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Protocol and reagents for pseudotyping lentiviral particles with SARS-CoV-2 Spike protein for neutralization assays

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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 Spike on biosafety-level-2 virions. Such pseudotyping has now been described for single-cycle lentiviral, retroviral and VSV virions—but the reagents and protocols are not widely available. Here we detail how to effectively pseudotype lentiviral virions with SARS-CoV-2 Spike, and then infect 293T cells engineered to express Spike’s receptor, ACE2. We also make all the experimental reagents available in the BEI Resources repository of ATCC and the NIH. Furthermore, we demonstrate how these pseudotyped virions can be used to measure the neutralizing activity of human sera 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.

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

1. Introduction

Infection with SARS-CoV-2 elicits antibodies that bind to the virus [1–6]. But as is the case for all viruses [7–10], only some of these antibodies neutralize the virus’s ability to enter cells [5,6,11,12]. While studies of immunity to SARS-CoV-2 are limited, for many other viruses neutralizing antibodies are more strongly correlated with protection against re-infection or disease than antibodies that bind but do not neutralize [7–10,13,14]. Indeed, for other coronaviruses, neutralizing antibodies are associated with at least some reduced susceptibility to re-infection or disease [15–18]—and anecdotal reports suggest that passive transfer of neutralizing antibodies to sick patients may help alleviate disease from SARS-CoV-2 and its close relative SARS-CoV [19–21].

But while there are now well-characterized and high-throughput methods (such as ELISA assays) to measure total antibody binding to SARS-CoV-2 or some of its key constituent proteins [1,3,22], quantifying neutralizing antibody activity is more difficult. Probably the most relevant method is to directly assay how antibodies or sera inhibit infection of cells by SARS-CoV-2 in the lab. Such live-virus assays have now been performed to quantify neutralizing activity in the sera of infected patients or characterize the potency of individual antibodies [2,12,23]. However, the throughput and accessibility of live-virus neutralization assays with SARS-CoV-2 is limited by the fact that the virus is a biosafety-level-3 agent that must be worked with in specialized facilities.

An alternative approach that alleviates these biosafety limitations leverages the fact that all known neutralizing antibodies to SARS-CoV-2 (and other coronaviruses that lack a HE protein) target the virus’s Spike protein [2,12,23]. Spike is the main protein on the surface of SARS-CoV-2, and is necessary and sufficient to enable the virus to bind and enter cells [24]. Spike from SARS-CoV-2 and other coronaviruses can be “pseudotyped” onto safer non-replicative virions in place of their endogenous entry protein, thereby making entry of these virions into cells dependent on Spike [25–31][32]. For SARS-CoV-2, such pseudotyping has recently been reported using HIV-based lentiviral virions [5,23,33], MLV-based retroviral virions [12,34], and VSV [25,35–37]. In the data reported to date, results from such pseudovirus neutralization assays correlate well with measurements made using live SARS-CoV-2 [2,12,23,35]. However, the detailed protocols and reagents to perform such assays are not yet widely available to the scientific community.

Here we fill this gap by providing a detailed description of how to pseudotype lentiviral virions with Spike. We explain how these pseudotyped virions can be used to conveniently measure Spike-mediated cell entry via fluorescent or luciferase reporters, and to quantify the neutralizing activity of human sera. Finally, we describe all the necessary experimental reagents in detail and make them available in the BEI Resources reagent repository.

2. Results

2.1. General approach for pseudotyping lentiviral particles with SARS-CoV-2 Spike.

Our basic strategy for pseudotyping lentiviral virions is shown in **Figure 1A**. It involves transfecting 293T cells with a lentiviral backbone plasmid encoding a fluorescent or luminescent protein, a plasmid expressing Spike, and plasmids expressing the other lentiviral proteins necessary to assemble virions. The transfected cells then produce Spike-pseudotyped lentiviral virions that can be used to infect permissive cells that express Spike’s receptor protein, ACE2 [CITE].

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**Figure 1.** General approach for lentiviral pseudotyping. (**A**) 293T cells are transfected with a plasmid encoding a lentiviral backbone (genome) expressing a marker protein, a plasmid expressing Spike, and plasmids expressing the other HIV proteins needed for virion formation (Tat, Gag-Pol, and Rev). The transfected cells produce virions with Spike on their surface. These virions can infect cells that express the ACE2 receptor for Spike. (**B**) We used three variants of Spike: the Spike protein sequence from SARS-CoV-2 strain Wuhan-Hu-1, a variant containing mutations K1269A and H1271A in the cytoplasmic tail (such that the C-terminal five amino acids are ALAYT), and a variant in which the cytoplasmic tail of Spike has been replaced with that from influenza hemagglutinin (HA).

We used a lentiviral system based on HIV strain X in which the backbone plasmid still depends on a Tat-driven LTR promoter (as for second-generation lentiviruses), but for which the HIV proteins needed to drive virion assembly are split across multiple plasmids (as for third-generation lentiviruses). Although we have not performed detailed comparisons with other lentiviral systems, anecdotally this system may support more efficient production of Spike-pseudotyped particles than some other common systems (Andrew McGuire and Abigail Powell, personal communication). We used two different lentiviral backbones: one that uses a CMV promoter to drive expression of just ZsGreen, and another that uses a CMV promoter to drive exprssion of luciferase followed by an internal ribosome entry site (IRES) and ZsGreen (hereafter referred to as the ZsGreen and Luciferase-IRES-ZsGreen backbones).

The Spike protein was from SARS-CoV-2 strain Wuhan-Hu-1 [CITE], with the nucleotide sequence codon optimized for expression in human cells. We used three variants of Spike (**Figure 1B**). The first variant was just the codon-optimized Spike. The second variant had two amino-acid mutations to basic residues in Spike’s cytoplasmic tail (K1269A and H1271A) that change the sequence of the five most C-terminal residues to ALAYT. This variant is hereafter referred to as Spike-ALAYT. The rationale for Spike-ALAYT was that for the original SARS-CoV, the two analagous mutations were shown to improve plasma-membrane expression of Spike by eliminating an endoplasmic reticulum retention signal [CITE]. The third variant had the cytoplasmic tail of Spike replaced with that from influenza hemagglutinin (HA); this variant is hereafter referred to as Spike-HAtail. The rationale for Spike-HAtail was that for the original SARS-CoV, replacing Spike’s cytoplasmic tail with that from other viruses was shown to improve pseudotyping efficiency [CITE]. The sequences of all of the Spike and lentiviral plasmids are in **File S1**, and the plasmids are available in the BEI Resources repository as items XXX.

2.2. Target 293T cells constititutively expressing Spike’s ACE2 receptor.

To create a target cell line that is efficiently infected by the Spike pseudotyped lentiviral virions, we transduced 293T cells to constitutively express Spike under control of an EF1a promoter. Specifically, 293T cells (ATCC CRL-3216) were transduced with a lentiviral vector expressing human ACE2 under a EF1a promoter (the plasmid sequence is in **File S1**, and the lentiviral backbone is available in the BEI Resources repository as item XXX). To create a clonal cell line from the bulk transduction, we sorted single transduced cells by flow cytometry and re-expanded into large populations. We identified an expanded clone that expressed high levels of ACE2 (**Figure 2A**). This clone is hereafter referred to as 293T-ACE2, and is available in the BEI Resources repository as item XXX.

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**Figure 2.** The Spike-pseudotyped lentiviral virions infect 293T-ACE2 cells. (**A**) The flow cytometry plot showing expression of ACE2 by the 293T-ACE2 cells (grey shaded) compared to not transduced 293T cells (grey line) as quantified by staining with antibody AF933 (R&D Systems). (**B**) Microscope image showing ZsGreen expression in 293T-ACE2 or 293T cells at X hours after incubation with equivalent amounts of Spike-pseudotyped or VSV G-pseudotyped lentivirus expressing ZsGreen. The Spike-pseudotyped virus efficiently infects the 293T-ACE2 but not the 293T cells, whereas the VSV G-pseudotyped virus efficiently infects both cell lines. Cells were infected with 10-fold less volume of VSV G-pseudotyped lentivirus than Spike-pseudotyped lentivirus.

We validated that the 293T-ACE2 cells were susceptible to infection by Spike-pseudotyped lentivirus by incubating 293T-ACE2 and parental 293T with equivalent amounts of virus carrying ZsGreen. As shown in **Figure 2B**, the virus efficiently infected the 293T-ACE2 but not the 293T cells. Virus pseudotyped with VSV G, an amphotropic viral entry protein that is not dependent on ACE2, efficiently infects both cell lines (**Fig. 2B**)**.**

2.3. Titers of pseudotyped lentiviral particles with different Spike cytoplasmic tail variants.

To quantify the titers of lentiviral virions pseudotyped with each of the Spike variants, we produced virions with each of these Spikes, as well as a positive control using the highly efficient VSV-G protein and a negative control in which we did not provide a viral entry protein in the transfected producing cells. We first produced virions using the ZsGreen backbone, and titered by flow cytometry to determine the number of transducing particles per ml. As shown in **Figure 1A**, all three Spike variants produced titers ≈104 transduction units per ml. These titers were about two orders of magnitude lower than those achieved with VSV-G, but we still considered them to be encouragingly high given that lentiviral virions can be further concentrated by a variety of methods [CITE]. We then produced virions using the Luciferase-IRES-ZsGreen backbone, and found that we could achieve titers of >106 relative luciferase units (RLUs) per ml in 96-well plate infections (**Figure 1B**). This titer was again about two orders of magnitude lower than that achieved using VSV-G. Of note, the magnitude of the fluorescent signal from ZsGreen is lower for the Luciferase-IRES-ZsGreen backbone than for the ZsGreen-only backbone (**Figure 1C**).

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**Figure 3.** Titers of Spike pseudotyped lentiviruses in 293T-ACE2 cells. (**A**) Titers of the ZsGreen backbone pseudotyped with the three Spike variants or VSV-G, as determined by counting green cells via flow cytometry analysis at 48 hours post-infection, and then calculating the titer of transduction-competent virus per ml from the percentage of green cells. The “n.d.” for None indicates that the titer was not detectable. (**B**) Titers of the Luciferase-IRES-ZsGreen backbone as determined by measuring the relative luciferase units (RLUs). The RLUs were determined at 48 hours post-infection infecting ~2.3e4 293T-ACE2 cells per well in 96-well plates. The RLUs per mL for the Spike-pseudotyped viruses are the average of three 3-fold serial dilutions of virus starting at 50 uL virus in a total volume of 150 uL. For the VSV G-pseudotyped virus, RLUs per mL were averaged from two 3-fold dilutions starting at 3 uL virus in a total volume of 150 uL. (**C**) Microscope images showing 293T-ACE2 cells infected with Spike pseudotyped virus with either the ZsGreen or Luciferase-IRES-ZsGreen backbone at 60 hours post-infection. As can be seen from the images, the ZsGreen backbone gives a stronger fluorescent signal than the Luciferase-IRES-ZsGreen backbone, presumably because this protein is expressed more strongly if it is the sole CMV-promoter driven transcript than if it is the second transcript driven by an IRES.

2.4. Neutralization assays with Spike-pseudotyped lentiviral particles.

We next proceeded to use the Luciferase-IRES-ZsGreen backbone viruses to perform neutralization assays in 96-well plates. Because such assays can be performed with <105 RLUs per well in 96-well plates, a relatively modest volume of virus is required to perform a full 96-well plate neutralization assay.

We performed neutralization assays using serum from a confirmed SARS-CoV-2 infected patient collected at 19 days post-symptom onset, and with soluble ACE2 protein (which has been reported to neutralize SARS-CoV-2 by acting as a decoy receptor [CITE]). For these assays, we first made serial dilutions of the serum or soluble ACE2 in a 96-well plate, then incubated with X RLUs pseudotyped lentiviral virions for 60 minutes, and then added the virus plus serum to a pre-seeded plate of 293T-ACE2 cells. We then measured the luciferase signal at 60 hours post-infection (see **Materials and Methods** for a more detailed protocol).

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**Figure 4.** Neutralization assays. (**A**) Neutralization assay using sera collected from a confirmed SARS-CoV-2 infected patient at 19 days post-symptom onset. (**B**) Neutralization assay using soluble ACE2 protein.

Both the serum and the soluble ACE2 effectively neutralized the virus (Figure 4). For the serum, the inhibitory concentration 50% (IC50) was X, which is in the range of values reported for sera from other patients at a similar time post-infection [CITE]. For soluble ACE2, the IC50 was X which is also in the range of previously reported values [CITE].

Of note, the virus pseudotyped with Spike-HAtail yielded lower IC50s than the virus pseudotyped with Spike (Figure 4). While the mechanism by which changing the cytoplasmic tail alters neutralization sensitivity is unclear, based on this finding we suggest performing the assays using the Spike without any cytoplasmic tail modifications, particularly since none of the modifications tested here greatly improved viral titers.

3. Discussion

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4. Materials and Methods

*4.1. Plasmids.*

*4.2 Creation of 293T ACE2 cells.*

VSV G-pseudotyped lentivirus packaging the hACE2 gene (plasmid \_\_\_\_) was generated via \_\_\_\_. This virus was used to infect \_\_\_\_. Single cells were stained with \_\_\_\_ ACE2 antibody and FITC secondary and sorted based on antibody staining. Once single cell clones had grown sufficiently, they were screened for ACE2 expression via flow cytometry and the clone with the best expression (**Fig. 2A)** was expanded.

These cells should be grown in D10 growth media: DMEM with 10% FBS, \_\_ L-glutamine, and \_\_\_ Pen/Strep.

*4.3 Detailed protocol for generation of pseudotyped lentiviral particles.*

Pseudotyped lentiviruses were generated from transfecting 293Ts as depicted in **Figure 1a.** First, 293T cells were seeded at a density of 5x105 cells per well (2.5x105 cells per mL) of a 6-well plate. The following day, the cells should be ~50-70% confluent. About 16-24 hours after seeding, the cells were transfected with the plasmids required for lentiviral generation according to the BioT (reagent info) protocol. Specifically, for each well, 1 ug of lentiviral backbone (ZsGreen or Luciferase-IRES-ZsGreen), 0.22 ug of HDM\_Gag/Pol (BEI \_\_\_\_), 0.22 ug of pC\_Rev (BEI \_\_\_\_), 0.22 ug of HDM\_Tat (plasmid \_\_\_\_\_), and 0.34 ug of either Spike, VSV G (positive control), or carrier DNA (Promega product info) (negative control) were combined with 100 uL DMEM and 3 uL BioT, gently mixed by pipetting up and down, briefly spun in a centrifuge, and allowed to sit at room temperature for 10-20 min before being gently added to the cells in a dropwise manner. At 18 to 24 hours post-transfection, the media was changed by removing the transfection supernatant and gently adding 2 mL fresh, pre-warmed D10 to each well. Virus was collected at 60 hours post transfection by harvesting the supernatant from each well and filtering it through a 0.45 um filter. Virus was then frozen at -80 C before use.

To titer this virus, we then infected 293T-ACE2 cells in a 96 well-plate with serial dilutions of virus in a final volume of 150 uL. Infection was measured 48-60 hours post infection either by measuring ZsGreen fluorescence using flow cytometry or by measuring RLUs using the Bright Glo luciferase reagent (reagent info) and Tecan plate reader.

To titer virus by ZsGreen expression, wells that appeared to yield ~1-10% positive cells were selected for flow cytometry. 30 uL of trypsin was added to each of these wells and incubated for 5 min or until cells visually detached. 70 uL of D10 was added to these wells and these cells were transferred to a V-bottom plate then centrifuged at 300xg for 4 min. Cells were washed 2x with 150 uL 3% BSA in PBS and then resuspended in 150 uL 1% BSA in PBS for flow cytometry.

To titer virus using luciferase, 100 uL of media was removed from the infected cells. Assuming an evaporation rate of about 10 uL per day, this leaves ~30 uL of media in each well. 30 uL of BrightGlo luciferase reagent was then added to each well and mixed well. The entire 60 uL well volume was then transferred to a black-bottom plate and allowed to incubate for 2 min before being analyzed using the Tecan plate reader (info and settings).

*4.3 Detailed protocol for neutralization assays.*

The following protocol was developed to streamline neutralization assays with Spike-pseudotyped lentiviruses:

1. Seed a poly-L-lysine-coated 96-well plate with 1.25x104 293T-ACE2 cells (BEI XXX) in 50 uL DMEM (2.5x105 cells per mL) in each well. Plan to infect this plate 8–12 hours post-seeding.
2. About 1.5 hours prior to infecting cells, begin preparing serum and/or ACE2 dilutions. In a separate 96-well plate, serially dilute serum samples leaving 60 uL diluted serum in each well. Add 60 uL D10 to the wells without serum.
3. Dilute virus to ~2-4x106 RLU per mL. Add 60 uL of diluted virus to all of the serum dilutions and several wells without serum as virus-only controls (see **Supplementary Figure 2** for an example plate layout).
4. Incubate virus and serum at 37 C for 1 hr.
5. Carefully add 100 uL of the serum + virus dilutions to the corresponding wells of the plate of 293T-ACE2 cells. Add 100 uL D10 to any wells without serum or virus.
6. Add 3 uL of 0.25 ug/uL polybrene for a final concentration of 5 ug/mL in each well.
7. Incubate at 37C for 48-60 hours before reading out luminescence or fluorescence.

*4.4 Human serum sample and soluble ACE2.*

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1: File S1: A zip file containing all the plasmid maps in Genbank format.

**Author Contributions:** Conceptualization, K.D.C. and J.DB.; investigation, K.D.C., R.E., A.S.D., K.M., and A.N.L.; resources and specialized reagents, A.B.B. and H.Y.C.; writing—original draft preparation, K.D.C and J.D.B.; writing—review and editing, all authors. All authors have read and agreed to the published version of the manuscript.

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