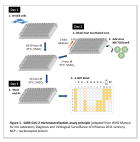

Version 2

Jul 29, 2022

SARS-CoV-2 live virus neutralization assay V.2

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ABSTRACT

Virus neutralization assays provide a means to quantitate functional antibody responses that block virus infection. These assays are instrumental in defining vaccine and therapeutic antibody potency, immune evasion by viral variants, and post-infection immunity. This protocol describe a means by which to measure neutralizing antibody titers in a live virus microneutralization assay specific for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The SARS-CoV-2 microneutralization assay is based on the influenza virus microneutralization assay described by WHO. In this assay, SARS-CoV-2 clinical isolates are pre-incubated with serial diluted antibody and added to Vero E6 cells. Replicating virus is quantitated by enzyme-linked immunosorbent assay (ELISA) targeting the SARS-CoV-2 nucleocapsid protein and the 50% virus inhibition titer calculated. The assay has been systematically optimized at critical test parameters that include virus titration, assay linearity, number of cells, viral dose, incubation period post-inoculation, and normalization methods.

This protocol has three parts:

Part I: Determination of the 50% tissue culture infectious dose per milliliter ($TCID_{50}$/mL).

Part II: ELISA.

Part III: Virus microneutralization assay.

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KEYWORDS

Neutralization, COVID-19, SARS-CoV-2, coronavirus, virus, antibody

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GUIDELINES

No specific guidelines.

MATERIALS TEXT

Equipment, Supplies, and Reagents

A	B
Equipment	
Water bath (37 °C)	Heating block (56 °C)
ELISA plate reader (FLUOstar Omega, BMG Labtech)	Incubator (humidified, 37 °C; 5% CO2)
Microscope	Benchtop centrifuge
Supplies	
Cell culture flasks (75 cm2, sterile, vented) **Nunc – cat. no. 156499**	Serological pipettes (1, 2, 5, 10, 25 ml; sterile)
96-well microtiter plate (flat bottom) **Thermo – cat. no. 167008**	Pipettes (assorted sizes, sterile)
Hemocytometer	Pipetman
Multichannel pipette **Biohit Sartorius**	Micropipette tips
Cells, media and buffers	
Vero E6 – passage (8-20 passages) low crowding (70-95% confluence)	Sterile cell culture growth medium (see below)
HEPES buffer (1 M) **Gibco – cat. no. 15630-056**	Wash buffer (see below)
DPBS (pH 7.2) without CaCl2 or MagCl2 **Gibco – cat. no. 14190-094**	Distilled water
Dulbecco's Modified Eagle's Medium (DMEM) **Gibco – cat. no. 41966-029**	Fetal Bovine Serum (FBS) **Gibco - cat. no. 10270-106**
Reagents	
Penicillin-streptomycin **Gibco – cat. no. 15140-122**	TrypLE (10X) **Gibco – cat. no. A12177-01**
Bovine serum albumin (BSA; 30% solution) **Sigma – cat. no. A8327**	Trypan blue stain **Bio-Rad – cat. no. 145-0013**
SARS-CoV-2 NP Antibody, Mouse Mab (clone 7E1B) **Bioss USA – cat. no. BSM-41414**	Goat anti-mouse IgG/HRP, highly cross-adsorbed **Invitrogen – cat. no. A16078**
Ethanol (70%)	Acetone **Fisher Scientific – cat. no. A18-500**
TMB (ready to use) **Kementec – cat. no. 4380A**	Sulfuric acid **SSI Diagnostica – cat. no. 24156**
Virus diluent (see below)	Fixative (see below)
Stop solution (see below)	

Preparation of media and solutions

A	B
Cell culture growth medium	*For 50 mL:* 44.5 mL DMEM, 0.5 mL Pen/Strep, 5 mL heat inactivated FBS
Virus diluent (make fresh)	*For 50 mL:* 46.5 mL DMEM, 0.5 mL Pen/Strep, 1.67 mL BSA, 1.25 mL HEPES
Fixative (make fresh)	*For 50 mL:* 10 mL PBS (1X), 40 mL acetone (store at -20 °C until just before use)
Wash buffer	PBS + 1% (v/v) Triton-X100 (SSI nr. 1335) or (0.2% Tween20 in PBS)
Dilution buffer	SSI Fortyndingsbuffer (SSI nr. 24212) + 2% Bovine Serum Albumin
Substrate	TMB (ready to use, equilibrate to room temperature)
Stop solution	0.2 M sulfuric acid

SAFETY WARNINGS

This assay uses live, replication competent severe acute respiratory syndrome virus 2 (SARS-CoV-2). This pathogen requires Biosafety Level 3 facilities and appropriate training for handling. The assay should be performed according to institutional and laboratory Biosafety Level 3 procedures.

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BEFORE STARTING

The assay requires several steps where liquids are added to and aspirated from live cell monolayers in a 96-well tissue culture plate. Ideally, optimize handling technique to minimize cell loss at these steps before running the full protocol. Cell loss will reduce assay signal and introduce variance.

Seeding Vero E6 cells into 96-well plates

1 **Seeding Vero E6 cells (from T75 flask)**

Perform all steps aseptically in a Biosafety 2 flow hood. Do not allow the cell monolayer to dry. Avoid touching the cell monolayer with pipette tips. Optimal cell passage number is between 8 and 20. Below passage 8, cells are more susceptible to infection and vary with each passage. Cell susceptibility is more stable between passage 8 and 20, to minimize inter-assay variation.

1.1 Verify that Vero E6 cells growing in maintenance media are actively growing (monolayer 70-95% confluent).

Do not use cells that are overgrown. **Maintenance media**: Dulbecco's Modified Eagle's Medium, Penicillin-Streptomycin (final concentration 10 U/mL), Fetal Bovine Serum (final concentration v/v 10%). Prepare fresh.

1.2 Remove the maintenance media from the flask with a serological pipette and discard.

1.3 Add 5 mL of phosphate buffered saline (PBS) to the cell monolayer and tilt the flask to cover and wash the whole monolayer. Remove the PBS with a serological pipette.

1.4 Add 2 mL 10X TrypLE dissociation media to the flask. Tilt the flask to ensure all cells are covered, and remove the dissociation media with a pipette.

Undiluted 10X TrypLE dissociation media is used for cells that are difficult to detach from the flask surface. It may be possible to use diluted TrypLE e.g. 1X or 2X, but this needs to be evaluated empirically.

1.5 Place the flask horizontally in a 37 °C, 5% CO_2 incubator and incubate for 10 minutes.

1.6 Add 10 mL **maintenance media** to the flask and gently wash cells from the flask surface. Pipette up and down to mix the single cell suspension.

1.7 Determine the cell number. Combine 10 µL of the resuspended cells with 10 µL Trypan Blue. Count the cells on a hemocytometer.

If using an automated cell counter, be aware of the cell counter's dynamic range and accuracy for different input cell numbers. To reduce cell number variation as a factor of inter-assay variance, it is important to use an accurate and consistent method for cell counting.

1.8 Adjust the cell concentration to 1×10^5 cells/mL with **maintenance media** and transfer 100 µL to each well in a 96 well plate (10 000 cells per well) using a multichannel pipette.

To ensure cell numbers are constant for all wells, mix the cell suspension well each time before

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transferring cells to a new column in the 96-well plate.

1.9 Incubate the 96-well plate containing the cells overnight under standard tissue culture conditions (37 °C, 5% CO_2) for use the following day.

SARS-CoV-2 virus titration

2 SARS-CoV-2 virus titration

Perform these steps aseptically according to institutional and laboratory Biosafety Level 3 procedures.

2.1 Thaw a vial of each virus stock just prior to use. Dilute 10 µL virus in 90 µL **virus diluent** (1:10 dilution).

Virus diluent: Dulbecco's Modified Eagle's Medium, Penicillin-Streptomycin (final concentration 10 U/mL), Bovine Serum Albumin (final concentration v/v 1%), HEPES buffer (final concentration 25 mM). Prepare fresh.

2.2 In a sterile 96-well dilution plate, prepare a ½ log (3.16-fold) serial dilution of the viruses in **virus diluent** (illustrated in the accompanying figure).

2.3 Add 158 µL of **virus diluent** to column 1 and 120 µL of **virus diluent** to columns 2 to 12.

2.4 For each virus stock to be titrated, allocate four rows on the 96-well plate for quadruplicate measurements. Add 17.6 µL of the 1:10 diluted virus to column 1, rows A to D (virus 1) and repeat for virus 2 for rows E to H.

Change tips between every 17.6 µL virus aliquot transferred to each well in column 1.

2.5 Using a multichannel pipette, mix column 1 thoroughly and transfer 55 µL from column 1 to column 2. Discard tips.

2.6 Using clean tips, mix column 2 thoroughly and transfer 55 µL from column 2 to column 3. Discard tips.

2.7 Continue with serial dilution to column 11 using clean tips between columns as described in step 2.6. In the final column with virus (column 11), mix thoroughly and discard 55 µL into a disinfectant (e.g. Virkon or sodium hypochlorite).

The final volume in all columns should be 120 µL. Column 12 is the negative control column.

2.8 Place plate(s) at 37 °C, 5%CO_2 for 1 hour.

This step simulates the virus neutralization assay conditions in which the titered virus will be used.

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2.9 Remove the 96-well tissue culture plate seeded with 10 000 Vero E6 cells per well the day before from the 37°C, 5%CO_2 incubator.

2.10 Aspirate the cell culture media from the cell monolayer.

Aspirate slowly to not disturb the cell monolayer. Keep monolayer intact by not scraping the well bottom with the pipette tip. Do not allow the cells to dry out.

2.11 Gently wash the cell monolayer twice with 100 µL sterile PBS. Remove the PBS with a pipette.

Observe the monolayer condition under a microscope before and after washing. It is very important to minimize cell loss at this step, since it will introduce variance. If substantial cell loss is observed, first practice and optimize the technique to preserve the monolayer and repeat the experiment.

2.12 Add 100 µL of serial diluted virus to the Vero E6 cell monolayer in the same order as in the dilution plate.

2.13 Incubate for 96 hours at 37°C, 5%CO_2. Thereafter, the cells are fixed to the plate for the ELISA (described in the Section 3 and 4 of this protocol).

2.14 Using the optical density values from the ELISA, calculate the 50% tissue culture infectious dose using the method described by Reed and Muench (1938), see attached file.

Fixation of SARS-CoV-2 infected cells

3 Fixation of cells

Perform these steps according to institutional and laboratory Biosafety Level 3 procedures. It is not necessary to work aseptically. Do not allow cells to dry out after removing medium and PBS.

3.1 Prepare fixative and pre-cool at -20°C until just before use.

Fixative: 80% acetone (v/v) in PBS (does not need to be sterile).

3.2 Remove the 96-well tissue culture plate with SARS-CoV-2 cells from the 37°C, 5%CO_2 incubator.

3.3 Remove the medium from all the cells in the tissue culture plate.

3.4 Gently wash the wells with 200 µL PBS.

3.5 Remove the PBS and add 100 µL cold fixative to each well.

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3.6 Cover the plate with a lid and incubate at room temperature for 10 minutes.

3.7 Remove fixative with a pipette or by flicking onto stacked paper towel and let the plate air dry (approximately 10-20 minutes).

3.8 Proceed with ELISA directly (step 4 in this protocol) or add 50 µL 50% glycerol, cover with an adhesive plate seal and store at -20°C until ELISA can be done.

Enzyme linked immunosorbent assay (ELISA)

4 Anti-SARS-CoV-2 nucleocapsid ELISA

4.1 Dilute the primary antibody (Cat # bsm-41414; Bioss USA; SARS-CoV-2 nucleocapsid mouse mAb, clone 7E1B) 1:4000 in **dilution buffer**.

Dilution buffer: SSI Diagnostica Cat. # 24212 + 2% Bovine Serum Albumin or equivalent.

4.2 Add 250 µL **wash buffer** per well to the 96-well plate with fixed cells and soak for 30 seconds at room temperature.

Wash buffer: 1% (v/v) Triton-X100 in PBS. Alternatively, 0.05% Tween20 (v/v) in PBS.

4.3 Flick wash buffer out over a sink.

4.4 Repeat wash steps twice [repeat steps (4.2 and 4.3) × 2]. After the last wash, gently tap the 96-well plate upside-down on stacked tissue paper to remove excess wash buffer.

4.5 Add 100 µL of the diluted primary antibody to each well.

4.6 Cover the plate(s) with a plate sealer and incubate for 5 minutes on shaking incubator (300 rpm) at room temperature and then for 1 hour at 37°C.

4.7 Dilute the horse radish peroxidase (HRP)-conjugated secondary antibody (Cat. # A16078; Invitrogen; goat anti-mouse IgG/HRP highly cross-adsorbed) 1:10000 in **dilution buffer**.

4.8 Wash the plate(s) three times with wash buffer as before [repeat steps (4.2 and 4.3) × 3]. After the last wash, gently tap the 96-well plate upside-down on stacked tissue paper to remove excess wash buffer.

4.9 Add 100 µL of the diluted HRP-conjugated secondary antibody to each well.

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4.10 Cover the plate(s) with a plate sealer and incubate for 5 minutes on shaking incubator (300 rpm) at room temperature and then for 1 hour at 37°C.

4.11 Wash the plate(s) five times with wash buffer as before [repeat steps (4.2 and 4.3) × 5].

4.12 Add 250 µL **distilled water** per well to the 96-well plate and flick out over the sink without an incubation. Repeat twice. After final wash with distilled water, gently tap the plate upside-down on stacked tissue paper to remove excess water.

4.13 Add 100 µL 3,3',5,5'-tetramethylbenzidine (TMB; Cat. # 4380; Kementec; TMB One) substrate to each well. Do not cover plate.

4.14 Incubate for 15-30 minutes in the dark at room temperature. Monitor colour development.

4.15 Add 100 µL **stop solution** to all wells.

Stop solution: 0.2 M H_2SO_4

4.16 Read the absorbance of the wells at 450 nm (OD_{450}) with 620 nm as reference.

SARS-CoV-2 virus neutralization

5 SARS-CoV-2 virus neutralization

Controls and test sera
a. Test and control sera should be heat-inactivated at 56 °C for 1 hour.
b. Store sera (before or after heat inactivation) at -20 to -70 °C.
c. If samples are to be tested repeatedly, it is best to **make several aliquots of the sera** so that the sample is not repeatedly frozen and thawed.
d. Positive control: Included in each plate for standardization. Stored at -20 to -70 °C as single-use aliquots. Negative control: Sera from immune naïve individuals e.g. collected before the pandemic. Stored at -20 to -70 °C as single-use aliquots.

5.1 Prepare sterile 96-well flat bottom plates with 10 000 cells per well as described under Section 1 '**Seeding Vero E6 cells (from T75 flask)**'.

5.2 Incubate cells overnight at 37°C, 5%CO_2.

5.3 Thaw a vial of virus and place in the biosafety cabinet, mix thoroughly and dilute to 300× $TCID_{50}$ per 60 µL. Approximately 6 mL/plate is needed. Store on ice or +4°C.

Dilute virus stock to 5000 $TCID_{50}$/mL in 6 mL/plate. This yields 300× $TCID_{50}$ per 60 µL.

5.4 Add virus diluent to a sterile dilution plate as follows (layout on next page):
a. 108 µL to row A, wells 1-10
b. 60 µL to rows B-H, wells 1-10

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c. 60 µL to wells A11 – D11 (virus control)
d. 60 µL to wells B12 – H12 (virus back titration)
e. 120 µL to wells E11 – H11 (cells control)

5.5 Do the serum dilution in a separate sterile 96-well plate
a. 12 µL serum to the appropriate wells in row A (1:10 serum dilution)
b. Do a 2-fold serial dilution. Sequentially transfer 60 µL from row A through to row H. Mix thoroughly by pipetting. Discard 60 µL from row H.
c. 60 µL diluted serum remains in each well.

5.6 Do the virus back titration.
a. In column 12, add 120 µL virus to well A12
b. Do a 2-fold serial dilution of virus - transfer 60 µL from one row to the next, start row A through to row 12. Change tips between dilutions.
c. Discard 60 µL from well H12
d. Add 60 µL virus diluent to all wells in column 12 (final volume 120 µL).

This virus back titration serves as a control for the virus input amount and changes in sensitivity of cells to the virus infection e.g. early passage cells (passage 1-8) can be more susceptible to infection and reduce the neutralization assay sensitivity and have more positive wells in the back titration, whereas late passage cells (passage >20) can be less susceptible to infection and have fewer positive wells in the back titration. This is also an important control to ensure that the input virus for different strains are the same when comparing antigenicity between different viruses in the same assay.

5.7 Add 60 µL diluted virus (300× $TCID_{50}$) to the following wells:
a. All wells in rows A-H, wells 1-10 (test wells)
b. Wells A11 – D11 (virus control)

5.8 Place plate(s) at 37 °C 5% CO_2 for 1 hour.

5.9 Approximately 45-50 minutes into the incubation period (step 5.8), remove the 96-well tissue culture plate(s) seeded with 10 000 Vero E6 cells the day before from the 37°C, 5%CO_2 incubator.

5.10 Aspirate the cell culture media from the cell monolayer. Do not allow cells to dry out.

Aspirate slowly to not disturb the cell monolayer. Keep monolayer intact by not scraping the well bottom with the pipette tip. Do not allow the cells to dry out.

5.11 Gently wash the cell monolayer twice with 100 µL sterile PBS. Remove the PBS with a pipette.

Observe the monolayer condition under a microscope before and after washing. It is very important to minimize cell loss at this step, since it will introduce variance. If substantial cell loss is observed, first practice and optimize the technique to preserve the monolayer and repeat the experiment.

5.12 Add 100 µL serum/virus mix to the Vero E6 cell monolayer.

5.13 Incubate for 22-24 hours at 37°C, 5%CO_2.

For Omicron that has a slower cell entry mechanism, incubate for 28-32 hours. Evaluate optimal

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incubation time in-house based on signal intensity in virus only cells and back titration.

5.14 After the incubation period, fix the cells as described in Section 3 '**Fixation of SARS-CoV-2 infected cells**'

5.15 Perform the ELISA as described in Section 4 '**Enzyme linked immunosorbent assay (ELISA)**'

Quality control, neutralizing antibody titer calculations, and normalization

6 **Quality control**

Perform the following quality control checks to determine successful execution of the neutralization assay. If the criteria below are not met, the assay should be repeated or optimized by the specific performing laboratory.

6.1 The average OD values for the virus controls must be equal to or above 1.00.

6.2 The signal-to-noise ratio calculated for the average OD values of the quadruplicate virus controls divided by the average OD values of the quadruplicate cell controls should preferably be above 6.

6.3 Determine if the virus test dose (300× $TCID_{50}$) is acceptable in the virus back titration. In most cases, the test dose of virus is acceptable if the back titration is **below or equal** to the 50% cut-off value in the 5-6 wells containing the lowest dilutions of test virus.

6.4 Confirm that non-specific inhibition is not present on the plate using the negative serum control. The OD450 of the negative serum control for all dilutions should be similar to that observed for the virus control wells.

In row A, be aware of a possible edge effect where fewer cells are present. This can lead to a reduced OD value that erroneously represents an inhibition at a serum dilution of 1:10. The negative control is useful for detecting this. If an edge effect is detected, optimize technique to avoid this.

6.5 Assess inter-run variability using the positive control. The serum positive control should give titres within +-1.5-fold of the average values obtained in previous tests.

7 **Determine the serum 50% neutralization titers as follows:**

7.1 Calculate the optical density value cut-off representing 50% virus infection that represents inhibition of 50% virus infection in the neutralization assay i.e. 50% neutralization.

$$= ((AverageODofVirusControlWells) + (AverageODofCellControlWells))/2$$

7.2 All wells containing diluted serum with an OD value **below or equal** to the 50% cut-off value are positive for neutralization activity. The reciprocal serum dilution corresponding to that well is the 50% neutralization antibody titre for that serum sample. Serum dilutions are: well A 1:10; well B 1:20; well C 1:40; well D 1:80; well E 1:160; well F 1:320; well G 1:640; and well H 1:1280.

Alternatively, fit a four parameter logistic regression line over all the dilutions for a single sample and calculate the exact 50% neutralization titer as the intercept of the curve with the 50% cut-off OD value.

Normalize 50% neutralization titers to minimize inter-assay variation

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8 *This method can only be applied to exact titers calculated from four parameter logistic regression curves fitted over all serum dilution data points. It requires an exact titer for the same positive control used in all neutralization assays. This exact positive control titer is obtained by running single-use aliquots of the positive control in the neutralization assay on 8-15 different days with optimal cell passage numbers between 8 and 20. The average positive control titer is referred to here as the nominal value.*

8.1 Use the following equation to determine the normalized 50% neutralization titer for each sample:

$$x \times (((x-1)/(Log(1280)-1) \times ([\![\ Pos]\!]\ nom - Pos)/Pos) + 1)$$

x is the $\log_{10}$ transformed titer of the sample

Pos is the $\log_{10}$titer of the positive control included on the same assay plate as the sample

Pos_{nom} is the average $\log_{10}$ titer of the positive control determined from 8-15 runs denoted as the nominal value of the positive control material.

The second fraction calculates the bias of the result from the positive control according to the nominal "expected" value. Thus, if the positive control in a given run has a titer identical to the nominal value, samples will not be corrected. The first fraction describes the relative size of the titer of the sample compared to the maximum titer in a normal assay (here 1:1280). This weighs each titer according to size. Range is adjusted according to the minimum positive value ($\log_{10}$ 10 = 1) to exclude normalization for positive samples at the minimum titer of 10, which is the lower limit of quantification. This exclusion is designed to diminish influence of normalization on the qualitative judgement of borderline samples. The correction increases with titer and is greatest at maximal titer (1280).

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