.).

- Stop the reaction by adding 300 μL complete medium onto the cells. Detach the cells by gently pipetting up and down. Collect the cells in 1.5 mL tubes.
- Centrifuge the cells at 200 × g for 4 min at RT and discard supernatant carefully.
- Resuspend the cell pellet with 1 mL 1× DPBS and centrifuge at 200 × g for 4 min.

- g. Carefully discard the supernatant and use a 200  $\mu$ L tip to completely remove the rest of the supernatant without disturbing the cell pellet.
- h. Add 55  $\mu$ L Luciferase Lysis Buffer (1 $\times$ ) to the cell pellet and mix by pipetting up and down few times. Avoid bubbles.
- i. Incubate the lysates for 15 min at RT. During incubation time the following steps should be accomplished:
  - i. Transfer 50  $\mu$ L lysate to 96-well white microtiter plate.
  - ii. Thaw the Luciferase Substrate 10–15 min prior to detection at RT.

**Note:** Colder temperatures can negatively influence luciferase activity therefore make sure that the substrate temperature reaches RT before usage. Protect the Luciferase substrate from light.

- iii. Start the luminometer and setup the detection software. We employ 500 ms integration time.

**Note:** We have tested various integration times. Integration times between 5 ms and 1000 ms showed no significance differences. Short, 1 ms integration time results in bigger variability of the data. Too long integration time can cause a delay in the sample detection of the subsequent samples leading to different incubation intervals.

- j. Add 90  $\mu$ L of Luciferase Substrate to 50  $\mu$ L lysate and mix the samples by carefully swirling the plate.
- k. Start measurement after 20 s incubation period at RT.

**Note:** Alternatively to the use of Luciferase Substrate from Promega, after cell lysis and transfer of the cell lysates into 96-Well microplate (see step 12i.), add 100  $\mu$ L of Firefly Luciferase Assay Buffer (Solution A, see [materials and equipment](#)) and 50  $\mu$ L Luciferin Substrate Solution (Solution B, see [materials and equipment](#)). Measure after 20 seconds incubation at RT with luminometer (see step 12k.).

**Note:** Since the peak intensity of detected light starts to slowly decline typically in 3–4 minutes after adding the substrate, it is recommended to use multichannel pipette for simultaneous sample detection of multiple samples. Alternatively, plate readers equipped with injectors can be employed, which ensure unified time interval between substrate injection and detection.

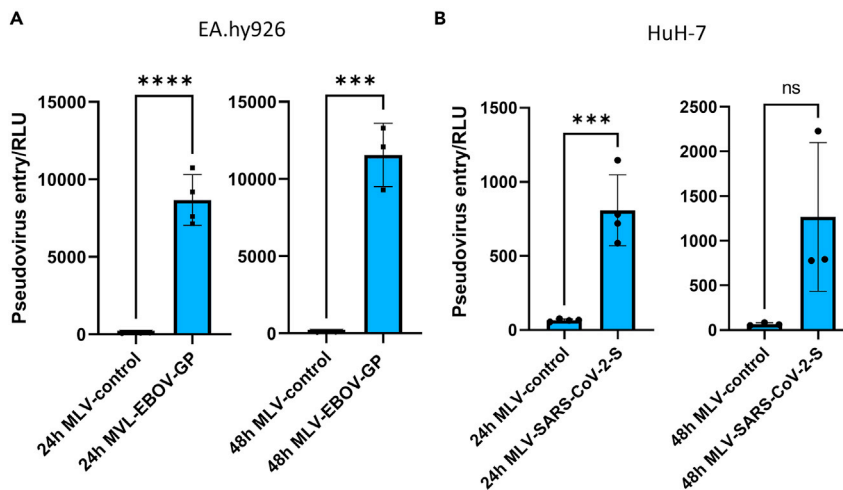
**Note:** In general, longer incubation time than 24 hours results in a minimal increase of the detected luminescence intensity. However, 48 hours incubation leads to higher variability of the results. Therefore, we typically used 24 hours for detection. [Figures 4A](#) and [4B](#) show the comparison between 24 and 48 hours measurements. Others reported that the luciferase activity peaks at 24 h (Ebola-GP) and 48 h (SARS-CoV-2-S) by employing VSV pseudotyped viruses ([Lay Mendoza et al., 2020](#)).

### Transduction of HuH-7 cells with MLV-SARS-CoV-2-S pseudoviruses

HuH-7 cells were selected for transduction experiments because they express angiotensin-converting enzyme 2 (ACE-2), the host binding site of the SARS-CoV-2-Spike protein and therefore these cells are susceptible to infection ([Hoffmann et al., 2020](#)). In these steps, human HuH-7 cells are transduced by MLV-SARS-CoV-2-S pseudoviral particles. The pseudovirus entry efficiency is quantified by measuring luciferase activity at 24 h.

⌚ **Timing: 3 days**

13. Day 1. Keep HuH-7 cells in cell culture for at least 2 passages before starting the experiment. The cells should grow exponentially and exhibit healthy morphology ([Figure 1C](#)). Wash HuH-7 cells



**Figure 4. Detection of luciferase activity on MLV pseudovirus transduced cell lines**

(A) EA.hy926 cells were transduced with 1-1 mL MLV-control and MLV-EBOV-GP pseudoviral supernatants. The samples were harvested after 24 and 48 h (24 h and 48 h) and luciferase activity was measured as described in the protocol. The data represent means  $\pm$  sd. To test significance Student's t-test was performed. \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns-non significant.  $n = 4$  (24 h) and  $n = 3$  (48 h).

(B) HuH-7 cells were transduced with 1-1 mL MLV-control and MLV-SARS-CoV-2-S pseudoviral supernatants. The samples were harvested and luciferase activity was measured at 24 and 48 h as described in the protocol. The data represent means  $\pm$  sd. To test significance Student's t-test was performed. \*\*\* $p < 0.001$ ; ns-non significant.  $n = 4$  (24 h) and  $n = 3$  (48 h).

with  $1 \times$  DPBS and detach the cells by trypsinization typically incubating less than 5 min at  $37^\circ\text{C}$ . Resuspend the cells in 10 mL prewarmed fresh complete DMEM medium.

**Note:** Here we describe the transduction of HuH-7 cells, which are permissive to corona viruses (Freythuth et al., 2005), however other permissive cell lines, such as Caco2 cells can be also transduced by MLV-SARS-CoV-2-S pseudoviruses (Hoffmann et al., 2020).

14. Seed  $2 \times 10^5$  cells/well in a 12-well plate to reach 70–80% confluency on the next day.
15. Day 2. Thaw MLV-control/MLV-SARS-CoV-2-S pseudoviral supernatants and prewarm them at  $37^\circ\text{C}$ . Completely remove medium on the cells and replace it with 1-1 mL pseudoviral supernatant. Incubate the cells at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ .
16. Day 3. Detection of the luciferase activity. The next steps are identical to step 12. Please follow the sub-steps described under step 12.

### EXPECTED OUTCOMES

The detection of luminescence typically results in approximately 2 orders of magnitude increase in relative luminescence unit (RLU) by employing the MLV-EBOV-GP particles in EA.hy926 cells (Figure 4A) and 1-2 orders of magnitude increase by employing the MLV-SARS-CoV-2-S particles in HuH-7 cells (Figure 4B).

### QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments have been repeated at least three times. To test statistical significance of the results Student's t test (unpaired, two tailed) was applied. The significance is indicated as \*\*\* $p < 0.001$  or ns-non significant.

### LIMITATIONS

The produced MLV pseudoviruses are replication-incompetent. Thus, this method is not suitable for studies aiming at investigating the processes of de novo virus production, viral replication and viral

budding. In addition, in combination application of chemical compounds that result in additional light emission/light absorption might interfere with luciferase activity measurement leading to false positive/negative results. For instance, employing the fluorescent compound Doxorubicin increases the detected light intensity (Calvert and Vohra, 2013) and the application of carbon nanotubes may lead to reduced measured light intensity in luminescence detection assays (Szymanski et al., 2020).

### TROUBLESHOOTING

#### Problem 1

No virus production due to poor plasmid yield.

If the luciferase measurement of the transfected HEK293T cells exhibited no sufficient intensity increase (see step 8), it can indicate low yield of plasmids.

#### Potential solution

Make sure that the amount of the plasmids is sufficient by measuring the concentration of the DNA. If necessary, prepare new plasmid stocks ("before you begin" steps 5–9). Check, if the plasmids harbor the correct, not mutated, inserts by employing gene sequencing.

#### Problem 2

No virus production due to poor transfection efficiency

If the luciferase measurement of the transfected HEK293T cells exhibited no sufficient intensity increase (see step 8), and the plasmid concentration is sufficient, it might indicate poor transfection efficiency of the plasmids.

#### Potential solution

- Make sure that HEK293T cells exhibit healthy morphology (Figure 1A) and reach 50–60% confluency at the time of transfection. If the cells exceed 20 passages, thaw new cells. Mycoplasma infection can substantially influence transfection efficiency. Make sure that the cells are mycoplasma free.
- Transfection reagent is expired or was stored at room temperature for longer period of time (>24 h). Use/prepare fresh transfection reagent.

#### Problem 3

Pseudovirus loss during filtration.

The Luminescence intensity is low in pseudovirus transduced cells due to not sufficient pseudovirus concentration. If the luciferase measurement of the transfected HEK293T cells exhibited sufficient intensity increase (see step 8), it indicates efficient transfection of the plasmids. Therefore, the problem might have occurred in the subsequent (steps 9–12) steps, possibly at the pseudovirus filtration.

#### Potential solution

Make sure that you use filter units with 0.45  $\mu\text{m}$  pores. Based on our experiments, application of 0.22  $\mu\text{m}$  filters might also work, however they result in significantly less yields. In addition, check the manufacturer's site for the specification of the filters. The application of filters with reduced binding capacity is required to avoid the attachment of viral particle to the filters. Furthermore, increased mechanical stress by intense up and down pipetting may also lead to destruction of the pseudovirus particles.

#### Problem 4

Too many freeze-thaw cycles of the pseudoviral supernatants.

In addition, repeated freeze-thaw cycles can result in loss of transduction efficiency of the pseudoviruses.

#### **Potential solution**

Please make sure to prepare aliquots as suggested at step 7. Try to avoid repeated freeze-thaw cycles. Repeated freeze-thaw cycles can strongly influence pseudovirus infectivity. Store the viral supernatants at  $-80^{\circ}\text{C}$ . The best infectivity is achieved, if the pseudoviruses used immediately after filtration without freezing.

#### **Problem 5**

Luminescence intensity is low

The luminescence intensity is low in pseudovirus infected cell lysates, however western blot analysis of the transduced lysates detects high amount of luciferase. This indicates that the pseudovirus transduction of the cells worked efficiently and the cells produced Luciferase protein. The problem might have occurred at step 12. Possible causes: Luciferase substrate does not work (a), trypsin does not work (b) or cells were lost during centrifugation steps (c).

#### **Potential solution**

To resolve problem (a), prepare small aliquots of the dissolved substrate and store them at  $-80^{\circ}\text{C}$ . Try to avoid repeated freeze-thaw cycles. Check the pH of the buffer solutions. The optimal pH is 7.8. Protect substrates from light.

To resolve problem (b), after 5 min incubation with Trypsin/EDTA (step 12c) check if the cells are completely detached by employing a cell culture microscope. If not, use fresh, prewarmed Trypsin/EDTA solution to detach the cells.

To resolve problem (c), check at steps 12e and f, if you see visible pellet after centrifugation. Make sure that you don't lose pellet during discarding the supernatants.

### **RESOURCE AVAILABILITY**

#### **Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gergely Imre ([gimre80@gmail.com](mailto:gimre80@gmail.com)).

#### **Materials availability**

This study did not generate new unique reagents.

#### **Data and code availability**

The published article includes all data generated or analyzed during this study.

### **ACKNOWLEDGMENTS**

We thank Professor Dr. Stefan Pöhlmann (Infection Biology Unit, German Primate Center - Leibniz Institute for Primate Research, Faculty of Biology and Psychology, University Göttingen) for the plasmids pCMV-Luc and pCMV-MLV-gag-pol. The project is supported by the Goethe Corona Fonds Frankfurt to J.P. (80301283) and German research foundation (DFG, SFB1039 to J.P., Projekt ID:204083920).

### **AUTHOR CONTRIBUTIONS**

G.I. adapted and modified the protocol from the original. M.E. and G.I. performed most of the experiments and wrote the protocol with the input of all authors. E.A. performed supporting experiments. J.P. acquired funding and resources and provided valuable discussion of the results.

## DECLARATION OF INTERESTS

The authors declare no competing interest.

## REFERENCES

- Bartosch, B., Dubuisson, J., and Cosset, F.L. (2003). Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J. Exp. Med.* *197*, 633–642. <https://doi.org/10.1084/jem.20021756>.
- Calvert, R.J., and Vohra, S. (2013). Doxorubicin-treated H9c2 cells: caution with luminescent ATP and Hoechst 33258 assays. *In Vitro Cell. Dev. Biol. Anim.* *49*, 95–96. <https://doi.org/10.1007/s11626-012-9573-1>.
- Chen, Y.-N., Hsu, H.-C., Wang, S.-W., Lien, H.-C., Lu, H.-T., and Peng, S.-K. (2019). Entry of scotophilus bat coronavirus-512 and severe acute respiratory syndrome coronavirus in human and multiple animal cells. *Pathogens* *8*, 259. <https://doi.org/10.3390/pathogens8040259>.
- Cosset, F.L., Marianneau, P., Verney, G., Gallais, F., Tordo, N., Pécheur, E.I., ter Meulen, J., Deubel, V., and Bartosch, B. (2009). Characterization of Lassa virus cell entry and neutralization with Lassa virus pseudoparticles. *J. Virol.* *83*, 3228–3237. <https://doi.org/10.1128/JVI.01711-08>.
- Edgell, C.J., McDonald, C.C., and Graham, J.B. (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. U S A* *80*, 3734–3737.
- Freymuth, F., Vabret, A., Rozenberg, F., Dina, J., Petitjean, J., Gouarin, S., Legrand, L., Corbet, S., Brouard, J., and Lebon, P. (2005). Replication of respiratory viruses, particularly influenza virus, rhinovirus, and coronavirus in HuH7 hepatocarcinoma cell line. *J. Med. Virol.* *77*, 295–301. <https://doi.org/10.1002/jmv.20449>.
- Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T.S., Herrler, G., Wu, N.H., Nitsche, A., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* *181*, 271–280.e8. <https://doi.org/10.1016/j.cell.2020.02.052>.
- Imre, G., Krähling, V., Eichler, M., Trautmann, S., Ferreirós, N., Aman, M.J., Kashanchi, F., Rajalingam, K., Pöhlmann, S., Becker, S., et al. (2021). The sphingosine kinase 1 activator, K6PC-5, attenuates Ebola virus infection. *iScience* *24*, 102266. <https://doi.org/10.1016/j.isci.2021.102266>.
- Lay Mendoza, M.F., Acciani, M.D., Levit, C.N., Santa Maria, C., and Brindley, M.A. (2020). Monitoring viral entry in real-time using a luciferase recombinant vesicular stomatitis virus producing SARS-CoV-2, EBOV, LASV, CHIKV, and VSV glycoproteins. *Viruses* *12*, 1457. <https://doi.org/10.3390/v12121457>.
- Millet, J.K., and Whittaker, G.R. (2016). Murine leukemia virus (MLV)-based coronavirus spike-pseudotyped particle production and infection. *Bio Protoc.* *6*, e2035. <https://doi.org/10.21769/BioProtoc.2035>.
- Millet, J.K., Tang, T., Nathan, L., Jaimes, J.A., Hsu, H.L., Daniel, S., and Whittaker, G.R. (2019). Production of pseudotyped particles to study highly pathogenic coronaviruses in a biosafety level 2 setting. *J. Vis. Exp.* *145*. <https://doi.org/10.3791/59010>.
- Pleet, M.L., Mathiesen, A., DeMarino, C., Akpamagbo, Y.A., Barclay, R.A., Schwab, A., Iordanskiy, S., Sampey, G.C., Lepene, B., Nekhai, S., et al. (2016). Ebola VP40 in exosomes can cause immune cell dysfunction. *Front. Microbiol.* *7*, 1765. <https://doi.org/10.3389/fmicb.2016.01765>.
- Ratnapriya, S., Chov, A., and Herschhorn, A. (2020). A protocol for studying HIV-1 envelope glycoprotein function. *STAR Protoc.* *1*, 100133. <https://doi.org/10.1016/j.xpro.2020.100133>.
- Shang, J., Ye, G., Shi, K., Wan, Y., Luo, C., Aihara, H., Geng, Q., Auerbach, A., and Li, F. (2020). Structural basis of receptor recognition by SARS-CoV-2. *Nature* *581*, 221–224. <https://doi.org/10.1038/s41586-020-2179-y>.
- Szymański, T., Kempa, M., Giersig, M., and Dalibor Rybka, J. (2020). Carbon nanotubes interference with luminescence-based assays. *Materials* *13*, 4270. <https://doi.org/10.3390/ma13194270>.
- Temperton, N.J., Hoschler, K., Major, D., Nicolson, C., Manvell, R., Hien, V.M., Ha, d., de Jong, M., Zambon, M., Takeuchi, Y., and Weiss, R.A. (2007). A sensitive retroviral pseudotype assay for influenza H5N1-neutralizing antibodies. *Influenza Other Respir. Viruses* *1*, 105–112. <https://doi.org/10.1111/j.1750-2659.2007.00016.x>.