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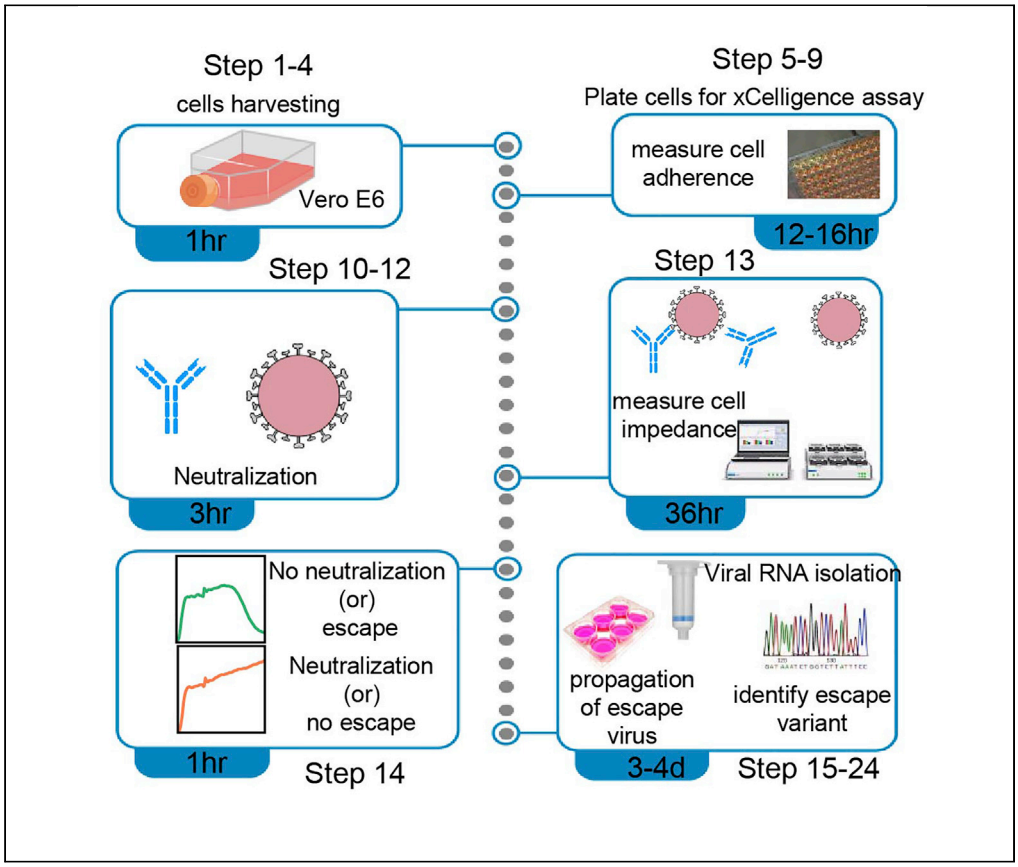


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Naveenchandra Suryadevara, Pavlo Gilchuk, Seth J. Zost, Nikhil Mittal, Li Leyna Zhao, James E. Crowe, Jr., Robert H. Carnahan

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## Highlights

Rapidly identify potent neutralizing antibodies from hundreds of candidates

Quickly identify and isolate virus variants that escape from antibody neutralization

Assay provides facile pathway to identify critical residues that mediate viral escape

Avoids virus-specific reagents (ex. reporter viruses) and labor-intensive approaches

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# Real-time cell analysis: A high-throughput approach for testing SARS-CoV-2 antibody neutralization and escape

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## SUMMARY

**Real-time cell analysis (RTCA) enables high-throughput, quantitative kinetic measurements of cytopathic effect (CPE) in virus-infected cells. Here, we detail a RTCA approach for assessing antibody neutralization. We describe how to evaluate the neutralizing potency of monoclonal antibodies (mAbs) and identify viral escape mutants to antibody neutralization for severe respiratory syndrome coronavirus 2 (SARS-CoV-2).**

**For complete details on the use and execution of this protocol, please refer to Zost et al. (2020) and Suryadevara et al. (2021).**

## BEFORE YOU BEGIN

### Protocol overview

Timing: 30 min for preparing culture medium

Timing: 8–12 days for antibody production and purification

Timing: 1–2 h for quantification of antibodies

Timing: 3–4 days for virus propagation and titration

Timing: 5 min for 70% ethanol

Timing: 25 min for equipment setup

In recent years, monoclonal antibodies (mAbs) increasingly have been deployed as therapeutic countermeasures against viral infections. Antibodies have been approved for respiratory syncytial virus (Johnson et al., 1997), Ebola virus (Corti et al., 2016a; Pascal et al., 2018) and human immunodeficiency virus (HIV) (Reimann et al., 1997). There are dozens of human mAbs in clinical trials against additional viral targets, such as influenza virus (Laursen and Wilson, 2013), Marburg virus (Flyak et al., 2015), Zika virus (ZIKV) (Sapparapu et al., 2016), Lassa virus (Robinson et al., 2016), Middle East respiratory syndrome coronavirus (MERS-CoV) (Corti et al., 2016b), poxviruses (Gilchuk et al., 2016), and Nipah virus (Geisbert et al., 2014). The current SARS-CoV-2 pandemic has further highlighted

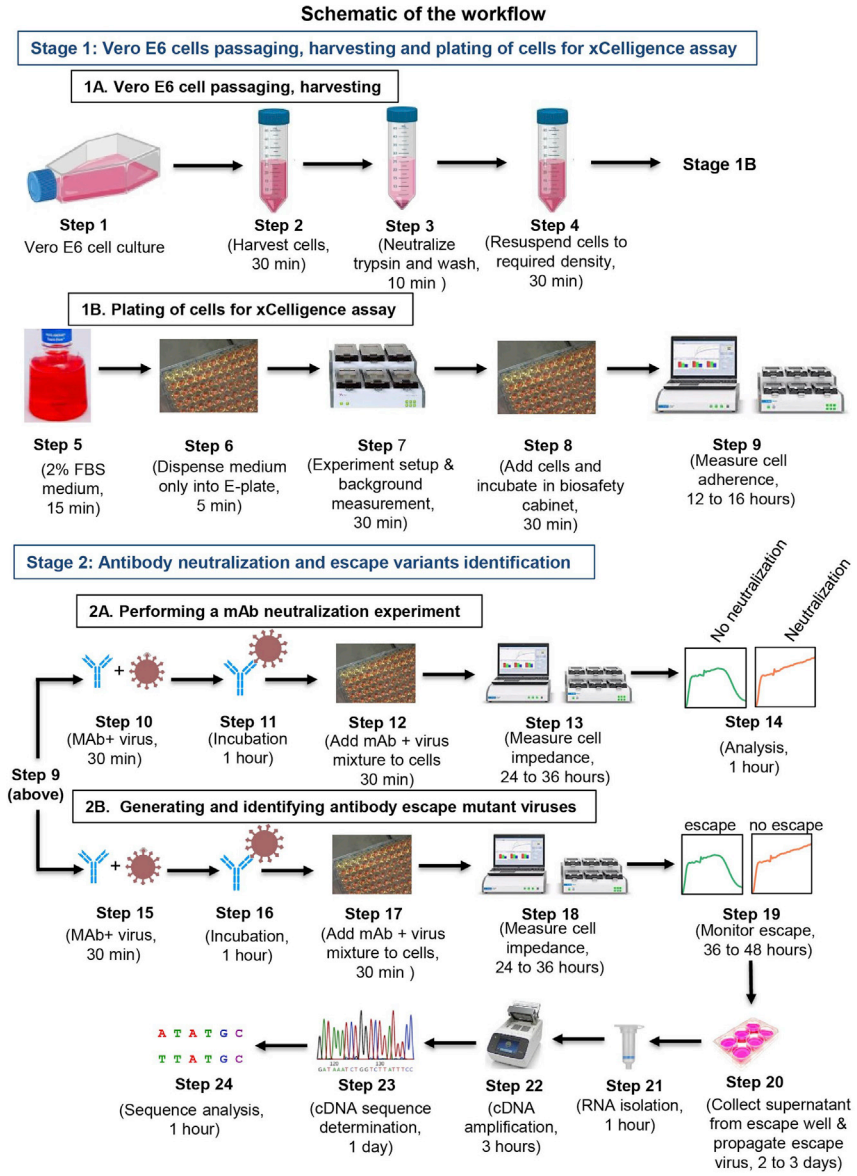


**Figure 1. Complete overview of the RTCA assay**

A schematic showing the overview of RTCA assay, starting from Vero E6 cells passaging, harvesting, and plating of cells for xCelligence assay, neutralization and escape variants identification.

the importance of the rapid development of human antiviral mAbs for COVID-19 prevention and treatment. The protocol describes *in vitro* monoclonal antibody neutralization assays and escape mutant identification using a RTCA assay-based workflow with a replication-competent recombinant vesicular stomatitis virus (rVSV) expressing the SARS-CoV-2 spike protein (rVSV-SARS-CoV-2) (Case et al., 2020; Gilchuk et al., 2022) (Figure 1). RTCA-based neutralization is monitored by measuring changes in cell impedance caused by virus infection of cell monolayers and displayed as cell index. Careful evaluation is required when selecting cell lines for RTCA, as many viruses do not replicate or cause cytopathic effects (CPE) in certain cell lines due to host-range restriction factors, such as the lack of an authentic cell-surface receptor to facilitate attachment and entry (Hoffmann et al., 2020). For instance, our laboratory has used this technique for identifying and characterizing neutralizing antibodies against Ebola virus (Gilchuk et al., 2020b, 2021b), Zika virus (Gilchuk et al., 2020a), influenza virus (Gilchuk et al., 2021a), and Nipah virus (Doyle et al., 2021).

1. Prepare cell culture medium.
   a. 10% fetal bovine serum (FBS) Dulbecco's Modified Eagle Medium (DMEM).
   Prepare cell culture medium in a biosafety cabinet. To make a 10% FBS solution, add 55 mL FBS into 500 mL high-glucose DMEM supplemented with 1% penicillin/streptomycin solution. Use 10% FBS DMEM for cell propagation.
   b. 2% FBS DMEM culture medium.
   To make 2% FBS DMEM, add 10 mL FBS into 490 mL high-glucose DMEM supplemented with 1% penicillin/streptomycin solution. Use 2% FBS DMEM for mAb dilutions, neutralization assays, and experiment to identify antibody escape mutant viruses.

2. Antibody production, purification, and quantification.
   a. Production and purification.
   Sequences encoding antibody variable genes are synthesized by a commercial DNA synthesis service and cloned into an IgG1 expression plasmid (pTwist CMV BetaGlobin WPRE Neo) were used for mAb expression in mammalian cell culture for secretion into cell supernatant. We previously described a microscale expression approach for mAbs in 1 mL Expifectamine™ Chinese Hamster Ovary (ExpiCHO; Thermo Fisher Scientific) cell cultures in 96-well plates (Gilchuk et al., 2020a). For larger-scale mAb expression, we performed transfection in larger volume suspensions (15–20 mL per antibody) of ExpiCHO cell cultures using the Gibco ExpiCHO Expression System and protocol for 50 mL Mini Bioreactor tubes (Corning), as described by the vendor. Culture supernatants were purified using HiTrap MabSelect SuRe (Cytiva, formerly GE Healthcare Life Sciences) on a 24-column parallel protein chromatography system (Protein BioSolutions). Purified mAb IgG1 proteins were buffer-exchanged into PBS, concentrated using Amicon Ultra-4 50-kDa centrifugal filter units (Millipore Sigma), and stored at 4°C until use (Gilchuk et al., 2020a).
   b. Quantification of antibodies;
   Quantification of purified mAbs was performed by UV spectrophotometry using a spectrophotometer (NanoDrop, ND-8000 thermo scientific) and accounting for the extinction coefficient of human IgG.

3. Virus propagation and titration.

The generation of a replication-competent rVSV expressing SARS-CoV-2 spike protein with a 21 amino-acid C-terminal deletion that replaces the VSV G protein (VSV-SARS-CoV-2) was described previously (Case et al., 2020). The spike protein-expressing VSV virus was propagated in MA104 cell culture monolayers (African green monkey, American Type Culture Collection [ATCC] CRL-2378.1) as described previously (Case et al., 2020), and viral stocks were titrated on Vero CCL81 cell monolayer cultures (ATCC CRL-1586). Titers are back calculated based on VSV plaque counts obtained by neutral red staining and visual counting. Figures 2A and 2B shows a virus titration using RTCA assay and the suggested dilution to choose for neutralization assays.

4. 70% ethanol.

Mix 150 mL nuclease-free water with 350 mL absolute ethanol in a 500-mL bottle and keep at 20°C–25°C, for use in sterilizing working surfaces of the laminar flow hood.

CAUTION 70% ethanol is still flammable. Keep stocks away from fire.

5. Equipment setup.

The xCELLigence RTCA MP (Multiple Plates) instrument (Agilent Technologies) uses impedance biosensors for continuous label-free monitoring of cell behaviors that alter cellular impedance. Depending on the exact model of instrument used, cells in each well of one or six parallel plates can be simultaneously monitored in real time. The multiple plate format is ideal for large screening

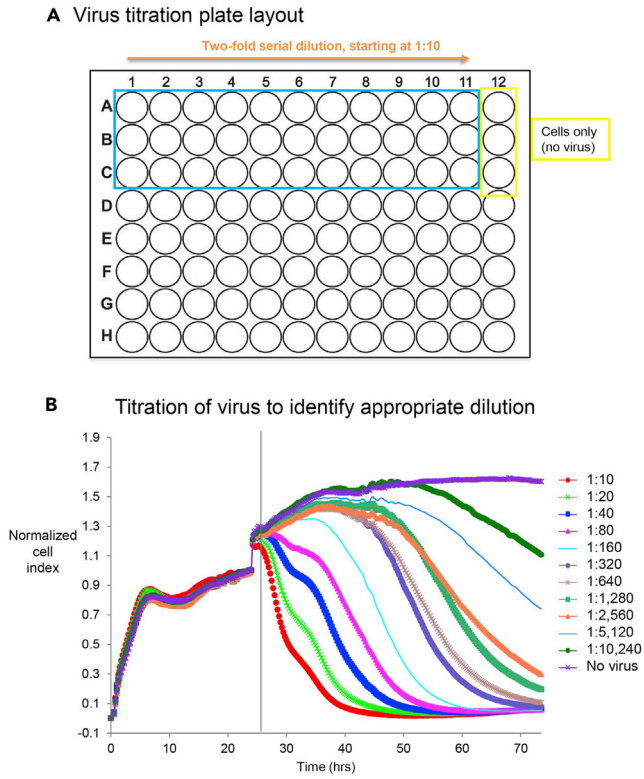


**Figure 2. Titration of rVSV-SARS-CoV-2 virus to identify appropriate dilution**

(A) Plate design for 96-well plate layout for titrating rVSV-SARS-CoV-2 virus. A two-fold serial dilution of virus stock is shown in cyan boxed wells and yellow boxed wells are the cells-only control. Determining the optimal dilution of virus to use in this assay is a critical step in establishing the experiment.

(B) Representative plot of the cytopathic effect (CPE) measurement caused by rVSV- SARS-CoV-2 at varying virus dilutions. Use of appropriate dilution of virus is important to obtain high quality results. Usually, to keep the assay duration as short as possible, the virus dilution associated with a curve that shows full CPE within 40 h of virus addition to cells is chosen. For instance, the 1:160 virus dilution shown here was chosen for subsequent assays.

workflows in numerous assay modalities. The instrument with E-plates operates in a standard $CO_2$ cell culture incubator with the control unit housed outside the incubator.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| COV2-2196 (10 μg/mL) | Zost et al. (2020) | N/A |
| COV2-2489 (10 μg/mL) | Zost et al. (2020) | N/A |
| COV2-2676 (10 μg/mL) | Zost et al. (2020) | N/A |
| COV2-2490 (10 μg/mL) | Zost et al. (2020) | N/A |
| r2 D22 (10 μg/mL) | Smith et al. (2012) | N/A |
| Bacterial and virus strains | | |
| rVSV-SARS-CoV-2 S | Case et al. (2020) | N/A |
| Competent cells | Takara Bio | CAT#636766 |
| Chemicals, peptides, and recombinant proteins | | |
| ExpiCHO Expression Medium | Thermo Fisher Scientific | CAT#A2910001 |
| Fetal Bovine Serum, ultra-low IgG | Thermo Fisher Scientific | CAT#16250078 |
| DMEM, high glucose, pyruvate | Gibco | CAT#11995-065 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Penicillin-Streptomycin | Gibco | CAT#15140122 |
| HEPES (1 M) | Gibco | CAT#15630080 |
| Trypsin-EDTA (0.25%), Phenol red | Gibco | CAT#25200114 |
| **Critical commercial assays** | | |
| MagMAX™-96 Viral RNA Isolation Kit | Thermo Fisher Scientific | AM1836 |
| MagMAX™ mirVana™ Total RNA Isolation Kit | Thermo Fisher Scientific | A27828 |
| TaqMan RNA-to-Ct 1-Step Kit | Thermo Fisher Scientific | 4392939 |
| SuperScript™ IV One-Step RT-PCR System | Thermo Fisher Scientific | 12594025 |
| QIAamp Viral RNA Mini Kit | QIAGEN | CAT#52904 |
| SPRI magnetic beads AMPure XP | Beckman Coulter | CAT#A63880 |
| **Experimental models: Cell lines** | | |
| Monkey: Vero E6 | ATCC | ATCC: CRL-1586, RRID: CVCL_0574 |
| Monkey: Vero E6 +TMPRSS2 | Diamond lab | N/A |
| Monkey: Vero E6 + ACE2 + TMPRS22 | A. Creanga and B. Graham (Vaccine Research Center, NIH) | N/A |
| Monkey: Vero CCL-81 | ATCC | CCL-81; RRID: CVCL_0059 |
| Monkey: Vero Furin | Mukherjee et al. (2016) | N/A |
| Monkey: MA104 | ATCC | ATCC CRL-2378.1 |
| **Oligonucleotides** | | |
| CACACTTTCCTCGTGAAGG | Suryadevara et al. (2021) | VSV DNA Sequencing |
| CACTATTAAATTGGTTGGCAATC | Suryadevara et al. (2021) | VSV DNA Sequencing |
| GTACAATCACTTCTGGTTGG | Suryadevara et al. (2021) | VSV DNA Sequencing |
| GAACAGCAACCTGGTTAGAAG | Suryadevara et al. (2021) | VSV DNA Sequencing |
| CAGAGACATTGCTGACACTAC | Suryadevara et al. (2021) | VSV DNA Sequencing |
| GTCTACAGCATCTGTAATGG | Suryadevara et al. (2021) | VSV DNA Sequencing |
| GATTCTTCTTCAGGTTGGACAG | Suryadevara et al. (2021) | VSV DNA Sequencing |
| Forward-AGCTTCTGAACAATCCCCGG | Suryadevara et al. (2021) | VSV RNA amplification |
| Reverse-GAGGCCTCTTTGAGCATGGT | Suryadevara et al. (2021) | VSV RNA amplification |
| **Recombinant DNA** | | |
| Plasmid: pTwist-mCis_G1 COV2-2489 | Zost et al., 2020a | N/A |
| Plasmid: pTwist-mCis_G1 COV2-2676 | Zost et al., 2020a | N/A |
| Plasmid: pTwist-mCis_G1 COV2-2490 | Zost et al., 2020a | N/A |
| Plasmid: pTwist-mCis_G1 COV2-2196 | Zost et al., 2020a | N/A |
| **Software and algorithms** | | |
| xCELLigence RTCA MP analyzer | Acea Biosciences, Inc | N/A |
| GraphPad Prism | GraphPad | v 9.0.0 |
| **Other** | | |
| xCELLigence E-Plate 96 PET cell culture plates | Acea Biosciences, Inc | Cat# 300601010 |
| Automated cell counter | Bio-Rad | CAT#1450102 |
| Counting slide | Bio-Rad | CAT#145-0011 |
| Falcon 15-mL conical tube | Corning | CAT#352096 |
| Falcon 50-mL conical tube | Corning | CAT#352070 |
| T175 flask | Corning | CAT#431080 |
| T-75 flask | Corning | CAT#430641 |
| ProFlex™ 3 × 32-well PCR System | Thermo Fisher Scientific | CAT#4484073 |
| PCR tube, 0.2 mL | Axygen | PCR-02-C |
| Axygen™ 96-well PCR Microplates | | 14-223-400 |
| Eppendorf benchtop centrifuges | Eppendorf | 5810R, 5424R, 5425 |
| Heracell™ VIOS 160i Tri-Gas $CO_2$ Incubator | Thermo Fisher Scientific | N/A |
| NuAire Class II Type A2 Biosafety Cabinet | Thermo Fisher Scientific | N/A |
| Water bath | VWR scientific | 1130A |
| Vortex | VWR Scientific | 58816-121 |

## STEP-BY-STEP METHOD DETAILS

Timing: 1–2 h culturing and passaging of Vero CCL-81 cells

Timing 1–2 h for passage of Vero CCL-81 cells

1. Culturing and passaging of Vero CCL-81 cells
   a. Thaw cryopreserved vial of Vero cells by gently swirling in 37°C water bath until few ice crystals are present; do not submerge the cap. Wipe the vial with 70% ethanol and place it into the biosafety cabinet.

⚠ CRITICAL: The water bath should be regularly calibrated for accurate temperature. Inappropriate temperature regulation may decrease cell viability. Mix 1 part of 0.4% trypan blue and 1 part cell suspension, load them onto cell counting chamber slide and insert the slide into counting chamber.

   b. Gently transfer the cells to a new 15 mL conical tube and add 9 mL of warmed growth medium, dropwise, to the cells. Mix gently.
   c. Centrifuge the cell suspension at 200 × *g* for 5 min at 20°C–25°C (using the full brake setting).
   d. Discard the supernatant and resuspend in 5 mL of warmed growth medium. Transfer to a new T-25 $cm^2$ flask.
   e. Grow cells to 70%–80% confluency in a 37°C incubator with 5% $CO_2$ atmosphere.
   f. Passage of Vero CCL-81 cells.
      i. Monitor cells until they reach 80%–90% confluency.
      ii. Aspirate growth medium from the flask and discard.
      iii. Wash cell layer with trypsin to remove residual growth medium (coat surface then immediately aspirate and discard).
      iv. Add 2 mL of 0.25% trypsin-EDTA to the flask to cover the cells and place at 37°C for 3–5 min (or until cells detach when viewed by bright-field microscopy).

⚠ CRITICAL: Use fresh cells at 80%–90% confluency. Do not over-incubate cells with trypsin.

      v. Add warmed growth medium (10% FBS DMEM) to dilute the original trypsin volume at least five-fold.

⚠ CRITICAL: For T-225 flasks, add ~10 mL of growth medium, then complete steps in (f). After the cells have been resuspended, add the remaining growth medium to ensure a five-fold dilution of trypsin.

      vi. Rinse flask surface to ensure all trypsin is deactivated.
      vii. Resuspend cells by gently aspirating and dispensing against the side of the flask.
      viii. Count the number of cells per volume in the cell suspension.
      ix. Seed flasks at minimum 0.4 × $10^4$ cells/$cm^2$ (~2–3 day passage) with fresh warmed 10% FBS DMEM.

2. For RTCA plate seeding:

Count the cell suspension and perform the following calculations:

   a. Use the viable cell density to calculate the total viable cells in the cell suspension (viable cell density * volume = total cells viable).
      i. Prepare cells at a density of 0.3–0.35 × $10^6$ cells/mL (which translates to ~18,000 cells/50 µL) and add 50 µL/well to the plate.

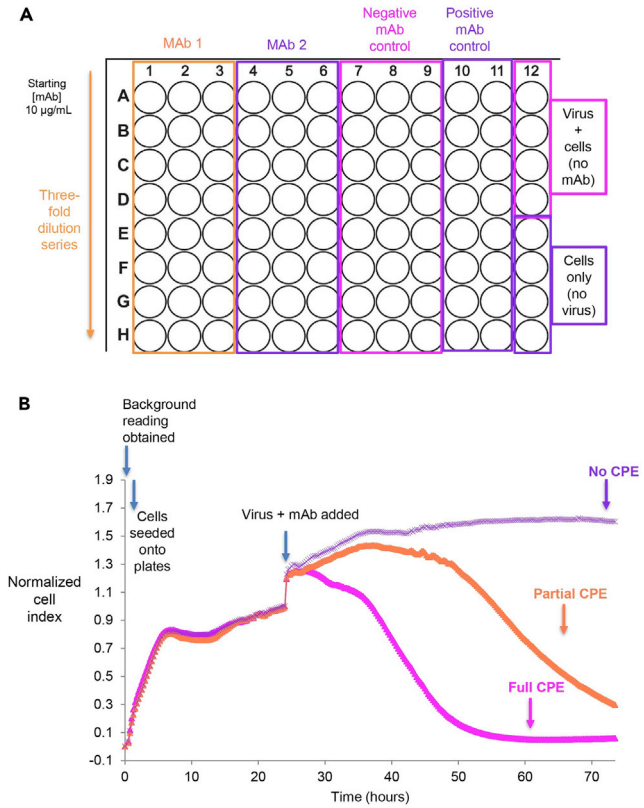


**Figure 3. Experimental design for running a neutralization assay using RTCA**

(A) Plate design for 96-plate layout for assessing mAb neutralization potency. Columns 1–6 highlighted wells are for the mAbs being assessed, columns 7–9 boxed wells are for a negative control mAb, columns 10–11 boxed wells for positive control mAb, pink boxed wells for virus-only control and purple boxed wells are for the cells-only control. It is important to include all controls in the plate so that the results can be interpreted properly.

(B) Graphic of representative results after analyzing data using neutralizing mAbs associated with lack of neutralization (full cytopathic effect [CPE] shown in the pink line), intermediate neutralization indicated by partial CPE (orange line) or lack of neutralization associated with no CPE (purple line).

⚠ CRITICAL: Use fresh cells at 80%–90% confluency. Seeding the plate at the proper cell density is essential. Using too many cells, cells at excess density, or cells that are excessively passaged could result in experimental artifacts. Also, use 2% FBS containing DMEM for assay.

3. Monoclonal antibody, serum or plasma neutralization experiment on xCELLigence:
   a. To start an experiment, open instrument software, select a cradle, go to the first tab labeled as "Experiment" and label the experiment to save in the desired location.
   b. In the "Layout" tab, prepare the scheme of the experiment as shown in Figure 3A.
   c. In the "Schedule" tab, add three steps using the 'Add Step' button or by right-clicking.
      i. The first step is to read the background with the medium only.
      ii. The second step is for cellular adherence (plating cells and allowing them to create a monolayer).
      iii. The third step is for measuring the impedance after virus addition.

⚠ CRITICAL: Always add extra time to this step. If the experiment reaches the end of the set hours, the machine will stop, causing a gap in readings.

   d. Unwrap 96 well E-Plate in a biosafety cabinet, while avoiding touching the contact pads (gold-colored) on the bottom of the plate.

⚠ CRITICAL: Fouling of the contact pads with residue from gloves could affect performance and data readout.

e. Add 50 µL of warmed 2% FBS DMEM to each well and tap gently on the sides to avoid bubbles.
f. Gently place the plate into the cradle, ensure the plate position A1 is located as indicated and check for any errors in the Messages tab.

⚠ CRITICAL: Ensure the message reads "Plate scanned. Connections okay". If an error is obtained, take the plate out and reseat it.

g. Measure the background of the medium alone.

⚠ CRITICAL: Background readings for all wells should read between -0.063 and 0.063. Avoid bubbles that may appear after addition the medium to obtain correct background readings.

h. Once background reading is recorded, remove the plate from the cradle. Return it to the biosafety cabinet and add 50 µL of cell suspension (~0.3–0.36 × $10^6$ cells/mL) to each well.

⚠ CRITICAL: If using multiple plates, mark each plate to ensure placement back onto the same cradle used to measure the background reading.

i. After seeding the cells, incubate plates with covers at 20°C–25°C in the biosafety cabinet for 30 min.

⚠ CRITICAL: Incubation is necessary to avoid edge effects and ensures uniform seeding.

j. Gently place each plate back into its cradle and ensure A1 is located as indicated on the cradle.
k. Ensure there are no error messages. Instrument report should read "Plate scanned. Connections okay".

⚠ CRITICAL: If any wells do not have a good connection, open the cradle and reseat the plate.

l. In the Schedule tab, select the second step to restart the reading. Monitor the sensograms on the "Plot" tab until cells reach a plateau which usually takes 12–16 h.

⚠ CRITICAL: Before running the actual assay, it is recommended that a titration of the virus be done to establish the dilution of the virus stock suitable for the assay. We found 0.1 multiplicity of infection (MOI, see Figure 2) (Case et al., 2020) is suitable for the applications we describe in this protocol. Start by calculating the volumes/concentrations/dilution of the antibody and virus required for executing the experiment. The Table below shows a serial dilution of an antibody to obtain half maximal inhibitory concentration ($IC_{50}$) estimates or dose-response curves.

| Dilution | 2% FBS DMEM (µL) | Volume to transfer (µL) | MAb concentration after dilution (µg/mL) |
|---|---|---|---|
| Starting | 990 + 10 µL of mAb (1 mg/mL) | 250 | 10 |
| 3-fold | 500 | 250 | 3.33 |
| | 500 | 250 | 1.11 |
| | 500 | 250 | 0.37 |
| | 500 | 250 | 0.12 |
| | 500 | 250 | 0.041 |
| | 500 | 250 | 0.013 |
| | 500 | 250 | 0.004 |

m. Perform virus + antibody or serum/plasma incubation steps. The Table below provides volumes and incubation time of heat-inactivated serum/plasma or mAb + virus mixture for neutralization.

| Component | Volume (μL) | Time of incubation at 37°C |
|---|---|---|
| Diluted antibody | 60 | 1 h |
| Diluted virus | 60 | |
| Total | 120 | |

n. When ready, go to the "Schedule" tab, select the current step and press the pause button. Press the lock/unlock button. Remove the plates from the cradle and place them in BSC.
o. Add 100 μL of serum, plasma or mAb + virus (or reagent to be tested) to the wells.

⚠ CRITICAL: Avoid any agitation to the cell monolayer.

i. Where possible, include serum-, plasma- or mAb-only, virus-only, and cell-only controls on each plate.

⚠ CRITICAL: Always include positive and negative controls such that positive control neutralizes the virus and does not cause CPE and vice versa for negative control.

p. Gently place the plates back into their cradles, ensure there are no error messages, and plates should read 'Plate scanned. Connections okay.'

⚠ CRITICAL: Ensure A1 is located as indicated. Open the cradle and reseat the plate in case of any errors.

q. Restart the experiment by initiating step three. Curves will stabilize once the temperature of the incubator returns to 37°C.
r. Change the axis to 'Normalized Cell Index' and move the cursor to the left side of the line at the point where cells reached a plateau prior to infection.
s. Monitor the curves for any changes due to virus infection. Typically, it takes 24–36 h to observe full CPE caused by the virus (See Figure 3B for example curves of full CPE (pink line), partial CPE (orange line), or no CPE (purple line).
t. At the end of the run, pause the step, unlock the instrument, and remove the plate(s), and electronically release the plates via the file menu.
u. Analyze data by normalizing the cellular index (CI) values at the endpoint (48 h after incubation with the virus) using the RTCA software (Agilent Technologies).
v. Results are expressed as percent neutralization in the presence of respective mAb relative to control wells with no CPE minus CI values from control wells with maximum CPE at the assay endpoint.

***Note:*** Endpoint can be defined by both time and CPE caused by virus, we in this assay defined endpoint to be where we see complete CPE between 36–40 h after virus addition. Endpoint can vary from virus to virus due to the level at which it causes CPE.

w. RTCA $IC_{50}$ values are determined by nonlinear regression analysis using Prism software. See Figure 4 for representative mAb neutralization curves are calculated using nonlinear fit with variable slope analysis (Gilchuk et al., 2020a).

4. Generating and identifying antibody escape variant viruses:
   a. To generate escape mutant virus, repeat steps 2a, and steps 3a through 3t and monitor for the escape of virus.

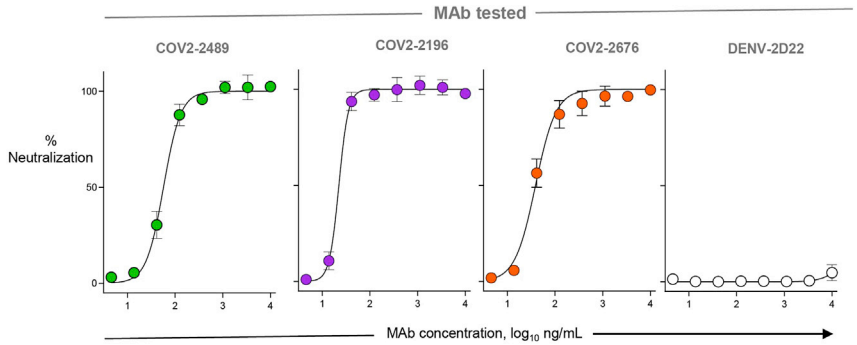


**Figure 4. rVSV-SARS-CoV-2 neutralization curves of mAbs using RTCA**

Representative rVSV-SARS-CoV-2 neutralization curves for COV2-2489, COV2-2196, COV2-2676 or negative control antibody rDENV-2D22. Error bars indicate mean ± SD for 3 technical replicates.

⚠ CRITICAL: It is important to mix the virus with a saturating neutralizing concentration of the antibody being evaluated.

*Note:* For escape mutant virus generation we used 10-fold excess virus (i.e., MOI:1) from the regular neutralization assay. Here in for escape selection experiment we used one concentration of antibody (i.e., 50 μg/mL for COV2-2489 and 10 μg/mL for COV2-2676) unlike, for IC50 calculation or neutralization experiment.

b. The escape variants are identified by delayed CPE in wells containing antibody, as shown in Figure 5A.

⚠ CRITICAL: It is essential to differentiate between CPE caused by the virus only and delayed CPE caused in the presence of antibody to identify escape virus.

c. To verify escaped viruses, isolated viruses are re-assessed in a subsequent RTCA experiment in the presence of 5 μg/mL of a distinct mAb that also neutralizes the virus, see Figure 5B.

⚠ CRITICAL: Validation of escape virus in the presence of a higher concentration (2-fold higher than saturating neutralizing concentration) of the corresponding mAb is critical.

d. To identify mutations that cause escape, escaped viruses are isolated after RTCA-based escape screening and propagated in 6-well culture plates with confluent Vero CCl-81 cells in the presence of 10 μg/mL of the corresponding mAb.

⚠ CRITICAL: Propagation of escaped virus and making stocks of escaped virus is important to isolate viral RNA.

e. Isolate viral RNA according to QIAamp Viral RNA extraction kit (QIAGEN) instructions from aliquots of supernatant containing a suspension of the selected virus population.

⚠ CRITICAL: Proper yields of RNA are required to get adequate amplification.

f. Pipet 560 μL of prepared Buffer AVL containing carrier RNA into a 1.5 mL microcentrifuge tube.

g. Add 140 μL supernatant to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.

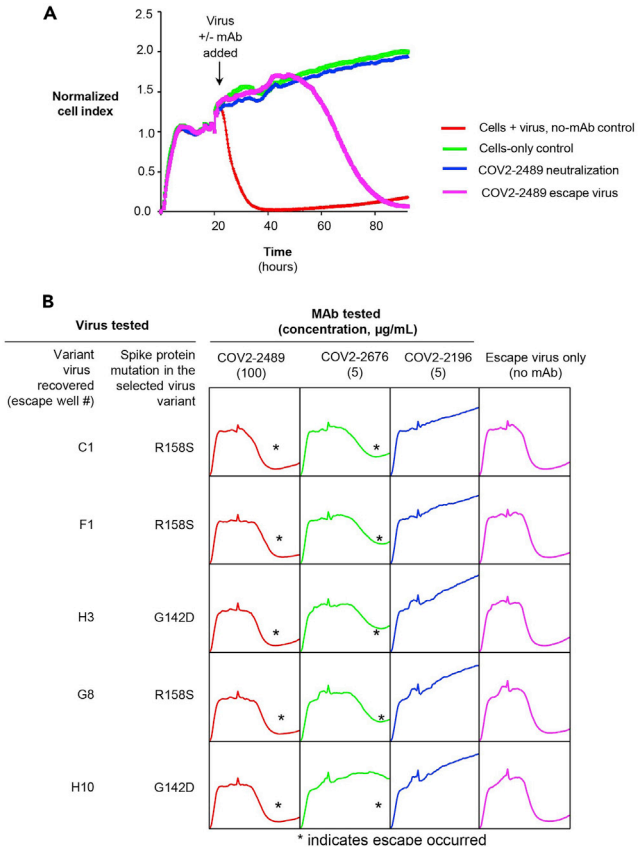


**Figure 5. Confirmation of the antibody escape phenotype for variants previously selected in the presence of a neutralizing mAb**

(A) RTCA sensogram for neutralization of parental virus by mAb COV2-2489 (blue), COV2-2489 escape virus (pink), cells + virus, i.e., the no-mAb control (red) or cells-only control (green).

(B) RTCA sensogram for neutralization of previously selected variant viruses that had replicated in the presence of COV2-2489 or COV2-2676. The COV2-2196 control mAb neutralized all variants (blue) but mAbs for which the virus variants were previously selected to escape did not neutralize (COV2-2489; red), COV2-2676; green). The pink curves show CPE caused by escape virus only (the no-mAb control).

h. Incubate at 20°C–25°C for 10 min.
i. Briefly centrifuge the tube to remove drops from the inside of the lid.
j. Add 560 µL 100% ethanol to the sample.
k. Mix by pulse-vortexing for 15 s. After mixing briefly, centrifuge the tube to remove drops inside the lid.
l. Carefully apply 630 µL of the solution from step 5 to the QIAamp Mini column (in a 2 mL collection tube).
m. Close the cap, and centrifuge at 6,000 × *g* (8,000 rpm) for 1 min.
n. Discard the collection tube and repeat steps i and m to apply the rest of the solution.
o. Carefully open the QIAamp Mini column and add 500 µL Buffer AW1.
p. Close the cap, and centrifuge at 6,000 × *g* (8,000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 mL collection tube and discard the tube containing the filtrate.
q. Carefully open the QIAamp Mini column and add 500 µL Buffer AW2.
r. Close the cap and centrifuge at full speed (20,000 × *g*; 14,000 rpm) for 3 min.
s. Discard collection tube and spin column again at full speed for 1 min to remove any residual buffer.
t. Place the QIAamp Mini column in a clean 1.5 mL Lobind microcentrifuge tube.

u. Discard the old collection tube containing the filtrate.
v. Carefully open the QIAamp Mini column and add 40 µL Buffer AVE equilibrated to 20°C–25°C.
w. Close the cap, and incubate at 20°C–25°C for 1 min.
x. Centrifuge at 6,000 × *g* (8,000 rpm) for 1 min. Store RNA samples in a clean labeled 1.5 mL Lobind microcentrifuge tube (Fisher Scientific, catalog no 13-698-791) at −80°C.

**Pause point:** Extracted viral RNA can be stored at −80°C for up to 1 year.

y. To amplify spike protein gene cDNA, use SuperScript IV One-Step RT-PCR kit (Thermo Fisher Scientific) using primers flanking the virus gene encoding spike protein.

⚠ CRITICAL: Before Sanger DNA sequence analysis, confirming the amplification is recommended. Add SPRI beads to each reaction at a 1:1 ratio.

z. Prepare 50-µL PCR reactions according to the instructions of the SuperScript IV One-Step RT-PCR kit. The table below has components and volumes required to amplify s-gene.

| Component | Volume (µL) |
|---|---|
| Nuclease-free water | Top up to 50 |
| 2× Platinum™ SuperFi™ RT-PCR Master Mix | 25 |
| SuperScript™ IV RT Mix | 0.5 |
| Forward primer | 2.5 |
| Reverse primer | 2.5 |
| Template RNA | to be determined (1–10 ng) |

aa. Incubate reactions in a thermal cycler according to the thermal cycling program outlined in the table below.

| Cycle step | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| Reverse transcription | 50 | 10 min | 1 |
| Initial denaturation | 98 | 2 min | 1 |
| Denaturation | 98 | 10 s | 35 |
| Annealing | 65 | 10 s | |
| Extension | 72 | 2 min | |
| Final extension | 72 | 5 min | 1 |
| Hold | 12 | – | – |

⚠ CRITICAL: Appropriate annealing temperature is required for amplification.

**Pause point:** PCR products can be stored at 4°C for several days or −20°C for months.

bb. Purify amplified PCR product (~4,000 base pairs) using SPRI magnetic beads (Beckman Coulter).

⚠ CRITICAL: Before Sanger DNA sequence analysis, confirming amplification is recommended. Add SPRI beads to each reaction at a 1:1 ratio.

i. 50 µL for a 50 µL reaction.
ii. Mix 15 times with a pipette.

cc. Add tubes to magnetic PCR tube holder and incubate for 5 min at 20°C–25°C. Do not remove tubes from the magnet and carefully remove the supernatant from tubes.

dd. Wash with 200 μL 80% ethanol.
    i. Dilute ethanol in nuclease-free water.
    ii. Do not mix, rather, pipette ethanol over beads while still on the magnet and remove supernatant, leaving beads undisturbed.
ee. Repeat step dd.
    i. Quick spin tubes and put them back in the magnetic holder.
    ii. Remove any residual ethanol from tubes.
    iii. Let beads air dry on the magnet for 2 min.
    iv. Remove from the magnet and resuspend beads in 50 μL of elution buffer.
    v. Add tubes back to the magnet and incubate for 2 min.
ff. Remove supernatant and place in clean, labeled 1.5 mL Lobind microcentrifuge tube and store at −20°C.

**Pause point:** Purified PCR products can be stored at 4°C for several days or −20°C for months.

gg. Analyze cDNA on 0.8% agarose gel to verify successful amplification, purity, and size before sequencing.
    i. Mix 1 μL of purified PCR sample with 10 μL loading dye supplied by Agilent.
    ii. Similarly, mix 1 μL of standard provided with 10 μL loading dye.
hh. Shake before loading samples to mix. Analyze the results. Usually, PCR will yield a single DNA band with a size of 4 kilobases.
ii. After obtaining the sequencing results, analyze the sequences by aligning to the wild-type virus sequence using appropriate software to identify the sequence changes in the variants.

*Note:* The inclusion of both positive (COV2-2196) and negative controls (rDENV-2D222) is critical in calculating the percentage of neutralization in the assay. An optimal negative control is vital for assay success for novel targets lacking a clear positive control. An mAb that recognizes an unrelated target antigen can be used as a negative control.

## EXPECTED OUTCOMES

This protocol can efficiently identify neutralizing mAbs against the SARS-CoV-2 virus and rapidly generate, isolate, and evaluate mutants that escape antibody neutralization. RTCA assay is a high-throughput technique that can identify SARS-CoV-2 neutralizing antibodies within 48–72 h, and results are highly correlated with conventional assays (Gilchuk et al., 2020a, 2022).

Analysis of data for $IC_{50}$ values calculation:

Normalized cellular index (CI) values at the endpoint (48 h after incubation with the virus) were determined using the RTCA software version 2.1.0 (Agilent Technologies). Results are expressed as percent neutralization in the presence of the respective mAb relative to control wells with no CPE minus CI values from control wells with maximum CPE. RTCA $IC_{50}$ values were determined by nonlinear regression analysis using Prism software.

## LIMITATIONS

Engineering and generating a replication-competent chimeric VSV reagent to study entry and neutralization of SARS-CoV-2 at biosafety level 2 is key to this protocol. Expression of the glycoprotein of interest is often accomplished by plasmid transfection, which requires optimization to minimize batch variation. It is also unknown how spike protein display on a heterologous virus impacts viral entry, antibody recognition, and antibody neutralization compared to authentic coronavirus. This question is important because neutralization assays are used to establish correlates of protection for vaccine and antibody-based countermeasures, and most manufacturers lack access

to high-containment laboratories to test antibody responses against highly pathogenic coronaviruses such as SARS-CoV-2.

To identify neutralizing mAbs, it is essential to have a cellular system that replicates or closely mimics the process of natural SARS-CoV-2 infection. Selecting appropriate cell lines is crucial. However, the availability of such cell lines immediately during the early stages of outbreak situations may be limited. Moreover, the adaptability and suitability of the identified cell line to RTCA assay needs to be tested.

## TROUBLESHOOTING

### Problem 1

A common problem with this assay is that cells may accumulate in the middle of the well rather than across the whole well after seeding. This distribution will result in inaccurate cell index values and increased variability between experiments (step 3i).

### Potential solution

Keep the plate at 20°C–25°C for 30 min after cell seeding to allow cells to settle to the bottom of the plate before plate is returned to the RTCA Station.

### Problem 2

The throughput using 6 ×96 well instrument is less (step 3).

### Potential solution

Using 8 × 384 well xCELLigence RTCA HT-BioSpa can increase throughput.

### Problem 3

The cytopathic property of the virus decreases from one experiment to another (step 3).

### Potential solution

The viability of SARS-CoV-2 is reduced when left too long in solution without cells or upon repeated cycles of freezing and thawing. Hence, do not re-freeze aliquots of virus for use in a later experiment. Prepare aliquots of virus in suspension for cryopreservation that are appropriate for the size of the experiments to be performed and re-establish infecting dose for each new batch of virus that is prepared and cryopreserved.

### Problem 4

At times $IC_{50}$ values for neutralizing mAb calculated using RTCA assay may vary from conventional PRNT and FRNT assays (step 4).

### Potential solution

It is good to cross validate IC50 value at least few mAbs using conventional assays.

### Problem 5

Data from well to well might vary due to several reasons (step 4).

### Potential solution

It is good to perform technical triplicates and experimental replicates to nullify the percent error.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Robert H. Carnahan (Robert.carnahan@vumc.org).

### Materials availability

Materials described in this paper are available for distribution for nonprofit use using templated documents from Association of University Technology Managers "Toolkit MTAs", available at: https://autm.net/surveys-and-tools/agreements/material-transfer-agreements/mta-toolkit.

### Data and code availability

The antibodies in this study are available by Material Transfer Agreement with Vanderbilt University Medical Center. Code – No custom computer code or algorithms to report.

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## AUTHOR CONTRIBUTIONS

N.S., P.G., and R.H.C. conceived the project. J.E.C. obtained funding. N.S., P.G., and S.J. performed laboratory experiments. N.M. and L.Z. provided technical support. R.H.C. and J.E.C. supervised the research. N.S., P.G., and R.H.C. wrote the first draft of the paper. All authors reviewed and approved the final manuscript.

## DECLARATION OF INTERESTS

J.E.C. has served as a consultant for Eli Lilly, GlaxoSmithKline, and Luna Biologics, is a member of the Scientific Advisory Boards of Meissa Vaccines, and is Founder of IDBiologics. The Crowe laboratory at Vanderbilt University Medical Center has received unrelated sponsored research agreements from Takeda, IDBiologics, and AstraZeneca. All other authors declare no competing interests.

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