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Pseudotyping lentiviral particles with SARS-CoV-2 Spike protein for neutralization assays V.2

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Coronavirus Method Development Community

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ABSTRACT

SARS-CoV-2 enters cells using its Spike protein, which is also the main target of neutralizing antibodies. Therefore, assays to measure how antibodies and sera affect Spike-mediated viral infection are important for studying immunity. Because SARS-CoV-2 is a biosafety-level-3 virus, one way to simplify such assays is to pseudotype biosafety-level-2 viral particles with Spike. Such pseudotyping has now been described for single-cycle lentiviral, retroviral and VSV particles, but the reagents and protocols are not widely available. Here we detail how to effectively pseudotype lentiviral particles with SARS-CoV-2 Spike and infect 293T cells engineered to express the SARS-CoV-2 receptor, ACE2. We also make all the key experimental reagents available in the BEI Resources repository of ATCC and the NIH. Furthermore, we demonstrate how these pseudotyped lentiviral particles can be used to measure the neutralizing activity of human sera or plasma against SARS-CoV-2 in convenient luciferase-based assays, thereby providing a valuable complement to ELISA-based methods that measure antibody binding rather than neutralization.

EXTERNAL LINK

<https://www.mdpi.com/1999-4915/12/5/513>

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ATTACHMENTS

Protocol and reagents for
pseudotyping lentiviral
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KEYWORDS

SARS-CoV-2, COVID-19, coronavirus, neutralization assay, lentiviral pseudotype, Spike, cytoplasmic tail, ACE2, 293T-ACE2, luciferase, ALAYT

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GUIDELINES

Plasmids

The sequences of all plasmids used in this study are available in Genbank format in File S1 and are also at https://github.com/jbloomlab/SARS-CoV-2_lentiviral_pseudotype/tree/master/plasmid_maps. The plasmids themselves are available in BEI Resources (<https://www.beiresources.org/>) with the following catalog numbers:

- **pHAGE2-EF1aInt-ACE2-WT (BEI catalog number NR52512)**: lentiviral backbone plasmid expressing the human ACE2 gene (GenBank ID for human ACE2 is NM_021804) under an EF1a promoter with an intron to increase expression.
- **HDM-IDTSpike-fixK-HA-tail (BEI catalog number NR52513)**: plasmid expressing under a CMV promoter the Spike from SARS-CoV-2 strain Wuhan-Hu-1 (Genbank NC_045512) codon-optimized using IDT, with the Spike cytoplasmic tail replaced by that from the HA protein of A/WSN/1933 (H1N1) influenza, and the Kozak sequence in the plasmid fixed compared to an earlier version of this plasmid.
- **HDM-IDTSpike-fixK (BEI catalog number NR-52514)**: plasmid expressing under a CMV promoter the Spike from SARS-CoV-2 strain Wuhan-Hu-1 (Genbank NC_045512) codon-optimized using IDT and the Kozak sequence in the plasmid fixed compared to an earlier version of this plasmid.
- **HDM-nCoV-Spike-IDTopt-ALAYT (BEI catalog number NR-52515)**: plasmid expressing under a CMV promoter the Spike from SARS-CoV-2 strain Wuhan-Hu-1 (Genbank NC_045512) codon-optimized using IDT, with the Spike containing two mutations in the cytoplasmic tail such that the last five amino acids are ALAYT.
- **pHAGE-CMV-Luc2-IRES-ZsGreen-W (BEI catalog number NR-52516)**: lentiviral backbone plasmid that uses a CMV promoter to express luciferase followed by an IRES and ZsGreen.
- **HDM-Hgpm2 (BEI catalog number NR-52517)**: lentiviral helper plasmid expressing HIV Gag-Pol under a CMV promoter.
- **HDM-tat1b (NR-52518)**: lentiviral helper plasmid expressing HIV Tat under a CMV promoter.
- **pRC-CMV-Rev1b (NR-52519)**: lentiviral helper plasmid expressing HIV Rev under a CMV promoter.
- **pHAGE2-CMV-ZsGreen-W (NR-52520)**: lentiviral backbone plasmid that uses a CMV promoter to express ZsGreen

**Note that all of these plasmids have ampicillin resistance. The only plasmid used in this study that is not in the BEI Resources catalog is the HDM-VSVG plasmid that expresses VSV G under a CMV promoter, and was used 8 of 15 to create the positive control lentivirus pseudotyped with VSV G. However, numerous VSV G expressing plasmids are available from AddGene and other repositories.

We now recommend using a Spike plasmid with a cytoplasmic tail truncation and a D614G mutation. This plasmid is available on AddGene as plasmid 158762 (<https://www.addgene.org/158762/>).

Creation of 293T-ACE2 Cells

VSV G-pseudotyped lentivirus packaging the human ACE2 can be generated via co-transfecting 293T cells (ATCC, CRL-3216) with the pHAGE2-EF1aInt-ACE2-WT plasmid (File S1) and lentiviral helper plasmids (HDM-VSVG, HDM-Hgpm2, HDM-tat1b, and pRC-CMV-Rev1b). The resulting lentivirus should be used to infect more 293T cells in the presence of 5 ug/mL polybrene. Stain the transduced cells with anti-human ACE-2 polyclonal goat IgG (AF933, R&D Systems) primary antibody at 1 ug/mL and donkey anti-goat IgG conjugated to Alexa Fluor 488 (ab150129, Abcam) secondary antibody at a 1:2500 dilution and sort based on antibody staining. Once single cell clones have grown sufficiently, they can be screened for ACE2 expression via flow cytometry and a clone with high expression should be expanded. For verifying expression via flow cytometry, cells should be harvested with enzyme-free dissociation buffer (ThermoFisher, 13151014) and stained with anti-human ACE-2 polyclonal goat IgG primary antibody at 2 ug/mL and donkey anti-goat IgG (Alexa Fluor 488) secondary antibody at a 1:1000 dilution. For each staining step, cells should be incubated with antibody in the dark at 4°C for 30 min. Cells should be washed 3 times with 3% BSA in PBS following each stain.

The 293T-ACE2 cells can be grown in D10 growth media (DMEM with 10% heat-inactivated FBS, 2 mM Lglutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin) at 37°C and 5% carbon dioxide. Note that there is not a selectable marker for the ACE2 expression. The 293T-ACE2 cells are available from BEI Resources as catalog number **NR-52511**.

Human plasma sample and soluble ACE2

Human plasma samples used for neutralization assays can be collected at 19 days post-symptom onset from a patient with a confirmed SARS-CoV-2 infection. Prior to use, the plasma should be heat-inactivated in a biosafety cabinet at 56°C for one hour. This duration of heat treatment has been shown to be sufficient to inactivate SARS-CoV-2 (see citations below: Amanat et al., Chin et al.), which is also not reported to be present at high titers in the blood (see citations below: Wang et al., Dodd et al.). The negative control serum pools came from Gemini Biosciences (Cat:100-110). The naïve serum pool collected in 2017-2018 is lot H86W03J. The age-matched negative control serum comes from serum residuals collected by Bloodworks Northwest. It was collected on 12/19/1989 and stored at -80°C.

Soluble human ACE2 protein fused to the Fc region of human IgG was produced as described in (see citation below: Walls et al.).

Amanat, F.; Nguyen, T.; Chromikova, V.; Strohmeier, S.; Stadlbauer, D.; Javier, A.; Jiang, K.; Asthagiri-Arunkumar, G.; Polanco, J.; Bermudez-Gonzalez, M.; et al. (2020). A serological assay to detect SARS-CoV-2 seroconversion in humans.. MedRxiv.
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Wang, W.; Xu, Y.; Gao, R.; Lu, R.; Han, K.; Wu, G.; Tan, W. (2020).
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<http://10.1001/jama.2020.3786>.

Dodd R.Y., Stramer S.L. (2020). COVID-19 and Blood Safety: Help
with a Dilemma.. Transfus Med Rev..
<http://10.1016/j.tmr.2020.02.004>.

Walls, A.C.; Park, Y.J.; Tortorici, M.A.; Wall, A.; McGuire, A.T.; Veesler,
D. (2020). Structure, Function, and Antigenicity of the SARS-CoV-2
Spike Glycoprotein. Cell.
<http://10.1016/j.cell.2020.02.058>

MATERIALS TEXT

MATERIALS

[FBS](#) **Invitrogen - Thermo Fisher**

[DMEM](#) **Invitrogen - Thermo**

Fisher Catalog #11885

[Penicillin-Streptomycin](#) **Sigma**

Aldrich Catalog #P4333

[L-Glutamine](#) **Sigma**

Aldrich Catalog #G8540-25G

[BioT \(1 ml\)](#) **Bioland**

Scientific Catalog #B01-01

[Poly-L-lysine solution](#) **Sigma**

Aldrich Catalog #P4707

[Polybrene Infection / Transfection Reagent](#) **Sigma**

Aldrich Catalog #TR-1003

[Bright-Glo Luciferase Assay](#)

System Promega Catalog #E2610

[Corning 96-Well Solid Black or White Polystyrene Microplates](#) **Fisher**

Scientific Catalog #07-200-590

[HEK 293T-ACE2 cells](#) **BEI**

Resources Catalog #NR-52511

D10 growth media

DMEM with 10% heat-inactivated FBS

2 mM L-glutamine

100 U/ml penicillin
100 ug/ml streptomycin

Plasmids Required

The sequences of all plasmids used in this study are available in Genbank format in File S1 and are also at https://github.com/jbloomlab/SARS-CoV-2_lentiviral_pseudotype/tree/master/plasmid_maps. The plasmids themselves are available in BEI Resources (<https://www.beiresources.org/>) with the following catalog numbers:

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 - **HDM-nCoV-Spike-IDTopt-ALAYT (BEI catalog number NR-52515)**: plasmid expressing under a CMV promoter the Spike from SARS-CoV-2 strain Wuhan-Hu-1 (Genbank NC_045512) codon-optimized using IDT, with the Spike containing two mutations in the cytoplasmic tail such that the last five amino acids are ALAYT.
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We now recommend using a Spike plasmid with a cytoplasmic tail truncation and a D614G mutation. This plasmid is available on AddGene as plasmid 158762 (<https://www.addgene.org/158762/>).

Machines Used

Becton Dickinson Celesta cell analysis machine with a 530/30 filter
Tecan Infinite M1000 Pro plate reader

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

Sera or plasma should be heat-inactivated in a biosafety cabinet prior to use.

BEFORE STARTING

Purchase 293T-ACE2 cells or generate them using the methods listed in Guidelines.

Prepare **D10 growth media**:

DMEM with 10% heat-inactivated FBS
2 mM L-glutamine
100 U/ml penicillin
100 ug/ml streptomycin

Generation of Pseudotyped Lentiviral Particles

1 

Seed 293T cell in **D10 Growth Media** so they will be 50-70% confluent the next day.

For a 6-well plate, this is 5×10^6 cells per well (2.5×10^6 cells per mL).

2

At 16-24 hours after seeding, transfect the cells with the plasmids required for lentiviral production.

This protocol transfects using BioT following the manufacturer's instructions and using the following plasmid mix per well of a 6-well plate:

- **1 µg lentiviral backbone** (ZsGreen or Luciferase-IRES-ZsGreen backbone)
- **0.22 µg plasmid HMD-Hgpm2**, **0.22 µg plasmid pRC-CMV-Rev1b**, and **0.22 µg plasmid HDM-tat1b**
- **0.34 µg viral entry protein**: either SARS-CoV-2 Spike (NR-52513, NR-52514, NR-52515, NR-53742, or NR-53765), VSV G (positive control), or transfection carrier DNA (Promega E4881) as a negative control.

Plasmid amounts should be adjusted for larger plates.

We now recommend making virus with the spike plasmid with a 21 amino acid cytoplasmic tail deletion and D614G mutation. This is plasmid NR-53765 at BEI or plasmid 158762 at AddGene.

3 At 18 to 24 hours post-transfection, change the media to **fresh, pre-warmed D10**.

4

At 60 hours post transfection, collect virus by harvesting the supernatant from each well and filtering it through a **0.45 µm SFCA low protein-binding filter**.

Virus can be stored at **4 °C** for immediate use or frozen at **-80 °C**.

The titers of Spike- and VSV G-pseudotyped lentiviruses were found to be unaffected by a freeze-thaw cycle (data not shown).

Titration of Pseudotyped Lentiviral Particles

5

To determine viral titers, use either flow cytometry (for viruses packaging the ZsGreen backbone) or a luciferase assay (for viruses packaging the Luciferase-IRES-ZsGreen backbone).

Coat a 96-well cell-culture plate with **25 µl poly-L-lysine** per well according to manufacturer's protocol (Millipore Sigma, P4707).

Poly-L-lysine improves cell adherence and prevents cell disruption during infection.

The assay should work without poly-L-lysine coated plates, but we think the poly-L-lysine coating should help ensure cell numbers are consistent between wells.

6

Seed a poly-L-lysine-coated 96-well plate with **1.25x10⁴ 293T-ACE2 cells** (BEI NR-52511) per well in **D10 media**.

We usually seed each well with **50 µl** of cells at a concentration of 2.5x10⁵/mL.

For the luciferase readout, we recommend seeding in a black-walled, clear-bottom, poly-L-lysine coated plate.

We use plates pre-coated with poly-L-lysine (Greiner Bio-One 655936) or non-coated plates (Greiner Bio-One 655090) that we then coat ourselves as described above.

7 The next day (12-24 hours post-seeding), examine cells. They should be fairly sparse (~15-20% confluency).

If you will be titrating via flow cytometry, count at least 2 wells of cells to determine the number of cells present at infection.

8 Prepare serial dilutions of the viruses to be titrated in D10 growth media. The final infection volume will be **150 µl D10 Growth Media**.

We usually titer by adding **100 µl of virus** to the **50 µl of D10 growth media** already in the wells from seeding the cells. The suggested dilutions below refer to the **final** dilution, taking into account the volume of media already in the wells. Note a 1:3 final dilution is only a 1:2 dilution in the **100 µl of virus** actually added to the wells.

The below dilutions are recommendations. Any similar dilution series would work.


We also recommend titrating "bald" virus particles with no viral entry protein as a negative control to get the best sense of background titers. Dilute these negative control viruses in the same way you dilute your spike-pseudotyped viruses.

8.1 For **ZsGreen** virus: start with a 1:3 dilution and make 4 serial 1:5 dilutions.

- 8.2 For **Spike-pseudotyped Luciferase_IRES_ZsGreen** virus: start with undiluted virus and make five serial 1:3 dilutions.

We now recommend using a Spike plasmid with a cytoplasmic tail truncation and the D614G mutation. For this virus, you want to start with a 1:5 or 1:10 dilution and then do five serial 1:2 to 1:3 dilutions.

- 8.3 For **VSV G-pseudotyped Luciferase_IRES_ZsGreen** virus: start with a 1:50 dilution.

- 9 Slowly add the virus dilutions to the cells. We add  **100 µl of the virus dilutions** to the **50 µl of D10 growth media** already in the wells from seeding cells.

In the first version of this protocol, we also added polybrene to a final concentration of 5 ug/mL. However, we determined this only has a very modest effect on titer and no longer add polybrene.

- 10 At 48-72 hours post-infection, collect cells for analysis:
Step 10 includes a Step case.

Flow Cytometry assay
Luciferase assay

step case

Flow Cytometry assay

Perform the following steps:

- 11 

Look at the cells under a fluorescent microscope and select wells that appear ~1-10% positive.

- 12 

Harvest cells from these wells using trypsin and transfer them to a V-bottom plate or microcentrifuge tubes.

- 13 

Pellet cells at 300xg for 4 min and wash twice with 3% BSA in PBS.

- 14 

After the final wash, resuspend in 1% BSA in PBS and analyze via flow cytometry. A Becton Dickinson Celesta cell analysis machine with a 530/30 filter was used to detect ZsGreen in the FITC channel. Resulting FCS files were

analyzed using FlowJo (v10).

15

Calculate titers using the Poisson formula. If P is the percentage of cells that are ZsGreen positive, then the titer per mL is:

$$-\ln(1 - P / 100) \times (\text{number of cells / well}) / (\text{volume of virus per well in mL})$$

Note that when the percentage of cells that are ZsGreen positive is low, this formula is approximately equal to: (% ZsGreen positive / 100) x (number of cells / well) / volume of virus per well in mL).

Furthermore, the titers using even the Poisson equation will only be accurate if the percentage of cells that are ZsGreen positive is relatively low (ideally 1-10%).

Neutralization Assay

5d

16

Seed a poly-L-lysine-coated 96-well plate with **1.25x10⁴ 293T-ACE2 cells** (BEI NR-52511) per well in **50 µl DMEM** (2.5x10⁵ cells per mL).

Plan to infect this plate 8–20 hours post-seeding. Essentially, cells can be seeded overnight or seeded first thing in the morning for infection that evening.

If you are reading out the assay using luciferase, we recommend using black-walled, clear bottom plates.

17 Approximately 1.5 hours prior to infecting cells, begin preparing serum and/or ACE2 dilutions in **D10**:

Example layouts are included below.


Note that recently we have only been including the NoVEP virus and infecting into 293Ts (without ACE2) when titrating virus. These are good controls to make sure your virus prep is good, but are probably not necessary for each neutralization assay plate (and add a fair amount of work per plate).

| Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus + 293Ts | Virus + 293Ts | Virus + 293Ts | Virus + 293Ts |
|------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|---------------|---------------|---------------|---------------|
| Cells Only | Virus+Cells | Serum 1 – A | Serum 1 – B | Serum 2 – A | Serum 2 – B | Serum 3 – A | Serum 3 – B | Serum 4 – A | Serum 4 – B | Virus+Cells | Cells Only |
| Cells Only | | | | | | | | | | | Cells Only |
| Cells Only | | | | | | | | | | | NoVEP (60 uL) |
| Cells Only | | | | | | | | | | | NoVEP (60 uL) |
| Cells Only | | | | | | | | | | | NoVEP (60 uL) |
| Cells Only | | | | | | | | | | | Cells Only |
| Cells Only | | | | | | | | | | | Cells Only |

Example layout for a neutralization assay with 4 samples and 7 dilutions per sample.

| | | | | | | | | | | | |
|-------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only | Virus Only |
| Virus+Cells | | | | | | | | | | | |
| Serum 2 – B | | | | | | | | | | | |
| Serum 2 – A | | | | | | | | | | | |
| Serum 1 – B | | | | | | | | | | | |
| Serum 1 – A | | | | | | | | | | | |
| Virus+Cells | | | | | | | | | | | |
| Cells Only | Cells Only | Cells Only | Cells Only | Cells Only | Cells Only | Cells Only | Cells Only | Cells Only | Cells Only | Cells Only | Cells Only |

Example layout for a neutralization assay with 2 samples and 12 dilutions per sample.

17.1 In a 96-well “setup” plate (separate from the plate containing cells), serially dilute serum samples leaving  **60 µl diluted serum** in each well. Example layouts for setup plates are above.

17.2 Add  **60 µl D10** to wells corresponding to virus only and virus plus cells control wells.

As seen in the above layouts, we recommend having two rows (if diluting samples horizontally across the plate) or columns (if diluting samples vertically down the plate) for the virus plus cells positive controls.

Running the positive controls in duplicate allows you to normalize RLU or fluorescence readings for each experimental sample by the average of the two positive control wells in the same row or column.

17.3 Add  **120 µl D10** to media only and cells only control wells.

18

Dilute virus to $\sim 2-4 \times 10^6$ RLU per mL. Add  **60 µl diluted virus** to all wells containing serum dilutions and the virus only and virus plus cells control wells.

After further optimization, you want to add enough virus such that each well without serum has a reading of $\sim 200,000-800,000$ RLUs.

However, we have also found RLUs can vary between plate readers, so the important thing is to have a luciferase reading well above background that is consistent between wells. You may need to optimize this amount for your assay and reagents.

19

Incubate the virus and serum at **37 °C** for **01:00:00**.

20

Carefully add **100 µl** from each well of the setup plate containing the sera and virus dilutions to the corresponding wells of the plate of 293T-ACE2 cells.

In the first version of this protocol, we also added polybrene to a final concentration of 5 ug/mL. However, we determined this only has a very modest effect on titer and no longer add polybrene.

21

5d

Incubate at **37 °C** for **48:00:00** to **72:00:00** before reading out luminescence or fluorescence as described in the previous section.

For luciferase neutralization assays, remember to add the luciferase such that it is added to all wells with the same dilution and the 2 positive control wells in that same row or column at the same time.

Cells should be completely confluent (but still adherent and not overgrown) when the assay is readout. This helps ensure that cell numbers are generally equal between wells.

22

Plot the data.

"This protocol first subtracted out the background signal (average of the "virus only" and "virus + 293Ts" wells) and then calculated the "maximum infectivity" for each plate as the average signal from the wells without serum ("virus + cells" wells). Then "fraction infectivity" was calculated for each well, by dividing the luciferase reading from each well by the "maximum infectivity" for that plate. For the curves shown in the associated manuscript, the fraction infectivity data was then plotted using the neutcurve Python package (<https://jbloomlab.github.io/neutcurve/>). This package fits a three-parameter Hill curve, with the top baseline being a free parameter and bottom baseline fixed to zero."

We now calculate the fraction infectivities per row or per column of the plate (depending on plate layout). We do this by dividing the RLU reading for each experimental well (containing plasma or antibody) by the average of the RLU readings for the two virus plus cells positive control wells in the same row or column as the experimental well.

