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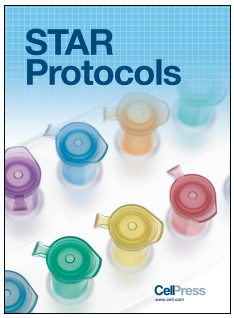

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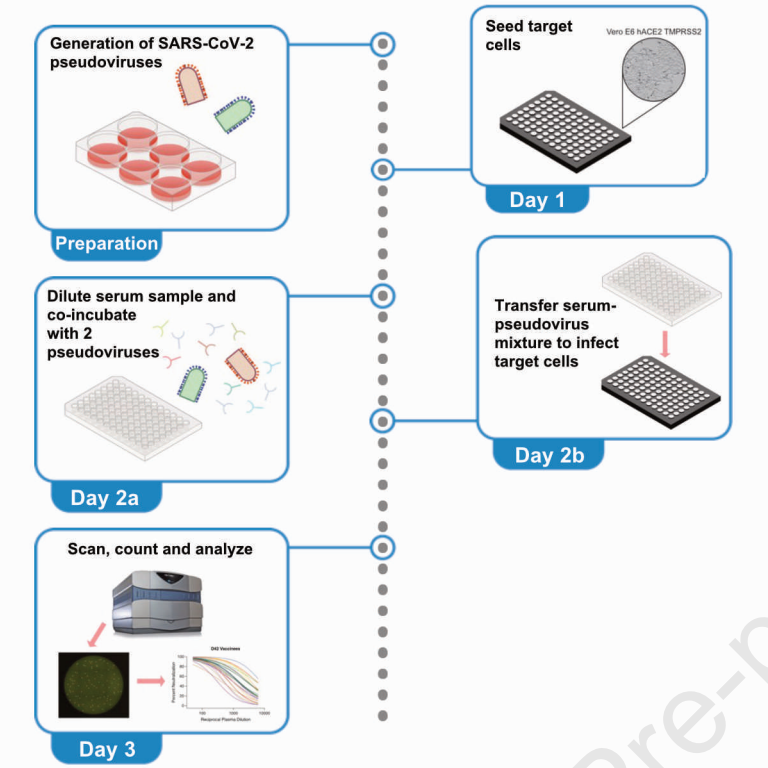

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A high throughput SARS-CoV-2 pseudovirus multiplex neutralization assay

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Summary

Evaluating the neutralizing antibody titer following SARS-CoV-2 vaccination is essential in defining correlates of protection. We describe an assay that uses single-cycle vesicular stomatitis virus (VSV) pseudoviruses linking a fluorophore with a spike (S) from a variant of concern (VOC). Using two fluorophores linked to two VOC S, respectively, allows us to determine the neutralization titer against two VOCs in a single run. This is a generalizable approach that saves time, samples and run-to-run variability.

For complete details on the use and execution of this protocol, please refer to Sievers et al. (2022).

Graphical abstract

Before you begin

Institutional Permissions

The propagation of single-replication vesicular stomatitis virus and the neutralization assay were approved by the J. Craig Venter Institute Institutional Biosafety Committee.

Generation of VSV pseudotyped with SARS-CoV-2 spike (S)

This step describes the production of pseudoviruses used for the neutralization assay described previously[1,2]

Timing: approximately 5 days

1. Coat 6-well plates with 0.05 mg per mL of poly-D-lysine for 1 to 2 hours at room temperature (RT). Wash three times (3X) with sterile water. Store at 4°C for up to a week until use.

Note: If you want to use the plates the day of seeding, coat at a minimum of 1 hour and wash 3X with water before use.

2. Seed the 6-well plates with human embryonic kidney (HEK) 293T at 1.2 x 10^6 cells per well in cell growth media [10% fetal bovine serum (FBS) 1X Dulbecco's modified eagle medium (DMEM)].

3. Grow overnight at 37°C and 5% CO_2.

4. Transfect HEK 293T cells with an expression plasmid encoding the S of SARS-CoV-2 with the last 18 amino acids in the carboxy terminus deleted (pCAGGS-SARS-CoV-2 SΔ18)[1,2].

    Lipofectamine 2000 protocol (per well):
    a. Mixture A: 1 µg of pCAGGS-SARS-CoV-2 SΔ18 + 249 µL of OPTI-MEM; incubate at RT for 5 minutes.
    b. Mixture B: 4 µL of Lipofectamine 2000 + 246 µL of OPTI-MEM; incubate at RT for 5 minutes.
    c. After the 5-minute incubation, combine mixtures A and B together and incubate at RT for 20 minutes.
    d. Aliquot 500 µL of the mixture (A and B) into a well.

Note: Depending on how many VOC pseudoviruses, transfect different set of cells with different plasmids encoding the S of VOCs.

Alternatives: Aside from Lipofectamine 2000, we have also tried other transfecting reagents, such as Polyethylenimine (PEI) Max described below.

    PEI Max protocol (per well):
    e. Add 2 µg of plasmid DNA to 300 µL of OPTI-MEM and vortex briefly.
    f. Then add 6 µg of PEI MAX to the plasmid DNA and OPTI-MEM mix and vortex
    g. Incubate at RT for 15 minutes.
    h. Add the transfection mixture into a well.

Note: Calculations noted above are calculated per well for a 6-well plate. For a full 6-well plate, scale calculation accordingly.

5. Two days following transfection, infect the HEK 293T cell monolayer with the seed VSV-eGFP-ΔG or VSV-mCherry-ΔG virus (a kind gift from Dr. Matthias J. Schnell, Thomas Jefferson University) with a multiplicity of infection (MOI) of 2 to 3. Doubling time for HEK 293T cells is around 24 hours; calculate the MOI based on a 48 hour cell growth timeline.

Note: VSV is not selective in incorporating other viral membrane protein[3,4] and does not require its own glycoprotein for viral egress[5]. Thus, viruses that lack their glycoprotein in their genome such as VSV-eGFP-ΔG and VSV-mCherry-ΔG viruses[6] can easily be pseudotyped with the glycoproteins of other highly pathogenic viruses such as the S of SARS-CoV-2.

6. Carefully aspirate the cell culture growth media off the HEK 293T cell monolayer and discard it.
7. Wash the cell monolayer by gently adding 1 mL of 1X phosphate buffered saline (PBS) to each well, then aspirate off the 1X PBS wash.
8. Infect with VSV-eGFP-ΔG or VSV-mCherry-ΔG with an MOI of 2 or 3 in a 300 µL volume of infection media (2% FBS, 1X DMEM, 1% glutamine, 1% sodium pyruvate**)**.
9. Place back in the incubator at 37°C and 5% CO_2. Rock every 10 to 15 minutes for at least 45 minutes to an hour
10. After infection, carefully remove inoculum and wash with 1X PBS four times to remove residual VSV-eGFP-ΔG or VSV-mCherry-ΔG.

11. Add 2 mL of infection media per well and incubate at 37°C and 5% CO_2.

    Note: To multiplex the assay, generate stocks of each VOC pseudovirus with a specific fluorophore. For example, infect cells transfected with pCAGGS Wuhan (D614) SΔ18 with VSV-eGFP-ΔG, and infect cells transfected with pCAGGS Omicron (B.1.1.529) with VSV-mCherry-ΔG[2]. Thus, when performing the neutralization assay described below (Steps 1-19 under the step-by-step method details section), one can differentiate the titers between Wuhan and Omicron based on fluorophore.

12. One day post infection, harvest and combine cell culture media containing the fluorescent VSV pseudotyped with SARS-CoV-2 S from all the wells and clarify at 2,325 *g* at 4°C for 30 minutes.

13. Aliquot in 1 to 2 mL aliquots and store at -80°C until use.

14. Take one aliquot and titer virus on Vero E6 cells stably expressing human angiotensin 2 (hACE2) and transmembrane serine protease 2 (TMPRSS2) (Vero E6 hACE2 T2A TMPRSS2) to determine the fluorescence focus units per mL (ffu) of the pseudovirus stock (see Steps 16 to 26 for titering procedure below).

15. Store pseudoviruses in -80ºC until use.

Titration of VSV pseudotyped with SARS-CoV-2 spike (S)

This step describes the titration of pseudoviruses on Vero E6 hACE2 T2A TMPRSS2 cells. The data generated from this step will allow the user to accurately generate a 1:1 virus mix ratio when performing the neutralization assay.

Timing: approximately 2.5 days

16. One day prior to titration, seed 96-well half area plates (Greiner BioOne, Cat. No. 675090) with Vero E6 hACE2 T2A TMPRSS2 cells.

17. Wash a confluent T75 flask of Vero E6 hACE2 T2A TMPRSS2 cells with 5 mL of 1X PBS making sure that the monolayer is thoroughly washed with the PBS.

18. Trypsinize Vero E6 hACE2 T2A TMPRSS2 cells with 2 mL of pre-warmed 0.25% Trypsin-EDTA (1X) (Gibco™ 25200056) and place back in the incubator for 3 to 5 minutes.

19. Manually count viable cells using trypan blue using a phase contrast hemacytometer.

20. Dilute cells to 5 x 10^5 cells per mL in cell growth media and transfer unto a sterile reservoir and aliquot 50 µL into each well of the 96-well half area Greiner plates using a multichannel pipette.

21. Incubate 96-well plates overnight or at least for 16 hours at 37ºC, 5% CO_2 prior to use.

22. The day of titration, remove virus from -80ºC storage and thaw at RT.

23. Dilute stock viruses with infection media starting at 1:10 and transfer to row A of a sterile U- or V-bottom (Corning Falcon; Cat. No. 08-772-54) 96-well plate. Serially dilute 3-fold thereafter down the plate to row H. Set aside at RT.

24. Discard the cell growth media from the 96-well half area plate containing the monolayer of Vero E6 hACE2 T2A TMPRSS2 cells and wash twice with 1X PBS.

25. Then transfer 50 µL of serially diluted virus from the U- or V-bottom 96-well plate (in triplicate) using a multichannel pipette. Change tips between different dilutions to avoid cross-contamination.

Note: To minimize plastic tip waste, transfer serially diluted virus from most dilute to least dilute (row H to row A) without changing tips.

26. Sixteen to 24 hours post infection, count focus fluorescence units (ffu) using a Celigo Imaging Cytometer as described below.

Note: Calculate the viral titer (ffu per mL) using the following equation: ffu per mL = number of ffu/dilution factor*volume of diluted virus[7]. For example, 5300 ffu counted of the 1 x 10^{-3} dilution (5300/0.001*0.050) would yield a titer of 2.65 x 10^5 ffu per mL. If viral titers are lower than expected, check expression level of the S following transfection of HEK 293T cells (Step 5) using a commercially available polyclonal antibody against the S (ThermoFisher Cat. No. PA1-41165). Cell surface expression immunostaining was described previously [8].

Materials and equipment

Growth Media (10% FBS, 1X DMEM)

Reagent	Final concentration	Amount
DMEM	88%	440 mL
FBS	10%	50 mL
Penicillin/Streptomycin	1%	5 mL

Glutamine	2 mM	5 mL
Total	**n/a**	**500 mL**

Infection Media (2% FBS, 1X DMEM, 1% glutamine, 1% sodium pyruvate)

Reagent	**Final concentration**	**Amount**
DMEM	94%	470 mL
FBS	2%	10 mL
Penicillin/Streptomycin	1%	5 mL
Glutamine	2 mM	5 mL
Sodium Pyruvate	1 mM	5 mL
Non-essential Amino Acids	1%	5 mL
Total	**n/a**	**500 mL**

Note: All media should be stored at 4°C and used within a month. Both infection media and growth media are suitable for both Vero E6 hACE2 T2A TMPRSS2 cells and HEK293T cells.

Step-by-step method details

Day 1
Seeding 96-well plates with target cells

This step explains the seeding of the target cells in 96-well plates used for the neutralization assay.

Timing: Approximately 30 min, depending on the number of plates seeded

This section describes the preparation of the plates.

1. Seed a 96-well half area plate with Vero E6 hACE2 T2A TMPRSS2 cells as described in steps 16-20 of the before you begin section.

2. Incubate 96-well plates overnight and at least for 16 hours at 37ºC, 5% CO_2 prior to use.

CRITICAL: Ensure that the cells are well trypsinized and that a single-cell suspension is obtained. This will result in more accurate cell counting and provide an even monolayer when seeding.

Note: The cell dilution above is equal to plating 2.5 x 10^4 cells per well. You will need approximately 5 mL of cells per plate.

Day 2
Sample Dilution

This step describes the serial dilution of patient samples in a separate 96-well plate (U- or V-bottom plate).

Timing: Approximately 2 h (add 10 min per extra plate)

3. In a sterile 96-well U- or V-bottom plate (Corning Falcon, Cat. No. 08-772-54), add 33.6 µL of infection media to row A (Columns 1 to 10) and row E (columns 1 to 10). Add 28 µL of infection media to the remaining wells. Add 1.4 µL of plasma sample to row A (columns 1 to 10) and row E (columns 1 to 10). Duplicates can be side by side (wells A1 and A2) or vertically (wells A1 and E1) (**Figure 1**).

Note: Dilution described above is 1:25 dilution.

4. Perform a 5-fold serial dilution by transferring 7 µL of the plasma/infection media from row A down to row D. Perform the same serial dilution for the duplicate from row E down to row H. Discard the last 7 µL so that all wells have a total volume of 28 µL.

5. Make a master mix of the two SARS-CoV-2 pseudoviruses, each containing a unique fluorophore (eGFP and mCherry) at a 1:1 ratio. Ideally, titers should be at 50 to 100 ffu per 25 µL in infection media.

Note: 2.5 mL of pseudovirus are required for each 96-well plate.

6. Add 25 µL of SARS-CoV-2 pseudoviruses from row D up to row A and from row H up to row E. For column 11 add 25 µL of virus; this will be the virus only control. For column 12, add 25 µL of infection media; this will be the cells only control.

CRITICAL: Add the virus from the most diluted plasma to the least diluted plasma (row D to A; row H to E) to prevent potential carry-over of highly neutralizing antibodies (if done the opposite way) that may skew the neutralization curve.

7. Cover the dilution plate and incubate at 37°C and 5% CO_2 for 1 hour.

Pre-incubation and infection

This step describes the infection of the target cells.

Timing: Approximately 15 minutes is required to prepare one 96-well plate; add 7 minutes per additional plate.

8. Wash the half area 96-well plate containing the Vero E6 hACE T2A TMPRSS2 target cells with 50 µL of 1X PBS twice to remove residual growth media, but gently without disturbing the cells.

9. Transfer 50 µL of the pre-incubated plasma-virus mixture from rows A to D of the U- or V-bottom plates to its corresponding wells of the 96-well plate containing Vero E6 hACE T2A TMPRSS2 target cells with a multichannel pipette. To decrease risk of cross-contamination, change tips for each row. In a similar manner, transfer plasma/virus mixture from rows E to H for the duplicate sample.

CRITICAL: To minimize plastic waste, one can transfer the plasma-pseudovirus mixture from the least diluted to the most diluted plasma sample (row A to D, row E to H) without

changing tips. The row with the least diluted plasma (row A or E), hypothetically has the least amount of infectious viruses and the most diluted (row D or H), has the largest amount of infectious virus. By contrast, if the mixture is transferred from the most diluted (row D or H) to the least diluted plasma (row A or E), non-neutralized virus is potentially transferred up the wells and can skew the neutralization data.

10. Incubate (infection) at 37°C and 5% CO_2.

11. Read plates at 16- to 24-hour post infection.

Day 3
Read plate using Celigo Image Cytometer

This section describes how to measure fluorescence via a Celigo Image Cytometer.

Timing: Approximately 20 min per plate

12. Approximately 16-24 hours post infection, place the plate in the Celigo Image Cytometer.
    a. Start the Celigo cytometer system according to standard protocol and select "Create New."
    b. Choose the correct plate type and create a unique Plate ID. Once completed, select "Load Plate."

Note: Consider using plates that are supported by the Celigo Image Cytometer.

13. In the "Scan" tab, under "Application," select "Expression Analysis," and "Target 1 + 2 + 3" as there are three channels needed for performing two-color fluorescence analysis. One channel is dedicated to brightfield (BF), and a second and third channel to the detection of the two fluorophores.
    a. Click the yellow finger icon next to "Application" to name your different channels.
    b. It is advisable to label them as: i) BF, ii) color 1 (*e.g.*, eGFP) and iii) color 2 (*e.g.*, red fluorescent protein for mCherry) as it will prevent confusion when assigning the color filter (**Figure 2**) and during analysis.

14. Select BF as your first channel and under "Motion Control" select "Focus Setup" and "Register Auto". Set a minor offset (-5 to -10) using the down arrow next to "Auto Focus."

Note: Setting a slight offset helps with the counting efficiency.

15. For the first fluorescence analysis, select your second channel, and specific to this protocol, we labeled it as "GFP VOC 1" since it was a pseudovirus that encodes the eGFP gene pseudotyped with the S of a VOC. Then choose the 'Green 483/536' under illumination and exposure time (**Figure 3**).
    a. Select "Find Focus" after proper exposure.

b. Then select “Set Offset”.

16. For the second fluorescence analysis, select your third channel, and specific to this protocol, we labeled it as “mCherry VOC 2” since it was a pseudovirus that encodes mCherry gene pseudotyped with the S of another VOC. Then choose ‘Red 531/629’ under illumination and exposure time (**Figure 4**).
    a. Select “Find Focus” after proper exposure.
    b. Then select “Set Offset”.

Note: We found an exposure time of 35,000 µs for eGFP (Green 483/536) and 100,000 µs for mCherry (Red 531/629) to be the optimal length of exposure time for our work.

17. Highlight all the wells using your cursor, ensuring that all wells light up yellow and select “Start Scan” (**Figure 5**).

18. To start the analysis, complete the following:
    a. Under the “Analysis Settings” select “Well Mask” and set the “% Well Mask” to 100%.
    b. Under “Identification”, ensure correct channel is selected and set the “Cell Diameter” to 15 µm.
    c. Under “Pre-Filtering” set the “Cell Area” to 40 μm^2.

19. Export the data by selecting “Export Well-Level Data.”

Expected outcomes

When performed properly, this protocol is designed to measure the neutralizing antibody titer from serum or plasma samples collected from infected and vaccinated patients. This can also be applied to testing the neutralizing capacity of monoclonal antibodies. The use of two pseudoviruses expressing unique fluorophores enables us to evaluate two different VOCs at one time. This saves time and patient samples. It hypothetically also allows for better direct comparison of the titers against different VOCs, eliminating variability of cell growth, passage and density. **Figure 6** shows data from an experiment against a SARS-CoV-2 VOC, where each square unit represents a well in the 96-well plate as described in **Figure 1**. After scanning and analysis, the numbers in each square represents a fluorescent-expressing cell that was infected with a pseudovirus. According to the layout described in **Figure 1**, the presence of high titers of neutralizing antibodies will yield no fluorescence units in target cells. However, as you dilute out the neutralizing antibodies in the plasma (from row A to D and E to H), there will be a concomitant increase of fluorescently-labeled cells representing viral infection due to lack of neutralization (**Figure 6**). A non-linear regression curve is then generated from the data, from which the half-maximal inhibition dose (ID_{50}) is calculated. The ID_{50} is the value that is typically reported in most studies[9-11].

In validating and optimizing our multiplex neutralization assays, we had first generated a panel of SARS-CoV-2 VOCs and titered them on different cell lines to determine the infection efficiency for each pseudovirus strain. During these experiments, we observed that Vero E6

hACE T2A TMPRSS2 cells are the most susceptible cell line for the majority of the VOCs and thus yielded the highest titers for our panel of pseudoviruses. Based on their titers on this cell line, we used a 1:1 mix ratio and have not seen any competition in infectivity between the different VOCs.

Quantification and statistical analysis

1. In GraphPad Prism, create an XY file with 2 replicate values in side-by-side subcolumns.

2. Label the X-axis as “Reciprocal Plasma Dilution” and input the descending dilution factors. The Y-axis should be labelled with the specific sample identification numbers.

3. For the input values, calculate the percent neutralization (pNT) with the following equation:

   pNT= 100-[(ffu in target well)/(average ffu of all virus only control)*100]

4. Input the percent neutralization values into GraphPad Prism sheet as shown below.

5. Under the button labelled “Analyze,” select “Transform” and ensure that all sample ID’s match to the ones selected for analysis.

6. For the parameters of the transformation, select the box next to “Transform x vales using” and select “X=Log(X)”. Also, select the box next to “Create a new graph of the results.”

7. On the transformed data, select “Analyze” and under “XY Analyses” select “Nonlinear regression (curve fit).”

8. When choosing an equation use “log(inhibitor) vs. response – Variable slope (four parameters).” Before selecting “OK,” under the tab labelled “Constrain” set a constant constraint equal to “0” on the bottom and “100” on the top.

9. Once analysis is complete, a graph will be generated as seen below. The graph generated in **Figure 7** reflects the data presented in **Figure 6**. Samples 7 and 8 have low to no neutralizing activity, respectively, while the non-linear curve of sample 2 is an example of a strong neutralizer. Lastly, samples 1, 3, 4, 5, 7, 9 and 10 are examples of potent neutralizers given that even at the highest dilution, neutralizing activity is still detected.

Note: Incorporation of a positive control (pooled from multiple samples) can be incorporated for each plate or run to validate the assay. However, note that the positive control may not neutralize equally against all VOCs.

10. The ID_{50} for each sample will be automatically calculated for each sample and reported under “Nonlin fit”.

Limitations

Serum samples with low to no neutralizing antibody titer may provide low and variable percent inhibition values, which at times may give minimally false positive values or ID_{50}. Thus, it is highly advisable to also include a negative serum or a no sample control in order to define the background of the run.

There are advantages and limitations with using the fluorescent-based neutralization assay rather than the traditional luciferase-based neutralization assay. Compared to the luciferase-based neutralization assay, the fluorescent reporter assay is not as sensitive with a smaller range of detection[12]. However, while there is a relative decrease in sensitivity, the fluorescent-based assay provides a method to multiplex the neutralization assay, which is not possible with luciferase-based assays. In the present manuscript, we used two fluorescent proteins to simultaneously measure the neutralizing titers against two different SARS-CoV-2 VOCs. It is possible to increase the throughput of the assays by increasing the number of fluorescent proteins and strains measured. Thus depending on the different channels or lasers found on your plate reader, more than two strains can be included in a future assay.

Troubleshooting

Problem 1: Low SARS-CoV-2 pseudovirus titer (step 16-26 under before you begin section)

Potential solution:

Low SARS-CoV-2 titer can be due to i) infection with a low MOI of the seed VSV-eGFP-ΔG or VSV-mCherry-ΔG, ii) transient expression of the SARS-CoV-2 S was not optimal, or iii) target cell is not the ideal cell line for viral entry.

- In our experience, increasing the MOI of the seed virus to at least 2 or more can dramatically increase the titer of the resulting pseudovirus.
- To generate a high titer of SARS-CoV-2 pseudovirus, transient expression of the S is crucial. We recommend that each laboratory perform their own optimization using their own experience with timing and transfection reagent to determine what works best. In our experience, we have used both Lipofectamine 2000 and PEI Max with equal success and harvested pseudoviruses at 2 days post infection.
- While optimizing our assay, we used a panel of different cell lines susceptible to SARS-CoV-2 infection. We observed that the cell lines used for target cells had different susceptibility to SARS-CoV-2 VOCs infection. Thus, we identified and chose the one cell line, Vero E6 hACE2 T2A TMPRSS2, which provided the optimal susceptibility of infection to most VOCs for the neutralization assays.
- Expression of S gene when making the pseudoviruses might be low. Check expression level of the S protein using a commercially available antibody (dilution factor of 1:200 to 1:500) to determine if transfection efficiency is optimal. If S expression is low then optimize transfection protocol by either switching transfection reagent, modify DNA and transfection reagent ratio, modify DNA plasmid input, use lower passage cell line for transfection and or check cell culture for mycoplasma.

Problem 2: HEK 293T cells are dislodge during transfection and/or infection (steps 1-15 under before you begin section)

Potential Solution:

- HEK 293T cells are highly transfectable cells, but are prone to dislodge if the tissue culture plate is not pre-treated with poly-D-lysine or similar matrices. An alternative cell line to use is the baby hamster kidney 21 cell line. While not as highly transfectable, their ability to adhere to the surface of tissue culture plates is highly desirable and we have used this cell line in the past to generate high titers of SARS-CoV-2 pseudoviruses.
- Increase the concentration of poly-D-lysine to 100 µg per mL or use a different matrix.

Problem 3: Inaccurate fluorescent forming unit counting (steps 12-19 under step-by-step method details)

Potential solution:

- Make sure to mask wells at least 100%. However, if there are still some extraneous fluorescence that are counted, mask the wells at a lower setting (*e.g.*, 98%).

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gene S. Tan (gtan@jcvi.org).

Materials availability

Plasmids encoding the spike of SARS-CoV-2 generated in this study will be made available upon completion of a material transfer agreement.

Data and code availability

This study did not generate/analyze [datasets/code].

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Author contributions

BLS, TG and GST wrote and edited the manuscript.

Declaration of interests

The authors declare no competing interests.

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Figure 1. A visual layout showing how much infection media (µL) to add to a sterile 96-well plate.

Figure 2. Label each channel with the appropriate nomenclature to prevent confusion when performing analysis. (*e.g.*, BF = brightfield, GFP = green fluorescent protein and RFP = red fluorescent protein)

Figure 3. Assigning and labeling Channel 2 with a specific laser that will detect a fluorophore (*e.g.*, GFP).

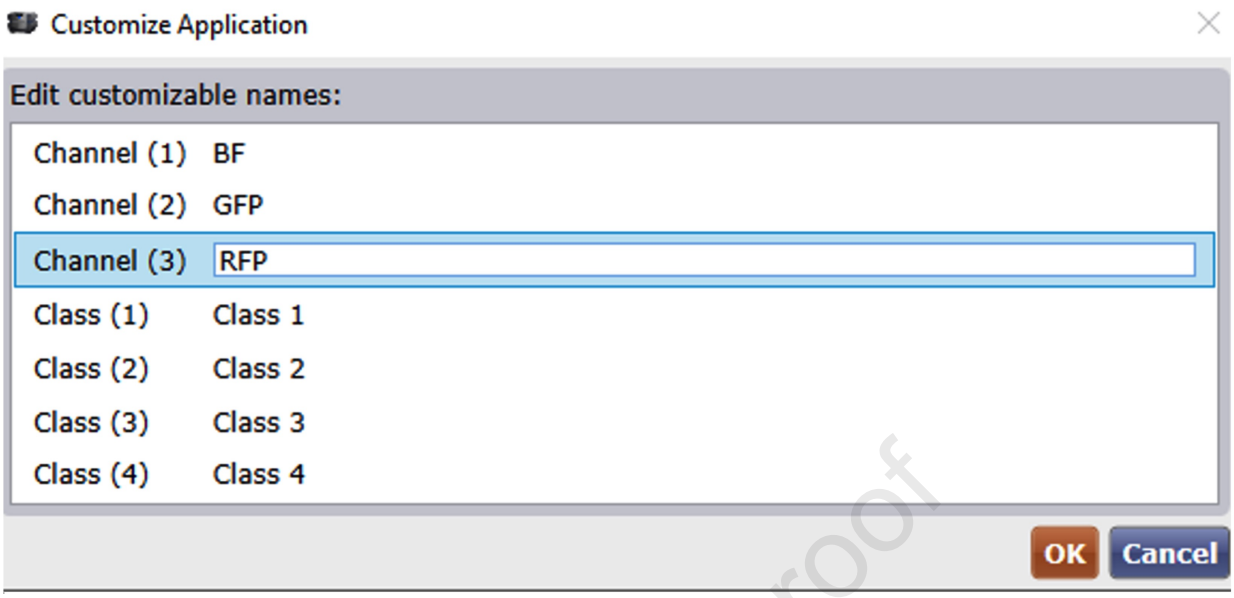
Figure 4. Assigning and labeling Channel 3 with a specific laser that will detect a second fluorophore (*e.g.*, RFP).

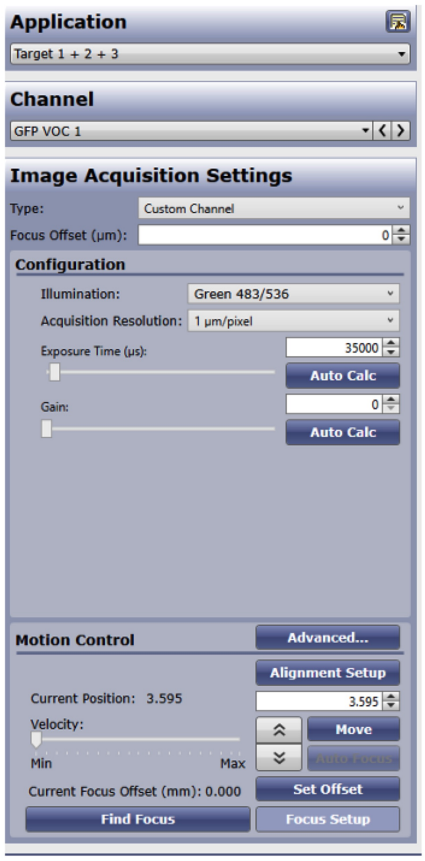
Figure 5. Press 'Selection' and then highlight by dragging the pointer to select all the wells in the 96-well plate. Selected wells will turn yellow as shown above.

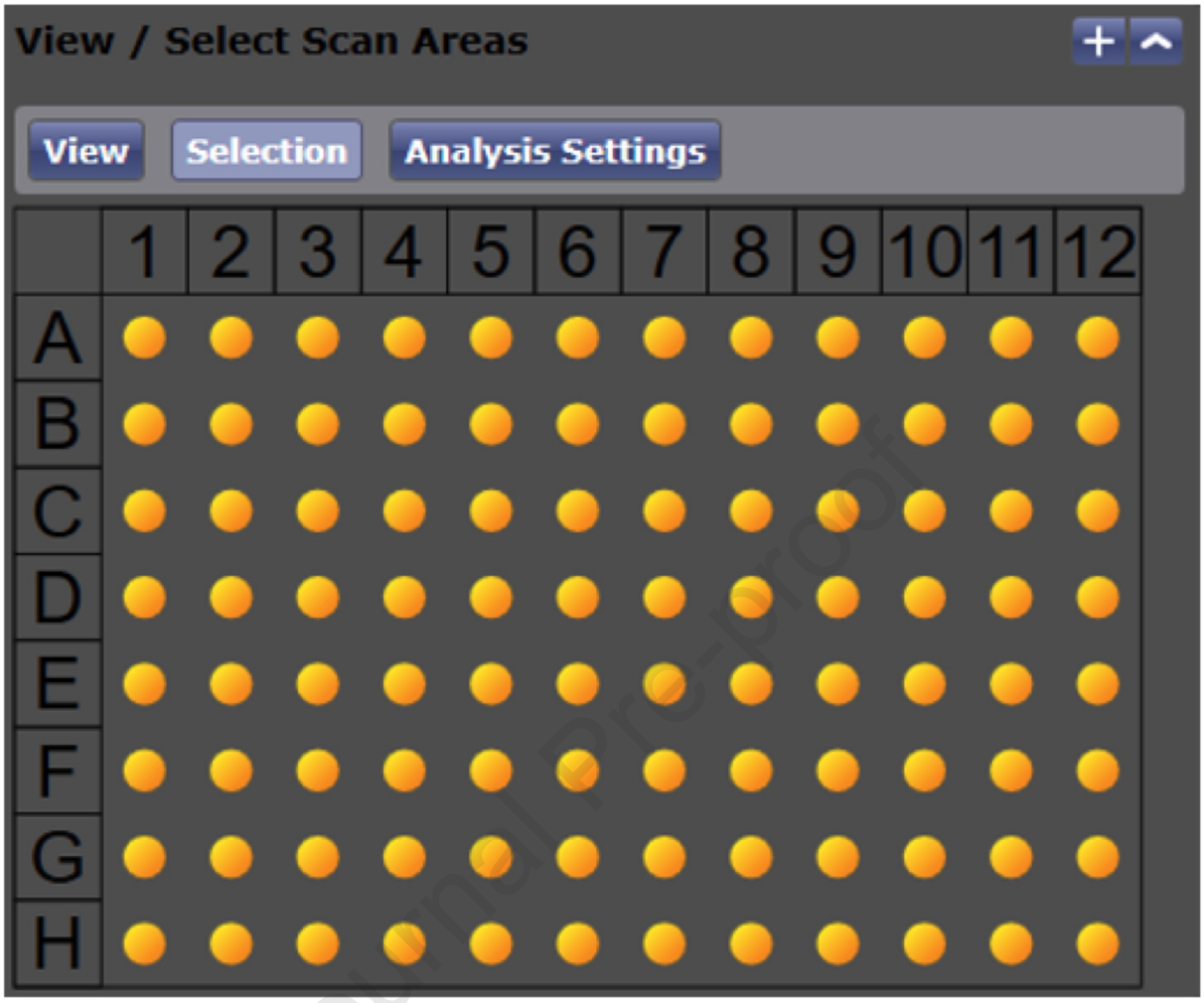
Figure 6. Example of results produced by the Celigo Cytometer of a neutralization assay. One square represents one well in a 96-well plate previously laid out in Figure 1. Columns 1 to 10 represents one sample performed in duplicate (*e.g.*, A1 to D1 and E1 to H1). Of note, plasma is diluted from A1 to D1 and from E1 to H1. Column 11 is the 'virus only' control in the absence of plasma. A positive control (typically pooled plasma from multiple samples) can be added in wells A12 to D12. Wells E12 to H12 is the 'target cell only' control, in the absence of pseudovirus and plasma. The numbers produced below each image are the fluorescence-forming units (ffu), indicating one cell infected with a viral particle.

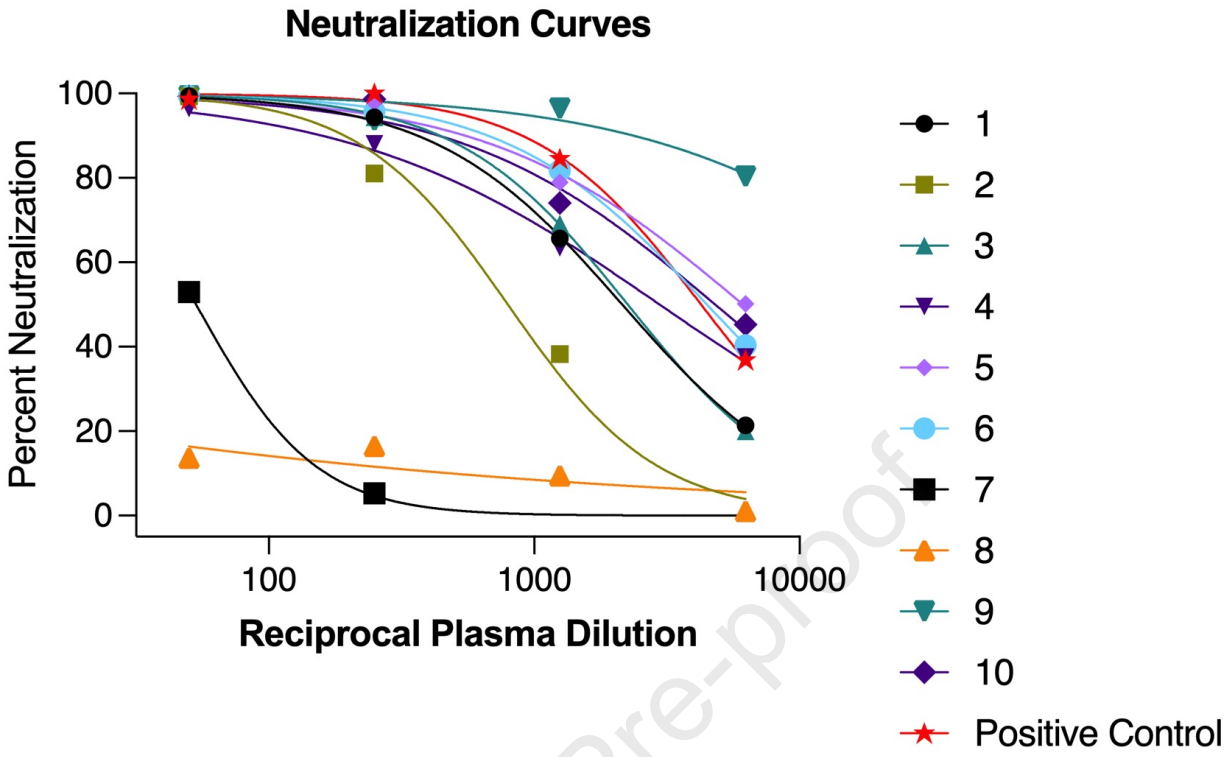
Figure 7. Neutralization curves generated using from data from Figure 6. For the sake of clarity, error bars are not displayed.

	1	2	3	4	5	6	7	8	9	10	11	12
A	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	28 µL	28 µL
B	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL
C	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL
D	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL
E	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	33.6 µL	28 µL	28 µL
F	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL
G	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL
H	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL	28 µL









Highlights

- Procedure for the titration of SARS-CoV-2 pseudoviruses.
- Steps to perform a multiplexed SARS-CoV-2 pseudovirus neutralization assay.
- Simultaneous analysis of two distinct fluorophore-linked pseudoviruses.

KEY RESOURCES TABLE

The table highlights the reagents, genetically modified organisms and strains, cell lines, software, instrumentation, and source data **essential** to reproduce results presented in the manuscript. Depending on the nature of the study, this may include standard laboratory materials (i.e., food chow for metabolism studies, support material for catalysis studies), but the table is **not** meant to be a comprehensive list of all materials and resources used (e.g., essential chemicals such as standard solvents, SDS, sucrose, or standard culture media do not need to be listed in the table). **Items in the table must also be reported in the method details section within the context of their use.** To maximize readability, the number of **oligonucleotides and RNA sequences** that may be listed in the table is restricted to no more than 10 each. If there are more than 10 oligonucleotides or RNA sequences to report, please provide this information as a supplementary document and reference the file (e.g., See Table S1 for XX) in the key resources table.

Please note that ALL references cited in the key resources table must be included in the references list. Please report the information as follows:

- **REAGENT or RESOURCE:** Provide full descriptive name of the item so that it can be identified and linked with its description in the manuscript (e.g., provide version number for software, host source for antibody, strain name). In the experimental models section (applicable only to experimental life science studies), please include all models used in the paper and describe each line/strain as: model organism: name used for strain/line in paper: genotype. (i.e., Mouse: OXTR$^{fl/fl}$: B6.129(SJL)-Oxtr$^{tm1.1Wsy/J}$). In the biological samples section (applicable only to experimental life science studies), please list all samples obtained from commercial sources or biological repositories. Please note that software mentioned in the methods details or data and code availability section needs to also be included in the table. See the sample tables at the end of this document for examples of how to report reagents.

- **SOURCE:** Report the company, manufacturer, or individual that provided the item or where the item can be obtained (e.g., stock center or repository). For materials distributed by Addgene, please cite the article describing the plasmid and include “Addgene” as part of the identifier. If an item is from another lab, please include the name of the principal investigator and a citation if it has been previously published. If the material is being reported for the first time in the current paper, please indicate as “this paper.” For software, please provide the company name if it is commercially available or cite the paper in which it has been initially described.

- **IDENTIFIER:** Include catalog numbers (entered in the column as “Cat#” followed by the number, e.g., Cat#3879S). Where available, please include unique entities such as RRIDs, Model Organism Database numbers, accession numbers, and PDB, CAS, or CCDC IDs. For antibodies, if applicable and available, please also include the lot number or clone identity. For software or data resources, please include the URL where the resource can be downloaded. Please ensure accuracy of the identifiers, as they are essential for generation of hyperlinks to external sources when available. Please see the Elsevier list of data repositories with automated bidirectional linking for details. When listing more than one identifier for the same item, use semicolons to separate them (e.g., Cat#3879S; RRID: AB_2255011). If an identifier is not available, please enter “N/A” in the column.
    - ***A NOTE ABOUT RRIDs:*** We highly recommend using RRIDs as the identifier (in particular for antibodies and organisms but also for software tools and databases). For more details on how to obtain or generate an RRID for existing or newly generated resources, please visit the RII or search for RRIDs.

Please use the empty table that follows to organize the information in the sections defined by the subheading, skipping sections not relevant to your study. Please do not add subheadings. To add a row, place the cursor at the end of the row above where you would like to add the row, just outside the right border of the table. Then press the ENTER key to add the row. Please delete empty rows. Each entry must be on a separate row; do not list multiple items in a single table cell. Please see the sample tables at the end of this document for relevant examples in the life and physical sciences of how reagents and instrumentation should be cited.

TABLE FOR AUTHOR TO COMPLETE

Please upload the completed table as a separate document. ***Please do not add subheadings to the key resources table.*** *If you wish to make an entry that does not fall into one of the subheadings below, please contact your handling editor.* ***Any subheadings not relevant to your study can be skipped.*** *(**NOTE:** For authors publishing in Cell Genomics, Cell Reports Medicine, Current Biology, and Med, please note that references within the KRT should be in numbered style rather than Harvard.)*

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
SARS Coronavirus Spike Protein antibody; stock is 1.0 mg/mL and working dilution is 1:500	ThermoFisher Scientific	PA1-41165
Bacterial and virus strains		
rVSV-GFP-ΔG	J. Craig Venter Institute Infectious Disease Department – a gift from Dr. Matthias J. Schnell	N/A
rVSV-mCherry-ΔG	J. Craig Venter Institute Infectious Disease Department – a gift from Dr. Matthias J. Schnell	N/A
Chemicals, peptides, and recombinant proteins		
Lipofectamine 2000	Thermo Fisher	11668019
PEI MAX – Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40K)	Polysciences	24765-1
1X OPTI-Modified Eagle Medium (MEM)	Thermo Fisher	51985091
Poly-D-Lysine	Thermo Fisher	A3890401
Penicllin and Streptomycin (PenStrep)	Gibco	15140-122
L-Glutamine	Sigma	G7513
Sodium Pyruvate (100 mM)	Gibco	11360-070
1X Dulbecco's Modified Eagle Medium (DMEM)	Gibco	10566-016
Fetal Bovine Serum Characterized (FBS)	HyClone	SH30071.03
0.25% Trypsin-EDTA	Gibco	25200-056
1X phosphate buffered solution (PBS), pH 7.4	Gibco	10010023
Nonessential Amino acids (100X)	Gibco	2428385
Experimental models: Cell lines		
Vero E6-TMPRSS2-T2A-ACE2; up to 20 passages	BEI Resources, National Institute of Allergy and Infectious Diseases	NR-54970
HEK 293T; up to 20 passages	American Type Culture Collection	CRL-3216
Recombinant DNA		

pCAGGS Wuhan (D614) SΔ18	J. Craig Venter Institute Infectious Disease Department (Sievers et al. 2022; Chakraborty et al. 2022).	N/A
pCAGGS Beta (B.1.351) SΔ18	J. Craig Venter Institute Infectious Disease Department (Sievers et al. 2022)	N/A
pCAGGS Delta (B.1.617.2) SΔ18	J. Craig Venter Institute Infectious Disease Department (Sievers et al. 2022)	N/A
pCAGGS Omicron (B.1.1.529) SΔ18	J. Craig Venter Institute Infectious Disease Department (Sievers et al. 2022)	N/A
Software and algorithms		
Prism 9.4.1	GraphPad	www.graphpad.com
Other		
96-well ½ area plates	Sigma	675090
96-well non-treated, U-shaped-bottom microplate	Corning	351177
6-well plates	Falcon	353046
Celigo Imaging Cytometer	Nexcelom	200-BFFL-5C