Article

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 that may help protect against re-infection or disease. Therefore, assays to measure how antibodies and sera affect Spike-mediated viral infection are important for studying immunity to SARS-CoV-2. 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 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 methods that simply measure antibody binding rather than neutralization.

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

1. Introduction

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

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 [CITE], quantifying neutralizing antibody activity is more difficult. Probably the most relevant method to quantify neutralizing activity 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 the neutralizing activity in the sera of infected patients [CITE] or to characterize the potency of individual antibodies [CITE]. 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 high-containment facilities [CITE].

An alternative approach that alleviates these biosafety limitations leverages the fact that all known neutralizing antibodies to SARS-CoV-2 and related coronaviruses target the virus’s Spike protein [CITE, add something about HE]. 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 [CITE]. 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 [CITE]. For SARS-CoV-2, such pseudotyping has recently been reported using HIV-based lentiviral virions [CITE], MLV-based retroviral virions [CITE], and VSV [CITE]. In the limited data reported to date, results from such pseudovirus neutralization assays correlate well with measurements made using live SARS-CoV-2 [CITE]. However, the papers describing these pseudotyping assays have generally focused on other biological questions, and only provided brief descriptions of the assays, which in many cases rely on reagents 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 how they can be used 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 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 as quantified by staining with antibody X. (**B**) Microscope image showing ZsGreen expression in 293T-ACE2 or 293T cells at X hours after incubation with equivalent amounts of Spike-pseudotyped lentivirus expressing ZsGreen. The virus efficiently infects the 293T-ACE2 but not the 293T cells.

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.

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 ZsGree-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 X 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 X hours post-infection using X cells per well in 96-well plates infected with X ul of virus in a total volume of X. (**C**) Microscope images showing 293T-ACE2 cells infected with serial dilutions of Spike or VSV-G pseudotyped virus with either the ZsGreen or Luciferase-IRES-ZsGreen backbone at X 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 as little as 105 RLUs per well in 96-well plates [??, Kate, is this correct], 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 X minutes, and then added X 293T-ACE2 cells per well. We then measured the luciferase signal at X 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.*

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

*4.3 Detailed protocol for neutralization assays.*

*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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**Conflicts of Interest:** The authors declare no conflict of interest.

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