Journal of Visualized Experiments

Detection of SARS-CoV-2 Neutralizing Antibodies Using High-throughput Fluorescent Imaging of Pseudovirus Infection

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62486R1
Full Title:	Detection of SARS-CoV-2 Neutralizing Antibodies Using High-throughput Fluorescent Imaging of Pseudovirus Infection
Corresponding Author:	Taha Azad, PhD Ottawa Hospital Research Institute Ottawa, Ontario CANADA
Corresponding Author's Institution:	Ottawa Hospital Research Institute
Corresponding Author E-Mail:	tazad@ohri.ca
Order of Authors:	Taylor Jamieson
	Joanna Poutou
	Ricardo Marius
	Xiaohong He
	Reza Rezeai
	Taha Azad, PhD
	Carolina Ilkow
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Immunology and Infection
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Ottawa , Ontario.
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

Detection of SARS-CoV-2 Neutralizing Antibodies Using High-throughput Fluorescent Imaging of Pseudovirus Infection

AUTHORS AND AFFILIATIONS:

Taylor R. Jamieson[1,2], Joanna Poutou[1,2], Ricardo Marius[1,2], Xiaohong He[1], Reza Rezeai[1,2], Taha Azad[1,2], Carolina S. Ilkow[1,2,*].

[1]Ottawa Hospital Research Institute, Ottawa, ON, K1H 8L6, Canada
[2]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H 8M5, Canada

Email addresses of co-authors:

Taylor R. Jamieson	(tjamieson@ohri.ca)
Joanna Poutou	(ypoutoupaumier@ohri.ca)
Ricardo Marius	(rmarius@ohri.ca)
Xiaohong He	(xhe@ohri.ca)
Reza Rezeai	(rrezaei@ohri.ca)
Taha Azad	(tazad@ohri.ca)

Corresponding author:

Carolina S. Ilkow	(cilkow@ohri.ca)

KEYWORDS:

SARS-CoV-2; COVID-19; Pseudovirus; Neutralizing antibody; Vesicular stomatitis virus; Spike glycoprotein

SUMMARY:

The protocol described here outlines a fast and effective method for measuring neutralizing antibodies against the SARS-CoV-2 spike protein by evaluating the ability of convalescent serum samples to inhibit infection by an enhanced green fluorescent protein-labeled vesicular stomatitis virus pseudotyped with spike glycoprotein.

ABSTRACT:

As the COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to evolve, it has become evident that the presence of neutralizing antibodies against the virus may provide protection against future infection. Thus, as the creation and translation of effective COVID-19 vaccines continues at an unprecedented speed, the development of fast and effective methods to measure neutralizing antibodies against SARS-CoV-2 will become increasingly important to determine long-term protection against infection for both previously infected and immunized individuals. This paper describes a high-throughput protocol using vesicular stomatitis virus (VSV) pseudotyped with the SARS-CoV-2 spike protein to measure the presence of neutralizing antibodies in convalescent serum from patients who have recently recovered from COVID-19. The use of a replicating pseudotyped virus eliminates the

necessity for a containment level 3 facility required for SARS-CoV-2 handling, making this protocol accessible to virtually any containment level 2 lab. The use of a 96-well format allows for many samples to be run at the same time with a short turnaround time of 24 h.

INTRODUCTION:

In December 2019, a novel coronavirus was identified, which we now know as SARS-CoV-2, the causative agent of coronavirus disease 2019 (COVID-19)[1]. SARS-CoV-2 is a betacoronavirus belonging to the *Coronaviridae* family. These enveloped viruses comprise a large positive-sense RNA genome and are responsible for respiratory and intestinal infections in both humans and animals[2]. As of December 13, 2020, there have been more than 72 million reported cases of COVID-19 globally and more than 1.6 million deaths[3]. The development of an effective vaccine has become the primary goal of researchers around the globe with at least 85 preclinical vaccines under investigation and 58 currently undergoing clinical trials[4].

Coronaviruses encode four structural proteins including the spike protein (S), nucleocapsid (N), envelope protein (E), and the membrane protein (M). Entry of SARS-CoV-2 requires interaction of the receptor-binding domain (RBD) of S with the host receptor, human angiotensin-converting enzyme 2 (hACE2), and subsequent membrane fusion following proteolytic cleavage by host cellular serine protease, transmembrane protease serine 2 (TMPRSS2)[5–10]. Humoral immunodominance of the S protein of SARS-CoV has been previously reported and has now been shown also for SARS-CoV-2[11–13]. Indeed, neutralizing antibody responses against S have been detected in convalescent serum from SARS-CoV patients 24 months after infection[14], highlighting their critical role in the long-term immune response. The S protein has been identified as a promising vaccine target and has thus become a key component of most vaccines under development[15,16].

While the rapid detection of neutralizing antibodies is a critical aspect of vaccine development, it may also shed light on the rate of infection and sero-epidemiologic surveillance in impacted areas[17]. A replication-competent VSV pseudotyped with the SARS-CoV-2 S glycoprotein, in place of the wild-type VSV glycoprotein, to study SARS-CoV-2 infection in biosafety level 2 settings was kindly donated by Whelan and co-workers[18]. VSV expressing spike (VSV-S) will be utilized to determine the neutralizing antibody response against SARS-CoV-2 spike protein. As the VSV-S used here also expresses enhanced green fluorescent protein (eGFP), eGFP foci may be detected within 24 h to quantify infection, whereas plaque formation can take 48 to 72 h. Summarized here is a simple and effective protocol to determine the ability of convalescent patient serum to neutralize VSV-S-eGFP infection. This method may also be easily adapted to interrogate other potential therapeutics that aim to disrupt the host-viral interaction of SARS-CoV-2 S protein.

PROTOCOL:

1. Plating cells (Day 1) for the production and quantification of SARS-CoV-2 pseudovirus

1.1. Preparation for tissue culture

1.1.1. Warm 1x Dulbecco's Phosphate-Buffered Saline (DPBS); Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (optional); and 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) to 37 °C in a water bath for approximately 15 min.

1.1.2. Disinfect a tissue culture hood with 70% ethanol, and place tissue culture dishes, Pasteur pipettes, and serological pipettes in the tissue culture hood as needed. Remove PBS, DMEM, and trypsin from the water bath, and disinfect with 70% ethanol prior to placing in the tissue culture hood.

1.2. Plating and maintaining cells

1.2.1. Grow Vero E6 cells in DMEM (containing 10% FBS) in T75 tissue culture flasks or 15 cm tissue culture dishes in a 37 °C incubator with 5% CO_2. Passage the cells as needed when cells are 80–90% confluent.

1.2.2. Wash the cells by adding 10 mL of 1x PBS to each dish and gently rocking 4–5 times; aspirate the PBS. Add 3 mL of trypsin-EDTA to each dish, rock the plate to ensure the entire surface is covered. Incubate the cells at 37 °C with 5% CO_2 for approximately 5 min, or until the cells have detached.

1.2.3. Add 7 mL of DMEM (containing 10% FBS) to deactivate the trypsin, and resuspend the cells by pipetting up and down several times. Spin down the cells to remove the trypsin using a benchtop centrifuge at 500 × *g* for 5 min, aspirate the media without disturbing the cell pellet, and resuspend the cells in 10 mL of DMEM (containing 10% FBS). If cells are required for future experiments, place 1 mL in a new dish containing 15 mL of fresh DMEM (containing 10% FBS).

1.2.4. Count the cells using an automated cell counter or hemocytometer, and add approximately 1×10^7 cells to each 15 cm plate, and incubate at 37 °C with 5% CO_2 until they are 100% confluent (1–2 days).

2. VSV-S-EGFP pseudovirus preparation

2.1. Infection

2.1.1. Infect cells at multiplicity of infection (MOI) 0.01 with VSV-S-eGFP stock virus in 12 mL of serum-free DMEM for 1 h at 37 °C with 5% CO_2, occasionally rocking the plates. Replace the inoculum with fresh DMEM (containing 2% FBS and 20 mM HEPES, pH 7.7), and move to an incubator set to 34 °C with 5% CO_2.

NOTE: A temperature of 34 °C is required for the propagation of VSV-S-eGFP in cell culture.

2.1.2. Collect cell supernatants upon observation of extensive cytopathic effect (CPE) and cell detachment, approximately 48 h post-infection. Use a fluorescent microscope to visualize the

extensive expression of eGFP by infected cells 24 h post-infection.

2.2. Collection

2.2.1. Centrifuge the supernatants using a benchtop centrifuge for 5 min at 1,000 × *g* at 4 °C to remove the cell debris. Aliquot the supernatant to avoid multiple freeze-thaw cycles, and store at -80 °C.

3. Titering the VSV-S-eGFP pseudovirus

3.1. Serial dilution to determine viral titer

3.1.1. Plate Vero E6 cells on 6-well plates at a seeding density of 6×10^5 cells per well in DMEM (with 10% FBS), and incubate overnight at 37 °C with 5% CO_2.

NOTE: Vero cells that overexpress TMPRSS2 and hACE2 can also be used.

3.1.2. Set up a 10-fold serial dilution series of the VSV-S-eGFP virus in cold serum-free DMEM by placing 900 µL of media in seven microcentrifuge tubes. Label tubes from 1 to 7. Add 100 µL of the viral stock to the first tube and vortex briefly. Transfer 100 µL of diluted virus from tube 1 to tube 2 and vortex briefly; continue until tube 7.

3.1.3. Aspirate the medium from all wells of the 6-well plate containing Vero E6 cells, and replace with 500 µL of dilutions 10^{-2} to 10^{-7}. Incubate the plates for 45 min at 37 °C with 5% CO_2, gently rocking every 15 min.

3.1.4. Aspirate the inoculum and replace with overlay containing a 1:1 mixture of 2x DMEM and 6% carboxymethyl cellulose (CMC), final concentration of 3% CMC, supplemented with 10% FBS; incubate at 34 °C with 5% CO_2 for 48 h.

NOTE: Pre-warm the overlay mixture in a 37 °C water bath for 15 min during the infection step. A 1:1 mixture of 1% agarose with 2x DMEM supplemented with 10% FBS may be used in place of CMC. To overlay with agarose, boil 1% agarose (in dH_2O) and mix with cold 2x DMEM. Ensure the mixture is a suitable temperature before adding to the cell monolayer (approximately 37–40 °C).

3.2. Staining and calculating viral titer

3.2.1. Visualize plaques by staining with crystal violet or direct visualization of eGFP under a fluorescent microscope.

3.2.2. To stain plates, aspirate the overlay, wash once with PBS, then add 2 mL of 0.1% crystal violet (in 80% methanol and dH_2O) to each well. Place on a plate rocker for approximately 20 min at room temperature prior to de-staining. Remove the crystal violet, and gently wash each well twice using dH_2O or PBS.

NOTE: Washing and staining steps should be performed gently by pipetting slowly towards the side of each well to ensure the cell monolayer remains intact throughout the procedure.

3.2.3. Allow plates to dry for at least 1 h prior to counting plaques. Ensure that plaque counts are obtained from wells containing 20 to 200 plaques. To calculate the titer of the virus in plaque-forming units (PFU) per mL, multiply the dilution factor of the well counted with the volume of infection, and divide this number from the plaques present in the respective well.

$$titer\ (pfu/mL) = \frac{number\ of\ plaques}{dilution\ factor\ \times\ volume\ of\ infection}$$

4. Plating cells (Day 1) for the measurement of neutralization of SARS-CoV-2 pseudovirus by commercially available antibodies and convalescent patient serum

4.1. Preparation for tissue culture

4.1.1. Prepare medium and tissue culture hood as described in section 1. In addition, prepare the necessary number of 96 well plates to accommodate a minimum of 2 replicates per sample (i.e., 1 plate per 6 samples); add additional replicates if sample volume permits.

4.2. Plating and maintaining cells

4.2.1. Grow and maintain Vero E6 cells as described in section 1. Prepare at least 10 mL of cell suspension per 96-well plate at a concentration of 2×10^5 cells per mL (plate 1.5×10^5 cells per mL for assays performed after 48 h, if needed). Add 100 µL of cell suspension to each well of a 96-well plate using a multichannel pipette, and incubate for 24 h at 37 °C with 5% CO_2.

NOTE: Mix the cell suspension well, and rock each plate gently in all directions to ensure even distribution of cells.

5. Antibody or serum dilutions and infections (Day 2)

NOTE: This protocol can be applied to measure the neutralization of VSV-S-eGFP by both commercially available antibodies and patient serum, as well as serum collected from animals for pre-clinical vaccine development studies. *Take note of the additional steps listed when handling patient/animal serum samples.

5.1. Serial dilution series

5.1.1. *Prior to diluting serum samples, heat-inactivate each sample in a 56 °C water bath for 30 min to inactivate complement. Ensure necessary safety precautions are taken when handling patient samples; only open sample containers when in the tissue culture hood, and ensure appropriate containment level 2 personal protective equipment is worn.

5.1.2. Set up the dilution series in an empty 96-well plate (Plate 1).

5.1.3. Begin all dilutions in Row A of the 96-well plate. *For patient samples, begin the dilution series at 1 in 10 by placing 8 µL of serum in 72 µL of serum-free DMEM (with 1% penicillin/streptomycin).

NOTE: The concentration of neutralizing antibody used will depend on the activity predicted by the manufacturer. For the SARS-CoV-2 Spike neutralizing antibody used here (see the **Table of Materials**), start with 5 µg/mL to observe at least 50% neutralization.

5.1.4. Add 40 µL of of serum-free DMEM (with 1% penicillin/streptomycin) to rows B to G, and 80 µL to row H. Do not add virus to row H, as it serves as a cell-only control.

5.1.5. Using a 12-well multichannel pipette, mix and transfer 40 µL of diluted serum or antibody from row A to row B. Mix and repeat until row F, discard 40 µL from row F. Do not add serum to row G as it represents the virus-only control row.

5.2. Infection and overlay

5.2.1. Prepare an appropriate volume of diluted VSV-S-eGFP to treat each well at MOI 0.05 (or 2000 pfu). (When completing this calculation, keep in mind that only 60 µL of the total 80 µL volume will be moved onto the cells; therefore, be sure to multiply the volume of the virus by 1.33 to maintain 2000 pfu).

NOTE: As VSV-S-eGFP is temperature-sensitive, ensure viral stocks remain on ice whenever not in use, and avoid multiple freeze-thaw cycles as titers will differ after repeated freezing.

5.2.2. Add 40 µL of diluted virus to each well in rows A to G of Plate 1 and mix by pipetting up and down 4–5 times. Incubate the plate for 1 h at 37 °C with 5% CO_2.

5.2.3. Remove the plate containing cells from the incubator (Plate 2), and carefully aspirate the media from all wells. Transfer 60 µL of antibody/virus mixture from Plate 1 to Plate 2, and incubate for 1 h at 37 °C with 5% CO_2, rocking the plate every 20 min.

5.2.4. Top up each well with 140 µL of overlay containing CMC in DMEM for a final concentration of 3% CMC (with 10% FBS and 1% penicillin/streptomycin). Place Plate 2 in an incubator set to 34 ˚C with 5% CO_2 for approximately 24 h before imaging

NOTE: Pre-warm the overlay mixture in a 37 °C water bath for 15 min during the infection step.

6. Imaging and quantification (Day 3)

6.1. Image plates using an automated fluorescent imager (using a fluorescein isothiocyanate

(FITC) filter or an alternative filter with an excitation wavelength of 488 nm). Quantify viral infection by creating a protocol that automatically identifies and counts individual eGFP foci. If an automatic counting feature is not available, use ImageJ software to quantify the number of eGFP foci.

REPRESENTATIVE RESULTS:

This protocol outlines a rapid and effective method for detecting neutralizing antibodies against SARS-CoV-2 S protein via inhibition of VSV-S-eGFP pseudovirus infection (quantifiable by loss of eGFP foci detected). A schematic representation of the protocol is depicted in **Figure 1**. It is recommended that a commercially available antibody be used as a positive control each time the assay is run to ensure the consistency of the assay. Here, we demonstrate a dilution curve using a commercially available neutralizing IgG antibody against SARS-CoV-2 Spike RBD compared to an IgG control (see the **Table of Materials** for details about both antibodies; **Figure 2**).

Convalescent patient samples were collected approximately three months post SARS-CoV-2 infection, and pseudovirus neutralization was determined using the method described above (**Figure 3**). Importantly, minimal background inhibition was observed using healthy donor serum. Furthermore, of note, this assay distinguishes between patients with high symptom severity versus those with milder disease based on the need for hospitalization. In line with other reports, we have observed within our small cohort that hospitalized patients tend to demonstrate increased neutralizing capacity than those who did not require hospitalization[19,20]. While this may not always be the case for hospitalized versus non-hospitalized patient samples, the ability of the assay to differentiate varying degrees of neutralization is an asset. There may also be cases in which the neutralizing ability of the patient serum is much higher than those demonstrated here. If necessary, the starting dilution of patient serum may be adjusted, or additional dilution steps may be carried out on an additional plate.

FIGURE AND TABLE LEGENDS:

Figure 1: Protocol outline demonstrating neutralization of VSV-S-eGFP by convalescent serum. Abbreviations: VSV = vesicular stomatitis virus; eGFP = enhanced green fluorescent protein; COVID-19 = coronavirus disease.

Figure 2: A commercially available neutralizing antibody against SARS-CoV-2 has been used as an example of a positive control alongside IgG as a negative control. The ability of neutralizing antibodies to inhibit viral infection will vary; refer to the information available for the specific antibody purchased to determine which concentration to begin the dilution curve (samples were run in triplicate, error bars represent ± SD). (**A**) Percent inhibition has been calculated based on the number of eGFP foci detected via (**B**) fluorescent imaging. Abbreviations: SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; IgG = immunoglobulin; SD = standard deviation; eGFP = enhanced green fluorescent protein; RBD = receptor-binding domain.

Figure 3: The ability of convalescent serum from patients to neutralize VSV-S-eGFP varies depending on the severity of symptoms. Patient serum samples were collected approximately 3

months post-SARS-CoV-2 infection, and control samples were collected from uninfected patients (samples were run in triplicate, error bars represent ± SD). (**A**) Percent inhibition has been calculated based on the number of eGFP foci detected via (**B**) fluorescent imaging. Abbreviations: SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; SD = standard deviation; eGFP = enhanced green fluorescent protein.

DISCUSSION:

The method described here may be adapted to suit varying lab environments and resources as needed. Importantly, the main limitation of this protocol is the necessity for a containment level 2 space and tissue culture hood. The application of a replicating RNA virus pseudotyped with the SARS-CoV-2 spike, such as VSV-S-eGFP, is a formidable alternative to the SARS-CoV-2 virus, which requires a containment level 3 working area, but may remain a limitation for some groups. All other steps described here are quite flexible and may be performed in nearly any containment level 2 lab. For example, the use of a fluorescent imager with an automated counting feature is not necessary. The 96-well format used here means that only approximately 4 fields of view are required to image nearly the entire well using the lowest objective available (2x).

A manual fluorescent microscope may be used to image multiple fields of each well, and ImageJ (free software) can then be used to quantify eGFP foci. Additionally, we have chosen to use the 96-well format to use the lowest volume of serum possible as well as keeping consumable usage to a minimum. If a fluorescent microscope is unavailable, this protocol may be scaled up to a 6- or 12-well format to detect plaque formation after crystal violet fixation and staining (similar to the viral titer protocol described above). The most critical consideration to keep in mind when performing this protocol is the temperature sensitivity of VSV-S-eGFP. When working with VSV-S-eGFP, always aliquot the virus into small volumes to avoid multiple freeze-thaw cycles, and keep the virus on ice whenever possible. Additionally, robust fluorescent signal may be detected after 24 h; however, we have acquired similar results after imaging at 20 to 28 h post infection.

There are several methods available to detect the presence of antibodies against SARS-CoV-2, including the gold-standard enzyme-linked immunosorbent assay (ELISA) assay, which quantifies the total amount of antibodies against S[21,22]. Here, we have outlined a fast and reliable method to specifically detect neutralizing antibodies against the immunodominant SARS-CoV-2 spike protein in convalescent serum from patients. We have improved the classical plaque-reduction neutralization test (PRNT) by successfully adapting to a 96-well format, which allows for the detection of neutralizing antibodies in a large number of samples within 24 h by automated quantification of pseudovirus infection, allowing for quick turnaround of final data reports. In addition to detecting neutralizing antibodies within serum, this method can be adapted to perform high-throughput screening of other therapeutic strategies, which aim to directly inhibit the SARS-CoV-2 spike protein interaction with hACE2, such as monoclonal antibodies, recombinant soluble hACE2, or protease inhibitors, to impede viral entry[23]. With the emergence of several SARS-CoV-2 spike protein variants, it is also important to note that this method may be applied to determine if similar neutralization levels occur following infection with different variants of the virus[24].

Other examples of available methods to detect antibodies against SARS-CoV-2 include ELISAs, lentivirus-based assays, and commercial kits to evaluate the neutralizing capacity of serum samples. While the commonly used IgM- or IgG-binding ELISAs are an effective method to determine the presence of antibody concentration to track previous infection or immunization, they are unable to distinguish the binding antibodies' neutralizing capacity[25]. Pseudotyped lentivirus-based neutralization assays have an improved safety profile, by using non-replicative viral particles as opposed to replicating VSV-S-eGFP; however, this creates a barrier in terms of testing capacity as lentiviral titers tend to be much lower[26,27]. There are several commercially available kits that measure the ability of antibodies to block infection via competitive inhibition of the SARS-CoV-2 spike protein (RBD specifically) binding with the hACE2 receptor following incubation with convalescent serum (e.g., CUSABIO, GenScript, Abnova). While many of these kits have reputable sensitivity and specificity, they also tend to be relatively expensive and therefore not ideal for a large volume of samples. The protocol provided here is fast, reliable, and inexpensive. This high-throughput method may be used to test many samples, achieving a robust readout within 24 h.

ACKNOWLEDGMENTS:

We would like to thank the Whelan lab for generously providing the VSV-S-eGFP virus used in this protocol (described in Case *et al.* 2020). We also thank Drs. Bill Cameron and Juthaporn Cowan (and team) for collecting the patient blood samples (REB protocol ID 20200371-01H). The authors disclose receipt of the following financial support for the research, authorship, and/or publication of this article: This work was funded by the generous support from the Ottawa Hospital Foundation and a grant from the Canadian Institutes of Health Research (#448323) and a Fast Grant from the Thistledown foundation for COVID-19 Science to C.S.I. T.R.J. is funded by an Ontario Graduate Scholarship and cluster Mitacs fellowship. JP is funded by a cluster Mitacs fellowship. T.A. is funded by a CIHR Banting Fellowship. We would also like to thank all the individuals who participated and donated their blood samples for this study.

DISCLOSURES: The authors have no competing financial interests related to this publication.

REFERENCES:

1. Hu, B., Guo, H., Zhou, P., Shi, Z.L. Characteristics of SARS-CoV-2 and COVID-19. *Nature Reviews Microbiology*. **19** (3), 141–154 (2021).
2. Burrell, C. J., Howard, C. R., Murphy, F. A. Coronaviruses. *Fenner and White's Medical Virlogy.* 437–446 (2017).
3. COVID-19 Map. Johns Hopkins Coronavirus Resource Center. https://coronavirus.jhu.edu/map.html.
4. Zimmer, C., Corum, J., Wee, S. L. Covid-19 vaccine tracker. The New York Times. https://www.nytimes.com/interactive/2020/science/coronavirus-vaccine-tracker.html.
5. Hoffmann, M. et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell*. **181** (2), 271–280.e8 (2020).
6. Letko, M., Marzi, A., Munster, V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nature Microbiology*. **5**, 562–569 (2020).
7. Azad, T. et al. Nanoluciferase complementation-based bioreporter reveals the

396 importance of N-linked glycosylation of SARS-CoV-2 Spike for viral entry. *Molecular Therapy*.
397 S1525-0016(21)00074-5, doi: 10.1016/j.ymthe.2021.02.007 (2021).
398 8. Brown, E. E. F. et al. Characterization of critical determinants of ACE2–SARS CoV-2 RBD
399 interaction. *International Journal of Molecular Sciences*. **22** (5), 2268 (2021).
400 9. Azad, T. et al. SARS-CoV-2 S1 NanoBiT: a Nanoluciferase complementation-based
401 biosensor to rapidly probe SARS-CoV-2 receptor recognition. *Biosensors and Bioelectronics*. **180**,
402 113122 (2021).
403 10. Azad, T. et al. Implications for SARS-CoV-2 vaccine design: Fusion of Spike glycoprotein
404 transmembrane domain to receptor-binding domain induces trimerization. *Membranes*. **10** (9),
405 215 (2020).
406 11. Cao, Z. et al. Potent and persistent antibody responses against the receptor-binding
407 domain of SARS-CoV spike protein in recovered patients. *Virology Journal*. **7**, 299 (2010).
408 12. To, K. K. W. et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples
409 and serum antibody responses during infection by SARS-CoV-2: an observational cohort study.
410 *The Lancet Infectious Diseases*. **20** (5), 565–574 (2020).
411 13. Gao, Q. et al. Development of an inactivated vaccine candidate for SARS-CoV-2. *Science*.
412 **369** (6499), 77–81 (2020).
413 14. Liu, W. et al. Two-year prospective study of the humoral immune response of patients
414 with severe acute respiratory syndrome. *Journal of Infectious Diseases*. **193** (6), 792–795 (2006).
415 15. Dong, Y. et al. A systematic review of SARS-CoV-2 vaccine candidates. *Signal Transduction*
416 *and Targeted Therapy*. **5** (1), 237 (2020).
417 16. Amanat, F., Krammer, F. SARS-CoV-2 vaccines: Status report. *Immunity*. **52** (4), 583–589
418 (2020).
419 17. Amanat, F. et al. A serological assay to detect SARS-CoV-2 seroconversion in humans.
420 *Nature Medicine*. **26** (7), 1033–1036 (2020).
421 18. Case, J. B. et al. Neutralizing antibody and soluble ACE2 inhibition of a replication-
422 competent VSV-SARS-CoV-2 and a clinical isolate of SARS-CoV-2. *Cell Host and Microbe*. **28** (3),
423 475–485.e5 (2020).
424 19. Garcia-Beltran, W. F. et al. Journal Pre-proof COVID-19 neutralizing antibodies predict
425 disease severity and survival. *Cell*. **184** (2), 476–488.e11 (2020).
426 20. Zeng, C. et al. Neutralizing antibody against SARS-CoV-2 spike in COVID-19 patients, health
427 care workers, and convalescent plasma donors. *JCI insight*. **5** (22), doi: 10.1172/jci.insight.143213
428 (2020).
429 21. Whitman, J. D. et al. Evaluation of SARS-CoV-2 serology assays reveals a range of test
430 performance. *Nature Biotechnology*. **38** (10), 1174–1183 (2020).
431 22. Ainsworth, M. et al. Performance characteristics of five immunoassays for SARS-CoV-2: a
432 head-to-head benchmark comparison. *The Lancet Infectious Diseases*. **20** (12), 1390–1400 (2020).
433 23. Sharifkashani, S. et al. Angiotensin-converting enzyme 2 (ACE2) receptor and SARS-CoV-
434 2: Potential therapeutic targeting. *European Journal of Pharmacology*. **884**, 173455 (2020).
435 24. Burki, T. Understanding variants of SARS-CoV-2. *The Lancet*. **397** (10273), 462 (2021).
436 25. Jayamohan, H. et al. SARS-CoV-2 pandemic: a review of molecular diagnostic tools
437 including sample collection and commercial response with associated advantages and
438 limitations. *Analytical and Bioanalytical Chemistry*. **413** (1), 49–71 (2020).
439 26. Nie, J. et al. Quantification of SARS-CoV-2 neutralizing antibody by a pseudotyped virus-

based assay. *Nature Protocols*. **15** (11), 3699–3715 (2020).
27. Crawford, K. H. D. et al. Protocol and reagents for pseudotyping lentiviral particles with SARS-CoV-2 spike protein for neutralization assays. *Viruses*. **12** (5), 513 (2020).

Figure 1

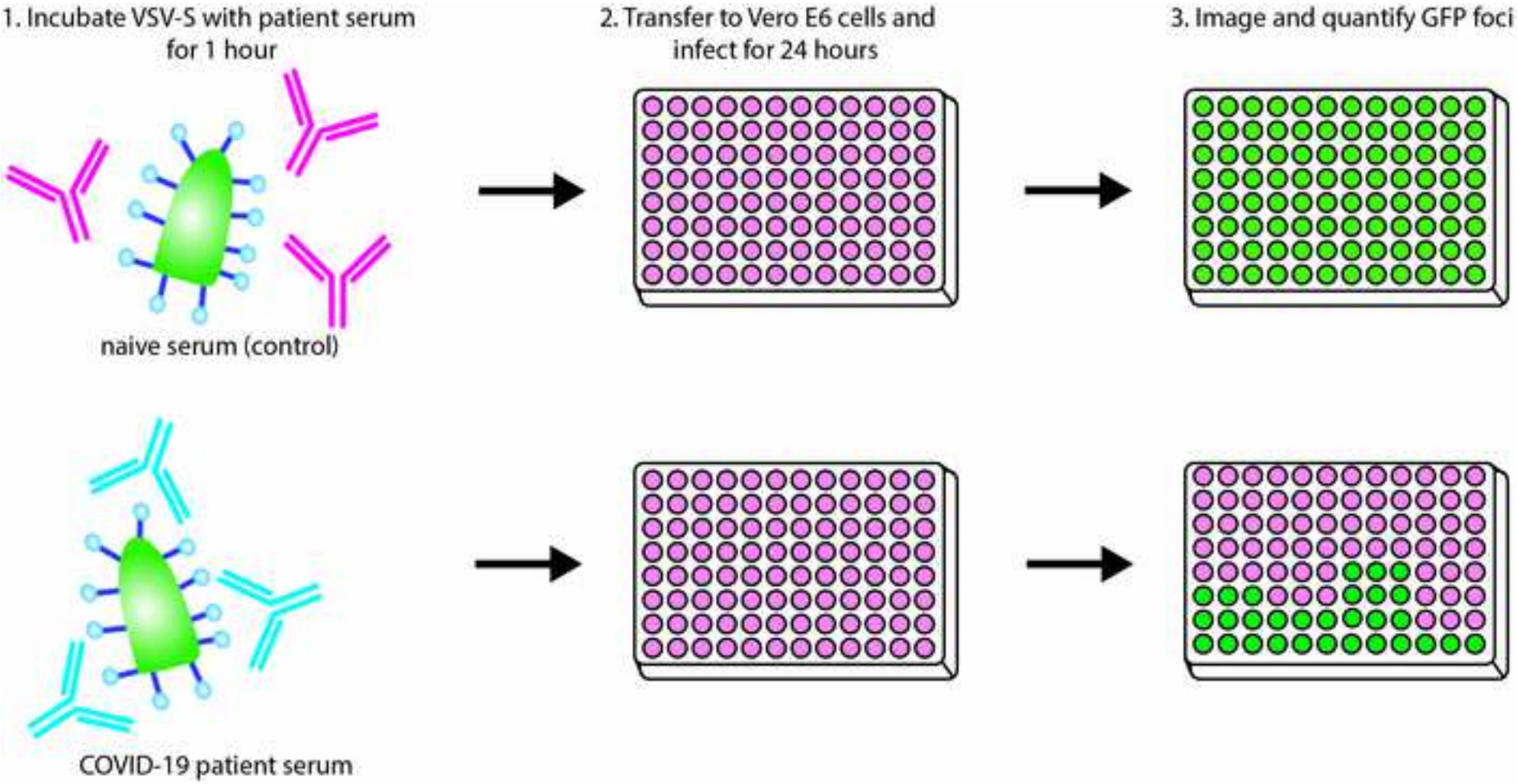

Figure 2

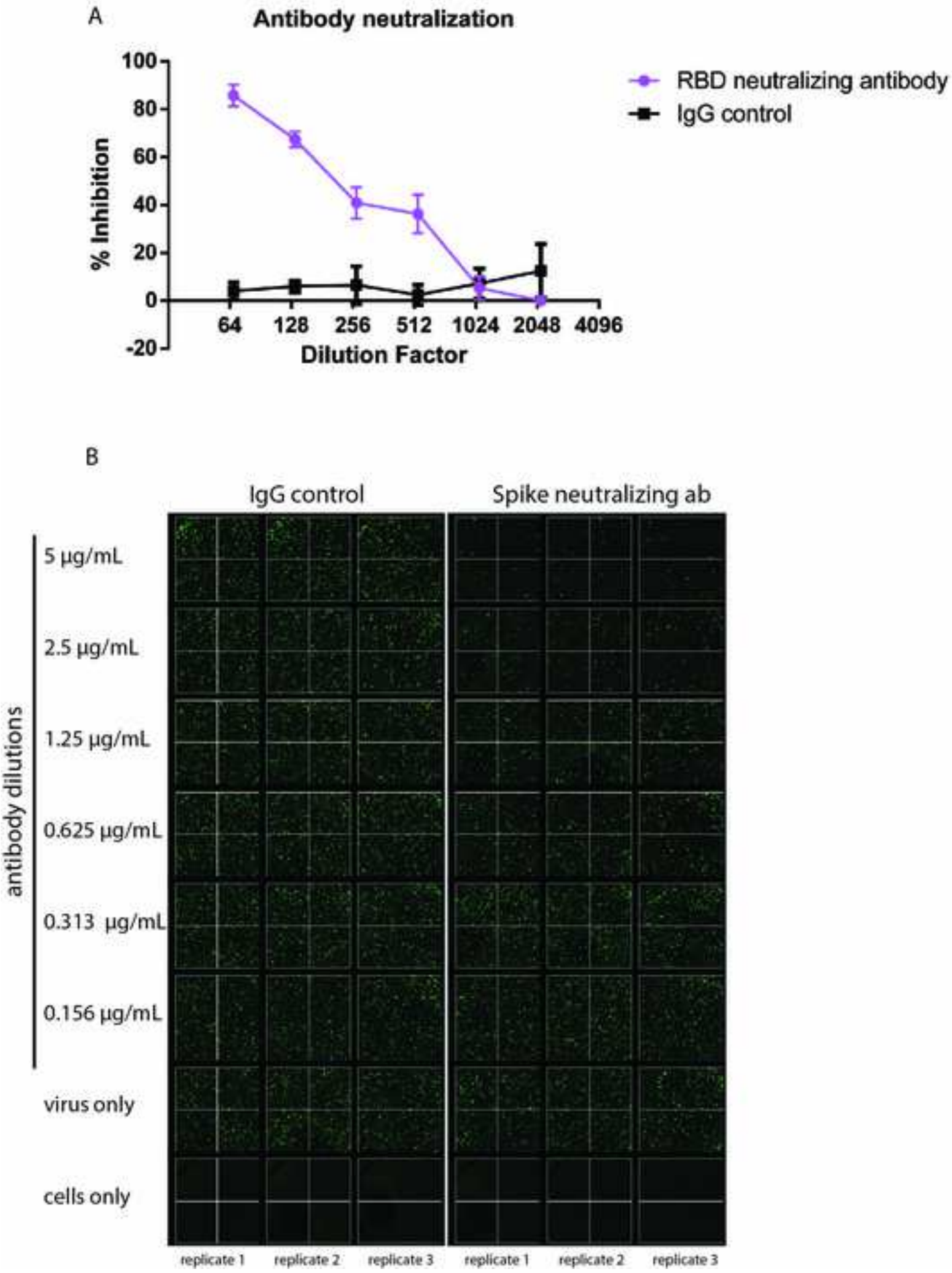

Figure 3

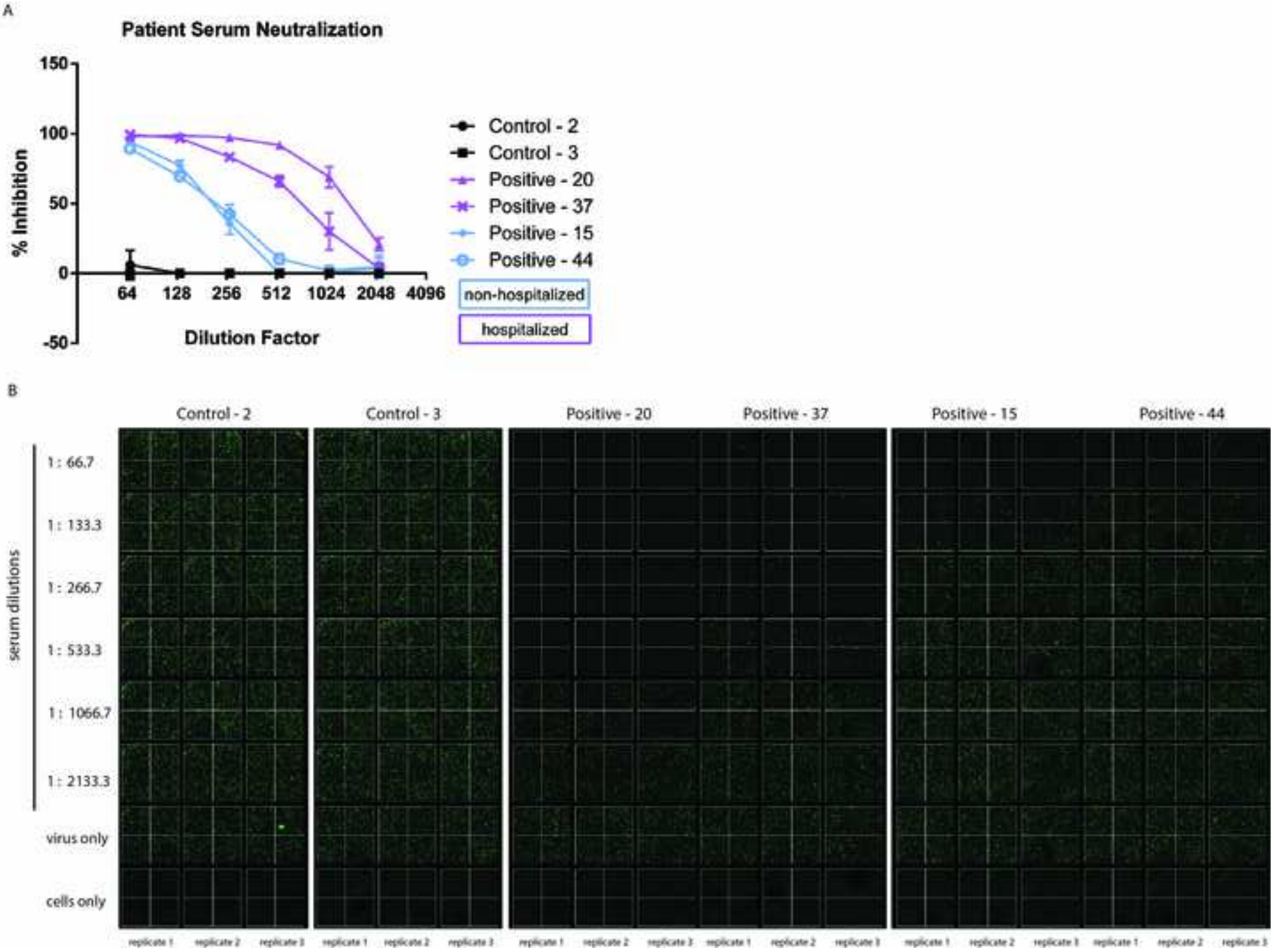

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials (7).xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25% trypsin-EDTA (Gibco)	Fisher scientific	LS25200114	
ArrayScan VTI HCS	Thermo Fisher Scientific		Automated fluorescent imager
carboxymethyl cellulose	Sigma	C5678	
Dulbecco's modified Eagle's medium (Gibco)	Fisher scientific	10-013-CV	
Dulbecco's modified Eagle's medium (Powder) (Gibco)	Thermo Fisher Scientific	12-800-017	
Dulbecco’s Phosphate-Buffered Saline (DPBS)	Fisher scientific	21-031-CV	
HEPES	Fisher scientific	BP-310-500	
IgG Isotype Control (mouse)	Thermo Fisher Scientific	31903	
Penicillin/streptomycin	Thermo Fisher Scientific	15070063	
SARS-CoV-2 (2019-nCoV) Spike Neutralizing Antibody, Mouse Mab	SinoBiological	40592-MM57	
Vero E6 cells	ATCC	CRL-1586	

Compatibility Report for JoVE_Materials.xls
Run on 2021-01-04 16:08

If the workbook is saved in an earlier file format or opened in an earlier version of Microsoft Excel, the listed features will not be available.

Minor loss of fidelity	# of occurrences
Some cells or styles in this workbook contain formatting that is not supported by the selected file format. These formats will be converted to the closest format available.	1

Version

Excel 97-2003

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Complete
2. Please provide an email address for each author. Complete
3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. Complete. Note – subsections (highlighted in red) have been added to make the protocol easier to follow.
4. Maintain a 0-inch left indent throughout the text and indicate new paragraphs using single-line spacing. Include a single line spacing between successive protocol steps. Complete
5. Use “µL” instead of “uL/uL”, “ x107” instead of “ E7”. Add a single space between the quantity and its unit. “5 mm” instead of “5mm”. Complete
6. Line 146: Wavelengths used for visualization? Added (line 292) – FITC filter or alternative 488 nm
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” E.g. line 169, etc. Complete
8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. Eppendorf, Cellomics, Vi Cell XR, ArrayScan, etc. Complete
9. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

10. Please include a Disclosures section, providing information regarding the authors’ competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included. Complete

---

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe a method to measure neutralizing antibodies in sera sample of infected or possibly vaccinated individuals.

Major Concerns:

While the broadly neutralizing antibody can be tested against the parental pseudotyped virus, how would variants virus derived Spike would be incorporated in the protocols need a mention. Furthermore a note on the in vivo relevance would put things in perspective as in the filed still there is a debate whether or not processing of spike is critical factor in the SARS-CoV2 infectivity.

Variants are now mentioned in discussion section (line 376) and a source describing spike processing is present in the introduction (line 65).

Reviewer #2:

Manuscript Summary:

The methods manuscript by Jamieson et al outlines describe a high-throughput protocol using VSV pseudotyped with the SARS-CoV-2 spike protein to measure the presence of neutralizing antibodies in convalescent serum from patients recently recovered from COVID-19. By using a replicating pseudotyped virus, the authors eliminate the necessity for a containment level 3 facility required for SARS-CoV-2 handling, making this protocol accessible to virtually any containment level 2 lab. The use of a 96-well format allows for many samples to be run at the same time with a short turnaround time of 24 hours.

Major Concerns:

1. The resolution of all the figures in the pdf document is poor. Not sure if this is the final resolution or the high resolution ones are with the journal. IF the latter, then there are no concerns. Higher resolution images have been submitted. Please let us know if you would prefer illustrator files.
2. The authors can cite Capcha et (PMID: 33521067) where they also reported on a similar high throughput method for SARS-CoV2 pseudovirus assays. This method is similar, however a transient pseudotype is used in the mentioned source. There are many similar protocols to ours, we would rather not choose one specific publication for comparison.

Minor Concerns:

1. Please include Ethical and safety considerations Complete (line 240 and acknowledgments).
2. Line 58 and 317. Indicate "human" ACE2. Complete.
3. Line 119-121. Please specify the time for GFP expression and supernatant collection. Complete.
4. Line 141-142. This part is not easy to follow. Therefore, if we have a mixture 1:1, the final concentration of CMC is 3%? Final concentration of 3% CMC has been noted.
5. Line 222-223. Is this solution the same used in lines 141-142? Yes, again final concentration of 3% therefore additional CMC should be added to account for the 60uL already in the well.
6. Figures 2a and 3a. I suggest transforming the dilution factor to a logarithmic scale. Also, indicate IC50 for each case. Dilution factor is shown as log2 scale as a 2-fold dilution series was performed. IC50 may be calculated using this method, we did not include it here as individual users may report results differently depending on the application.
7. In Table of materials, please include the source of the VSV-S. Is this purchased or donated. If the latter, acknowledgement is missing. The Whelan lab is mentioned in the acknowledgments, we have added the acknowledgment to the introduction to ensure it is not missed (line 77).

Reviewer #3:

Authors demonstrated a safe, easy, and inexpensive method for measuring neutralizing antibody capacity against SARS-CoV-2 using Spike bearing pseudoviruses. After a minor revision, this protocol would be ready to publish.

Line 86 (and later) - Would it be a problem if Pen/Strep is used in the media? Pen/strep should be used especially when testing human/animal serum, we have not encountered any issues with infection by adding pen/strep. It may also be used for cell plating and virus production (listed as optional for these

steps) (line 96).
Line 114 - From a methods article, it is expected to explain how pseudovirus is prepared as well. However, one should have stock pseudovirus to perform this protocol. This needs to be mentioned earlier and sources to find VSV-S stock virus need to be defined. Is it commercially available or need to be prepared based on a reference article? The source of VSV-S has already been noted in the acknowledgments and cited in the introduction, an explicit mention of the donor lab has been added (line 77).
Line 118 - Does the temperature have to be 34 oC? If it only increases efficiency, and not an absolute requirement, this needs to be mentioned as many labs do not typically use an incubator set to 34 oC. Yes, 34 degree incubation is necessary. This has been highlighted for clarity. Any standard incubator can be changed from 37 to 34 degrees.
Line 159-161 - An example calculation or formula will be more explanatory. Formula has been added (line 202).
Line 210 - 30 ul of this 60 ul aliquot will be virus, so calculation needs to be done accordingly. A note has been added (line 266).
Line 229-233 - Mention about duplicate/triplicate wells, negative/positive control conditions and relative infection calculations/normalizations. Positive, negative and replicates have been highlighted.

Discussion: This method can be used for not only patient serum neutralization, but also neutralization by other drug candidates (targeting Spike-Ace2 interaction). Therefore comments on this protocol's other potential applications will strengthen the manuscript. An additional statement has been added to the discussion (line 372).

cover letter

Figure 1 High Quality

Figure 2 High Quality

Click here to access/download

Supplemental Coding Files

JoVE_Figure 2.ai

Figure 3 High Quality

Click here to access/download

Supplemental Coding Files

JoVE_Figure3.ai