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Determination of SARS-CoV-2 Infectious Titer

1. Scope

This method is applicable to determine the infectious titer of SARS-CoV-2 in Vero E6 cells.

2. Method

Cell debris of SARS-CoV-2 samples had been removed by centrifugation. After serial dilution, inoculate each diluted test solution with Vero E6 cells. After 3 - 5 days incubation, observe the cytopathic effect (CPE) of cells by phase contrast microscope, record and calculate the infectious titer of SARS-CoV-2.

2.1. Work environment (note 1)

Experiments with SARS-CoV-2 should be conducted in a biosafety level 3 (BSL-3) laboratory. Procedures that handle viruses, such as infection or serial dilution, should be performed in a certified class II, type B2 or A2 biological safety cabinet (BSC). All the process should follow the regulations approved by the Taiwan Centers for Disease Control (CDC) (note 2)

Note 1: SARS-CoV-2 is classified as a risk group 3 (RG3) human pathogen. Personnel 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; Guidelines for the Implementation of Laboratory Biorisk Management; Safety Guidelines for the Use of Point-of-Care Testing (POCT) for SARS-CoV-2; Biosafety Guidelines for Laboratory Handling SARS-CoV-2.

2.2. Equipment

2.2.1. Biological safety cabinet (BSC): Class II, Type B2 or A2.

2.2.2. Autoclave: capable of operating at 121°C or higher temperature, and 15 pounds per square inch or above pressure.

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 an equivalent product.
2.2.8. Phase contrast microscope: up to 400X magnification.
2.2.9. Real-time PCR amplification and detection instrument: Roche LightCycler 480 Instrument II or an equivalent product.
2.2.10. Refrigerated centrifuge: fit for 15 mL and 50 mL centrifuge tubes; centrifugal force 2000 ×g and with temperature control at 4℃
2.2.11. Photo equipment: with photograph and transmit file function.

2.3. Reagents

2.3.1. Vero E6 cell line (ATCC® CRL-1586™): lower passage number (below 15) should be used. Cells are tested and found free of bacteria, fungi and mycoplasma.
2.3.2. Virus: SARS-CoV-2, for example, Wuhan strain, Omicron (BA.1) or Omicron (BA.4 / BA.5). The whole genome sequence of the virus must be known, and the subculture should be below 3 passages.
2.3.3. Fetal bovine serum (FBS).
2.3.4. Dulbecco's Modified Eagle's Medium (DMEM): Sigma-Aldrich D5796, or an equivalent product.
2.3.5. Phosphate buffered saline (PBS).
2.3.6. Trypsin: Thermo Fisher Scientific 25300054, or an equivalent product.
2.3.7. Antibiotics: with 10,000 units/mL penicillin and 10,000 units/mL streptomycin.
2.3.8. Dimethyl sulfoxide (DMSO).

2.3.9. QIAamp Viral RNA Mini Kit, or an equivalent product.

2.3.10. Roche LightCycler Multiplex RNA Virus Master, or an equivalent product.

2.4. Materials and Labware

2.4.1. Micropipettes: with volume ranges of 2 μL, 10 μL, 20 μL, 200 μL and 1000 μL.

2.4.2. Filter tips for micropipettes: 10 μL, 20 μL, 200 μL and 1000 μL.

2.4.3. Serological pipette: 5 mL, 10 mL pipette with 0.1 mL graduation line and 25 mL pipette with 1 mL graduation line.

2.4.4. Microcentrifuge tube: 1.5 mL.

2.4.5. Centrifuge tube: 15 mL and 50 mL, PP.

2.4.6. Cryogenic vial: 2.0 mL.

2.4.7. Cell culture flask/plate: T75 flask, T150 flask and 96-well plate.

2.4.8. 96 deep well plate for virus dilution.

2.4.9. Cell freezing container: stable cool down in -80℃, for storing cryogenic vials (2.0 mL).

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 with gently shaking.

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

2.5.3. DMEM-5% FBS: Add 50 mL of FBS to 950 mL of DMEM, and mix with gently shaking.

2.5.4. Cell freezing medium: Add 0.5 mL of DMSO to 9.5 mL of DMEM-5% FBS, and mix with gently shaking.

2.6. Cell culture

2.6.1. Culture Vero E6 cells with DMEM-10% FBS in T75 flask, and incubate with 37℃, 5% CO_2 in a humidified incubator, and then use for follow-up experiments.

2.6.2. Observe Vero E6 cells by a phase contrast microscope. When the cells are approximately 90% confluent, remove the spent medium, wash once with 5 mL of PBS, remove

and discard the wash solution, and add 2 mL of trypsin carefully. Gently shake the cell flask until completely covered by trypsin. Remove trypsin and incubate the cell flask in a humidified 37℃ incubator with 5% CO_2 for 2 minutes, then inactivate the trypsin with 10 mL DMEM-10% FBS. When cells are detached, collect the cell suspension to centrifuge tube.

2.6.3. Determine the cell numbers using a cell counter. Freeze the cultured cells or seeding in 96-well plate according to experimental needs.

2.6.3.1. Cryopreservation of cells

Prepare cell freezing container and fresh cell freezing medium on ice. Centrifuge the cell suspension from step 2.6.2. at 750 ×g for 5 minutes. Remove the spent medium, then gently resuspend cell pellet with appropriate amount of freezing medium to prepare a concentration of 1×10^6 - 5×10^6 cells/mL. Dispense of the resulting cell suspension into cryogenic vials, 1 mL/vial. Place the vials in the cell freezing container. Cover the freezing container and place at -80ºC for at least 1 day, then transfer the vials to liquid nitrogen.

2.6.3.2. Cells for virus titration

Count Vero E6 cells, and dilute the cells to 1×10^5 cells/mL with DMEM-10% FBS, then inoculate 100 µL to each well of 96-well plate. Incubate with 37℃, 5% CO_2 in a humidified incubator for 16-18 hours, and then use the plate for the following step 2.7.

2.7. Virus titration (note 3)

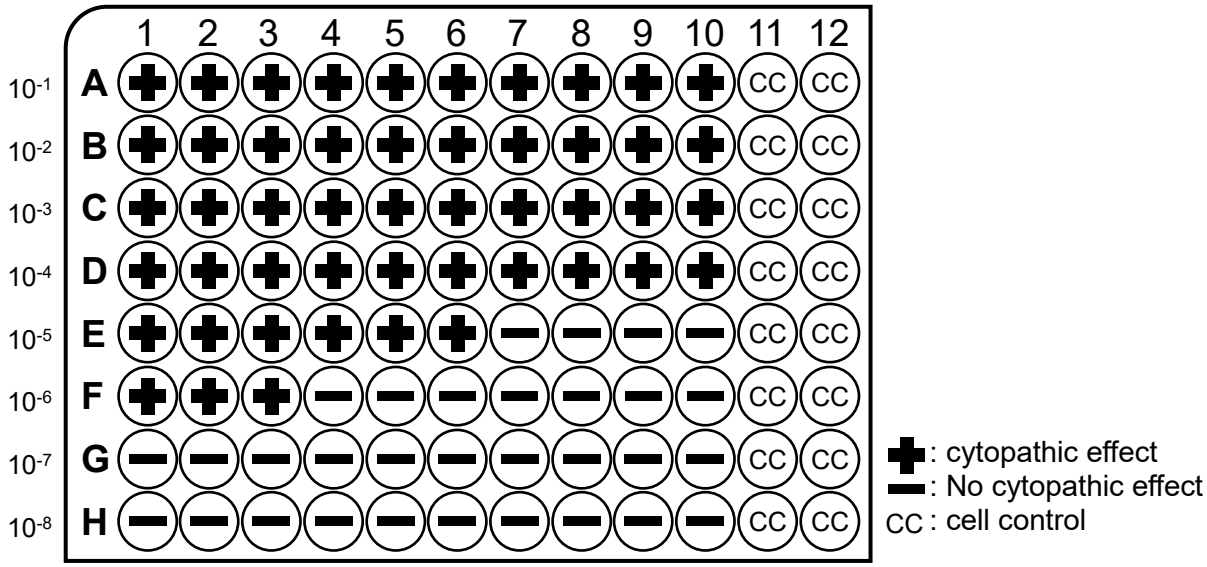
2.7.1. Approximately 1 hour before infection, remove the spent medium of 96-well plate from step 2.6.3.2. and wash once with 100 µL/well of PBS. Remove the PBS, and refill with fresh prepared 100 µL/well of DMEM-2% FBS.

2.7.2. Take out the virus sample to be tested for infectivity from the freezer and place it in the BSC until thawed.

2.7.3. Prepare 10-fold serial dilution (10^{-1}-10^{-8}) of virus sample in a 96 deep well plate. Add 1350 µL of DMEM-2% FBS into each well of A1-H1. Take 150 µL of virus sample from step 2.7.2. into A1, then replace the filter tip and pipetting 20 times. Take 150 µL of the 10^{-1} dilution from A1 into B1, then replace the filter tip and pipetting 20 times. Take 150 µL of the 10^{-2} dilution from B1 into C1. Repeat the same process to make more dilutions (10^{-3}-10^{-8}).

2.7.4. After pipetting 20 times by multichannel pipettes, take 100 µL of A1-H1of dilutions (10^{-1}-10^{-8}) form step 2.7.3. into vero E6 cells (A1-H1) of step 2.7.1. Perform ten replicates for each virus dilution. Add 100 µL of DMEM-2% FBS to each well of A11-H11, A12-H12 for cell control (CC). Incubate the cells in a humidified incubator with 35℃, 5% CO_2 for 3-5 days. Observe the cells by a phase contrast microscope, and check the CPEs. Calculate the fifty-percent cell culture infectious dose/mL ($CCID_{50}$/mL) using the Reed–Muench method on day 5.

Calculation of infectious titer:



Dilution	CPE negative	CPE positive	Cumulative		Infection rate (%, B/A+B)
			Negative (A)	Positive (B)	
10^{-1}	0	10	0	49	100.0
10^{-2}	0	10	0	39	100.0
10^{-3}	0	10	0	29	100.0
10^{-4}	0	10	0	19	100.0
10^{-5}	4	6	4	9	69.2
10^{-6}	7	3	11	3	21.4
10^{-7}	10	0	21	0	0

Determination of cytopathic effect: When the morphology of virus-infected cells different from cell control (CC) on the day 5, it is determined the cell have a cytopathic effect (CPE).

1. Calculate proportionate distance between the two dilutions in between 50% death.

   Example above: $\frac{\text{(\% next above 50\%) - 50\%}}{\text{(\% next above 50\%) - (\% next below 50\%)}} = \frac{(69.2\% - 50\%)}{(69.2\% - 21.4\%)} = \frac{19.2\%}{47.8\%} = 0.402$

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 $CCID_{50}$ = $10^{-5.402}$
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 = $(1 / 10^{-5.402}) / 0.1 = 2.52 \times 10^{6}$

Note 3: It is recommended that to use reverse pipetting at step 2.7.1. and 2.7.4. Add the liquid to 96-well plate by leaning the top filter tips against the wall of well, don't rinse cells directly.

2.8. Quantification of viral nucleic acids

2.8.1. After serial dilution, each dilution level of viral RNA was extracted by nucleic acid extraction method, and quantify the concentration of viral nucleic acids by using real-time PCR.

2.8.2. Calculate the average and standard deviation of viral nucleic acids, at least 3 independent tests of the above experiment.

2.8.3. The concentration of viral nucleic acids can refer to SARS-CoV-2 National standards produced by Taiwan Food and Drug Administration (TFDA) or International standards produced by World Health Organization (WHO).

References

1. Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27: 493-497.
2. Mautner, L., Hoyos, M., Dangel, A., Berger, C., Ehrhardt, A. and Baiker A. 2022. Replication kinetics and infectivity of SARS-CoV-2 variants of concern in common cell culture models. Virol. J. 19: 76.
3. Taiwan Food and Drug Administration. 2020. SARS-CoV-2 neutralization assay (RA05I001.001). Published, Dec 10, 2020.
4. Taiwan Food and Drug Administration. 2022. Method of test for *in vitro* diagnostic device for SARS-CoV-2 antigens (RA04B001.001). Published, Dec 30, 2022.