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SARS-CoV-2 Neutralization Assay

1. Scope

This method is applicable to determine the neutralizing antibody titer against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in serum collected from patients infected with SARS-CoV-2 or participants in SARS-CoV-2 vaccine trials.

2. Method

After serial dilution, serum samples are preincubated with a specified amount of SARS-CoV-2 allowing sufficient time for neutralization prior to inoculation of the mixture to Vero E6 cells. After 3~5 days of incubation, the Vero E6 cells infected with SARS-CoV-2 start forming visible cytopathic effects (CPEs). Detection of neutralizing antibodies is determined by the appearance of CPEs, which are observed under a phase contrast microscope. The highest dilution of the serum sample that prevents infectivity of virus is defined as 100% neutralization titer (NT_{100}) and the dilution that blocks half-maximal infectivity of virus is defined as 50% neutralization titer (NT_{50}).

2.1. Work environment (note 1)

For personnel safety, all experiments with SARS-CoV-2 should be conducted in a Biosafety Level 3 (BSL-3) laboratory. Procedures that handle viruses, such infection or serial dilution, should be performed in a certified class II, type B2 biological safety cabinet (BSC). All the process should follow the regulations approved by the Taiwan Centers for Disease Control and Prevention (CDC) (note 2).

Note 1: SARS-CoV-2 is classified as a Risk Group 3 (RG3) human pathogen. People who handle and process specimens associated with SARS-CoV-2 should follow related laboratory biosafety guidelines.

Note 2: Regulations Governing Management of Infectious Biological Materials; Operation Directions Governing Management of Infectious Biological Materials, Ministry of Health and Welfare; Interim Guidelines for Clinical Management of SARS-CoV-2 Infection.

2.2. Equipment

2.2.1. Biological safety cabinet (BSC): class II, type B2.
2.2.2. Autoclave: reach and maintain a temperature of 121°C or above.
2.2.3. Refrigerator: maintain a temperature of 5 ± 3℃℃.
2.2.4. Freezer: maintain a temperature of -30 ± 3℃.
2.2.5. Ultra-low temperature freezer: maintain a temperature of -80 ± 5℃.
2.2.6. CO_2 incubator: with temperature control at 35℃ or 37℃, and the level of CO_2 at 5%.
2.2.7. Cell counter: Beckman Coulter cell counter Z2 or similar apparatus, or an equivalent product.
2.2.8. Phase contrast microscope: up to 400X magnification.
2.2.9. Refrigerated centrifuge: appropriate for 15 mL and 50 mL centrifuge tubes; centrifugal force ≥ 2000 ×g and with temperature control at 4 ℃.

2.3. Reagents
2.3.1. Phosphate buffered saline (PBS)
2.3.2. Trypsin
2.3.3. Dulbecco's Modified Eagle's Medium (DMEM) : Sigma-Aldrich D5796, or an equivalent product.
2.3.4. Fetal bovine serum (FBS)
2.3.5. Antibiotics: with 10,000 units/mL penicillin and 10,000 units/mL streptomycin.
2.3.6. Vero E6 cell line (ATCC® CRL-1586™): lower passage number (below 15) should be used. Cells are tested and found free of bacteria, fungus and mycoplasma.
2.3.7. Virus: SARS-CoV-2, clade L.

2.4. Apparatus
2.4.1. Micropipette: 2 µL, 10 µL, 20 µL, 200 µL and 1000 µL.
2.4.2. Filter pipette tip: 10 µL, 20 µL, 200 µL and 1000 µL.
2.4.3. Serological pipette: 5 mL, 10 mL and 25 mL.
2.4.4. Glass bottle: 100 mL, 250 mL, 500 mL and 1000 mL.
2.4.5. Microcentrifuge tube: 1.5 mL.
2.4.6. Centrifuge tube: 15 mL and 50 mL, PP.
2.4.7. Cryogenic vial: 2.0 mL.
2.4.8. Cell culture flask/plate: T75 flask, T150 flask and 96-well plate.

2.4.9. 96 deep well plate.

2.5. Preparation of reagents

2.5.1. DMEM-10%FBS: Add 100 mL of FBS and 10 mL of antibiotics to 890 mL of DMEM, and mix well.

2.5.2. DMEM-2%FBS: Add 20 mL of FBS and 10 mL of antibiotics to 970 mL of DMEM, and mix well.

2.6. Cell culture

2.6.1. Culture Vero E6 cells with DMEM-10%FBS in a humidified 37℃ incubator with 5% CO_2. Vero E6 cells are applied in the following steps 2.7., 2.8., 2.9. and 2.10.

2.6.2. Remove the spent medium from the Vero E6 cells. Collect the cells and determine the cell numbers. Dilute the cells to 1 × 10^5 cells/mL with DMEM-10%FBS, and then inoculate 100 μL to each well of 96-well plate. Maintain the 96-well plate in a humidified 37 ℃ incubator with 5% CO_2 for 16-18 hours, and then use the plate for the following steps 2.8., 2.9., and 2.10.

2.7. Virus preparation

2.7.1. Take the Vero E6 cells from step 2.6.1., and determine the cell numbers. Dilute the cells to 1 × 10^6 cells/mL with DMEM-10%FBS, and then inoculate 5 mL to a T75 flask. Fill the flask with 5 mL of DMEM-10%FBS and mix the medium thoroughly with the cells. Maintain the T75 flask in a humidified 37℃ incubator with 5% CO_2 for 16-18 hours, and then use the flask for the following step 2.7.2.

2.7.2. Take the Vero E6 cells from the incubator in step 2.7.1., and remove the spent medium. Wash once with 5 mL of PBS, and then discard the used PBS. Infect the cells with 3 mL of SARS-CoV-2 (multiplicity of infection, MOI=0.01), and then incubate the cell-virus flask in a humidified incubator with 5% CO_2 for 1 hr at 35℃. After incubation, add 9 mL of DMEM-10%FBS to the flask, and maintain in a humidified 35℃ incubator with 5% CO_2 for 3~5 days.

2.7.3. Observe the cytopathic effects (CPEs) by a phase contrast microscope. If 70% of CPEs are observed, harvest the culture medium into a 15-mL centrifuge tube, and centrifuge at 2000 ×g for 10 min at 4℃. After centrifugation, transfer the supernatant to

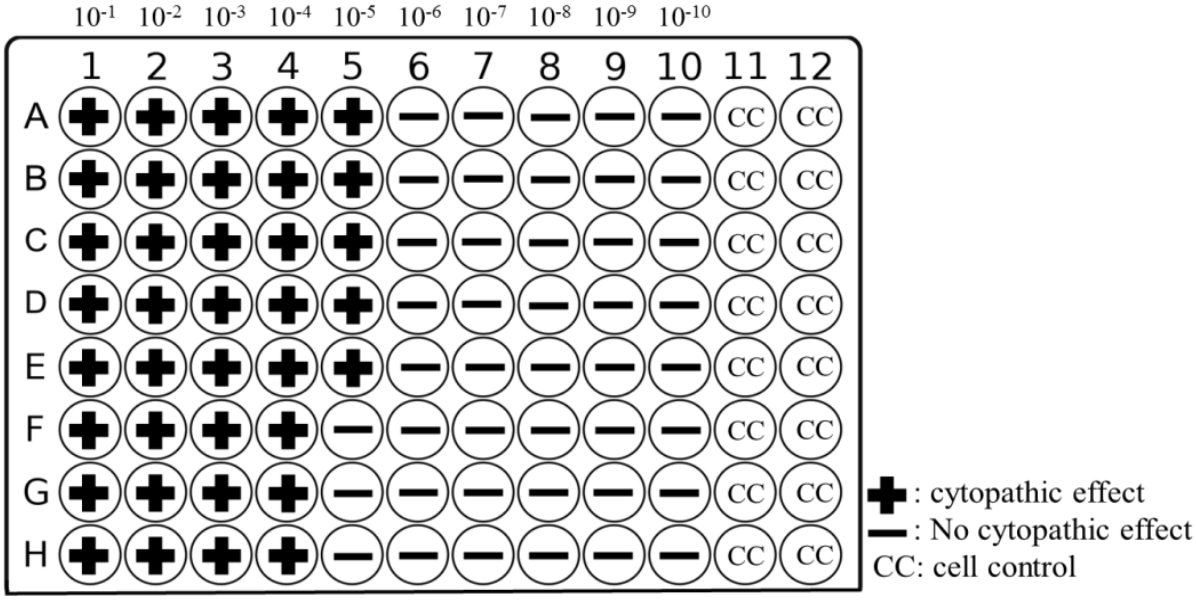
another 15-mL centrifuge tube. Aliquot 500 μL ~ 1 mL of the supernatant into each cryovial, and store in a freezer at -80℃ as the virus stock. Keep the vials for the following steps 2.8., 2.9., and 2.10.

2.8. Virus titration

2.8.1. Approximately 1 hour before infection, take the cells from step 2.6.2. Remove the spent medium, wash once with 100 μL/well of PBS, and refill with freshly prepared 100 μL/well of DMEM-2%FBS.

2.8.2. Prepare 10-fold serial dilution (10^{-1}~10^{-10}) of viral culture supernatant. Take 100 μL of the virus stock, and add 900 μL of DMEM-2%FBS as the 10^{-1} dilution. Take 100 μL of the 10^{-1} dilution and add 900 μL of DMEM-2%FBS as the 10^{-2} dilution. Then repeat the same process to make more dilutions (10^{-3}-10^{-10}). Add 100 μL of each dilution to Vero E6 cells from step 2.8.1. Perform eight replicates for each virus dilution. Incubate the infected cells in a humidified 35℃ incubator with 5% CO_2 for 3~5 days. At the end of incubation, observe the cells by a phase contrast microscope, and check the CPEs. Calculate the fifty-percent cell culture infective dose/mL ($CCID_{50}$/mL) using the Reed–Muench method.

Example



1. Calculate proportionate distance between the two dilutions in between 50% death.
   Example above:
   (% next above 50%)-50% / (% next above 50%)-(% next below 50%)
   = (5/8 × 100% - 50%) / (5/8 × 100% - 0%)
   = (62.5% - 50%) / (62.5% - 0%) = 0.2
2. Calculate 50% end point.
   Example above:
   Log lower dilution
   = dilution in which position is next above 50%
   = $\log 10^{-5}$
3. Apply proportionate distance to Log lower dilution.
   Example above:
   Log $CCID_{50}$ = $10^{-5-0.2}$ = $10^{-5.2}$
4. Calculate $CCID_{50}$/mL.
   Example above:
   Divide by the mL of viral inoculum added to column 1.
   According to the protocol, the viral volume added to column 1 is 0.1 mL .
   $CCID_{50}$/mL = (1 / $10^{-5.2}$) / 0.1 = 1.6×10^{6}

2.9. Neutralization procedure (note 3)

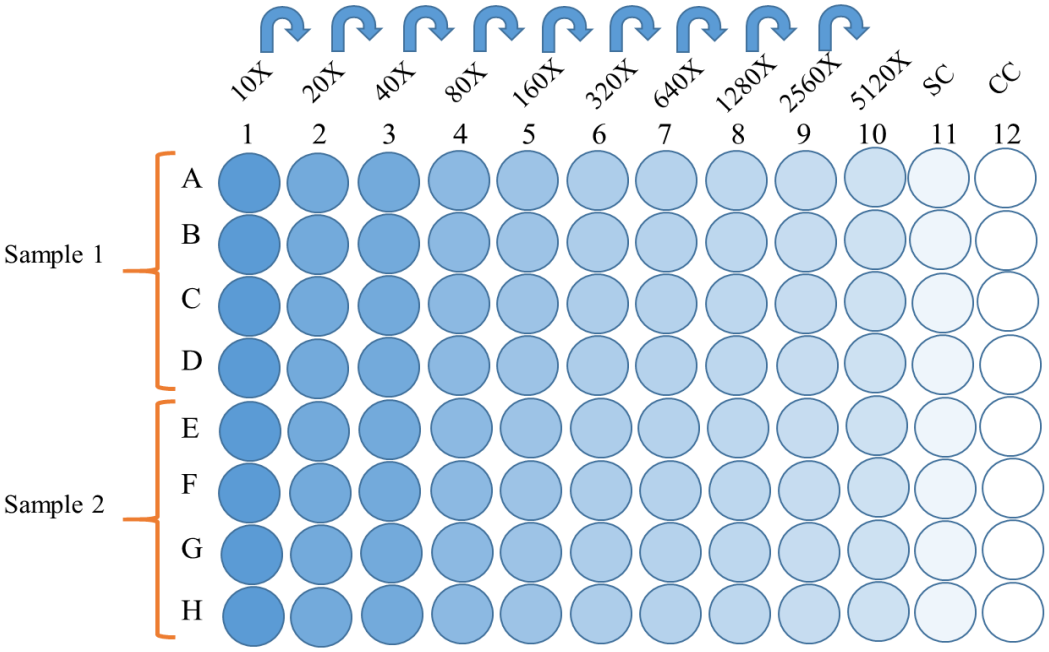
2.9.1. Before applying the virus onto neutralization, the virus titer must be determined following the procedures of step 2.8.

2.9.1.1. Approximately 1 hour before infection, take the cells prepared from step 2.6.2. Remove the spent medium, wash once with 100 µL of PBS, and refill with freshly prepared 100 µL/well of DMEM-2%FBS.

2.9.1.2. Heat inactivation of serum samples at 56℃ for 30 min before neutralization.

2.9.1.3. Take a new 96-well plate, pipetting 60 µL of DMEM into each well, and then use this plate as the sample dilution plate. Please use the following illustration as sample arrangements on the 96-well dilution plate. Briefly, use wells A1-10~D1-10 for sample dilution, wells A11~D11 for serum control (SC) and wells A12~D12 for cell

control (CC). Prepare 5-fold dilution of the testing sample by mixing 100 µL of the heated serum sample with 400 µL of DMEM. Pipette 60 µL of 5-fold dilution sample to wells A1~D1. Perform two-fold serial dilutions horizontally using 60 µL starting from column 1 and transferring dilutions horizontally until column 10 (column 1 = 1:10 through column 10 = 1:5,120) using an multi-channel pipette. Discard the final 60 µL from column 10 after completing dilutions in each row (note 4).



2.9.2. Dilute stock virus to a final concentration of 2000 $CCID_{50}$/mL for 10 mL as the virus working dilution (note 5). Add 60 µL of the virus working dilution to wells A1-10~D1-10 on the plate prepared from step 2.9.1.3. Meanwhile, add 60 µL for 5-fold dilution serum sample to wells A11~D11 as the serum control and 60 µL of DMEM to wells A12~D12 as the negative control. For neutralization to occur, keep the plate in a humidified 37℃ incubator with 5% CO_2 for 2 hours.

2.9.3. Transfer 100 µL of the serum-virus mixture from step 2.9.2. to infect Vero E6 cells prepared from step 2.9.1.1. Keep the cells in a humidified 35℃ incubator with 5% CO_2 for 3~5 days. Determine the results of neutralization by the appearance of CPEs with a phase contrast microscope observation. Then follow the instructions in step 2.11. to calculate the neutralization titer.

Note 3: Virus once removed from the freezer must be used in the same

day.

Note 4: Each 96-well plate can be used to test two serum samples. Every sample runs four replicates for each dilution.

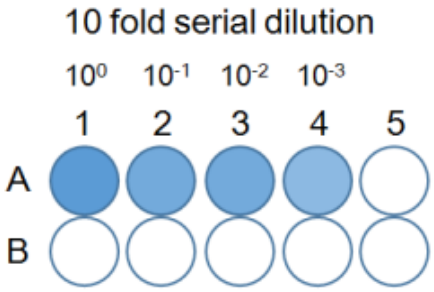
Note 5: The virus working dilution (2000 CCID50/mL) would also be applied at step 2.10.1.1.

2.10. Back titration

2.10.1. Follow the instructions to prepare virus dilution. Then, run eight replicates for each virus dilution.

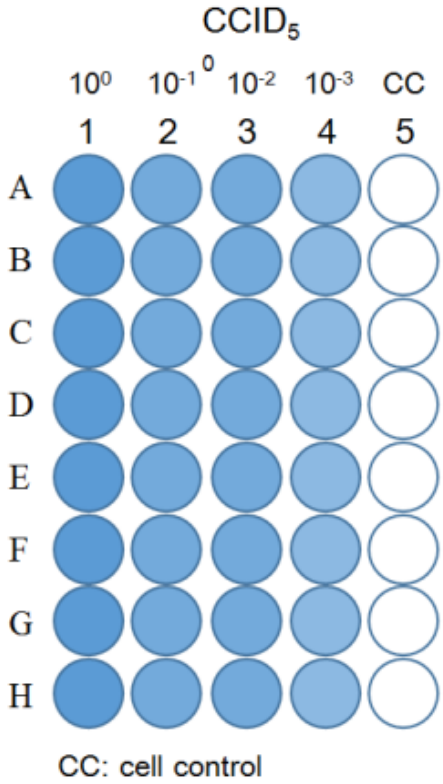
2.10.1.1. Add 900 µL of DMEM to wells A2~A4 of a 96 deep well plate. Fill well A1 with 1000 µL of the virus working dilution prepared from step 2.9.2.

2.10.1.2. Perform ten-fold serial dilutions horizontally using 100 µL starting from well A1 and transferring dilutions horizontally until well A4 (well A1 = 10^0 through well A4 = 10^{-3}). Discard the final 100 µL from well A4 after completing dilutions.



2.10.2. In a new 96-well plate, add 60 µL of DMEM to column 1 through column 5. Transfer 60 µL of the virus dilution from deep well A1, step 2.10.1.2 to column 1, deep well A2 to column 2, deep well A3 to column 3 and deep well A4 to column 4. Then, add 60 µL of DMEM to column 5. Keep the plate in a humidified 37℃ incubator with 5% CO_2 for 2 hours.

2.10.3. Before infection, take the cells prepared from step 2.6.2. Remove the spent medium, and wash once with 5 mL of PBS, and then



discard the used PBS. Refill with freshly prepared 100 μL/well of DMEM-2%FBS, and transfer 100 μL of the mixture from step 2.10.2 in order. Keep the cells in a humidified 35℃ incubator with 5% CO_2 for 3~5 days. Observe the appearance of CPEs by a phase contrast microscope and then calculate the virus titer according to the Reed-Muench method (note 6).

Note 6: Virus back titration should be performed in each batch of neutralization assay to verify the working virus titer. The working virus titer must be within the range of 50-150 CCID50 to meet the requirement.

2.11. Calculation of neutralization titer

2.11.1. Follow the step 2.9.2, and observe the test plates by a phase contrast microscope for the status of virus-induced formation of CPEs. The 100% neutralization titer (NT_{100}) is expressed as the highest dilution of the serum sample capable of completely preventing virus-induced CPEs.

2.11.2. At the same time, calculate logarithm 50% endpoint according to the

Reed-Muench method. The 50% neutralization titer (NT_{50}) is expressed by calculating the antilogarithm of logarithm 50% endpoint.

References

1. Manenti, A., Maggetti, M., Casa, E., Martinuzzi, D., Torelli, A., Trombetta, C., Serena, M. and Montomoli, E. 2020. Evaluation of SARS-CoV-2 neutralizing antibodies using a CPE-based colorimetric live virus micro-neutralizaiont assay in human serum samples. J. Med. Virol. 92: 2096-2104.
2. Loeffen, W., Quak, S., de Boer-Luijtze, E., Hulst, M., van der Poel., W., Bouwstra, R. and Maas, R. 2012. Development of a virus neutralisation test to detect antibodies against Schmallenberg virus and serological results in suspect and infected herds. Acta Vet. Scand. 54: 44-51.

Experiment flow