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Method of Test for *In vitro* Diagnostic Device for SARS-CoV-2 Antigens

1. Scope

This method is applicable to evaluate the performance of the "*In vitro* Diagnostic Device for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Antigens" for pre-, post-market or border inspection. (Note 1)

2. Method

Take the infectious SARS-CoV-2 with measured infectivity titer, dilute it to the specific concentration, apply the viral solution to the diagnostic device, complete the observation and record the results within the defined time frame according to the instructions of the diagnostic device for SARS-CoV-2 antigens, and record the images with photographic equipment and record the results with hard copy.

2.1. Work environment (Note 2)

SARS-CoV-2 related experiments should be carried out in biosafety level 3 (BSL-3) laboratories, and related operations such as SARS-CoV-2 infection and dilution should be carried out in biological safety cabinets which must comply with Class II, Type B2 or A2. The operations should follow the relevant regulations (Note 3) of Taiwan Centers for Disease Control, Ministry of Health and Welfare.

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℃ 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 400 X magnification.

2.2.9. Ribonucleic acid analysis system: 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 camera and capable of file transmission

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), and the full gene sequence must be known. It is recommended that the virus strain is subcultured for no more than 3 generations after it is obtained.

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.

2.4. Apparatus

2.4.1. Pipette: 2 μL, 10 μL, 20 μL, 200 μL, and 1,000 μL.

2.4.2. Filter pipette tips: 10 μL, 20 μL, 200 μL and 1,000 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 1,000 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 for dilution.

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.

2.6. 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. and 2.8.

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 step 2.8.

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 at multiplicity of infection (MOI)

equal to 0.01, and then incubate the cell-virus flask in a humidified 35℃ incubator with 5% CO_2 for 1 hr. 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 an ultra-low temperature freezer at -80℃ as the virus stock (hereinafter referred to as the working virus seed). Apply the vials for the following steps 2.8., 2.9., 2.10. and 2.11.

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^{-8}) of the virus stock. 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^{-8}). Add 100 µL of each dilution to Vero E6 cells from step 2.8.1. Perform ten repetitions 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.

Calculation method for virus titration:

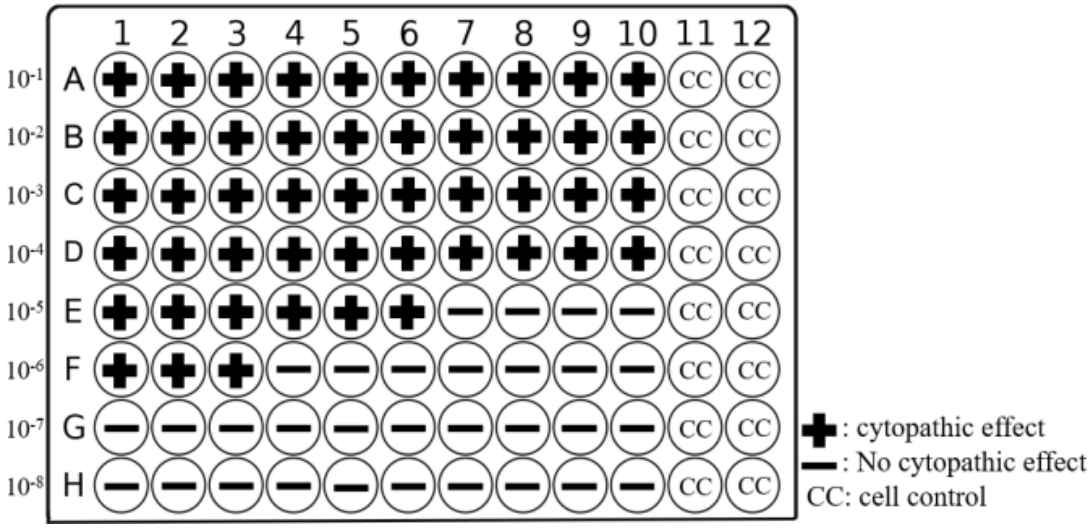

Figure: results of 10-fold serial dilution of virus titration ($CCID_{50}$)

Table 1: calculation of virus titration ($CCID_{50}$) 10-fold serial dilution results

Dilution	Alive (CPE-)	Dead (CPE+)	Total		Mortality (%, B/A+B)
			Alive (A)	Dead (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

1. Calculate proportionate distance between the two dilutions in between 50% death:
   [(% next above 50%) - 50%] / [(% next above 50%) - (% next below 50%)]
   = (69.2% - 50%) / (69.2% - 21.4%)
   = 19.2% / 47.8%
   = 0.402

2. Calculate the log value of the lowest dilution ratio for 50% cell death: $\log 10^{-5}$
3. Apply proportionate distance to Log lower dilution: Log $CCID_{50} = 10^{-5 - 0.402}$
4. Calculate $CCID_{50}$ /mL:
   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 .
   $CCID_{50}/mL = (1 / 10^{-5.402}) / 0.1 = 2.52 \times 10^6$

2.9. Positive test sample preparation

Take the working virus seed which is frozen at -80°C in section 2.7.3. and the virus titration has been determined as described in section 2.8., thaw in a biological safety cabinet and dilute to appropriate dilution ratio with PBS or DMEM. (For example, 2,000, 1,000, 500 $CCID_{50}$/mL, refer to the table below.)

Table 2: Example of viral dilution

Dilution No.	Source and volume	PBS /DMEM	Total volume	Final concentration ($CCID_{50}$/mL)
1	Working virus seed (5×10^5 $CCID_{50}$/mL) 1 mL	9 mL	10 mL	50,000
2	No.1 dilution 2 mL	3 mL	5 mL	20,000
3	No.1 dilution 1 mL	4 mL	5 mL	10,000
4	No.1 dilution 1 mL	9 mL	10 mL	5,000
5	No.4 dilution 4 mL	6 mL	10 mL	2,000
6	No.5 dilution 5 mL	5 mL	10 mL	1,000
7	No.6 dilution 5 mL	5 mL	10 mL	500
8	Previous dilution 5 mL	5 mL	10 mL	…

2.10. Consistency of positive test sample preparation

According to the experimental requirements, use the diagnostic device for validation or RT-qPCR to confirm the consistency of the preparation. It is recommended to choose either one for each test.

2.10.1. Diagnostic device for validation

2.10.1.1. Mix 150 μL of serial diluted SARS-CoV-2 with the buffer provided by the SARS-Co V-2 antigen diagnostic device. Apply the mixture to the diagnostic device, follow the instructions and record the lowest detectable concentration. For example, 1,000 $CCID_{50}$/mL indicates a positive test and 500 $CCID_{50}$/mL indicates a negative test. Then, the lowest detectable concentration of this batch of diagnostic device is 1,000 $CCID_{50}$/mL.

2.10.1.2. For follow-up tests, prepare serial dilutions of SARS-CoV-2, and test the diluted viral solutions using the above-mentioned validation diagnostic device with determined lowest detectable concentration. It is recommended that the lowest detectable concentration shall not be higher or lower than this determined value.

2.10.2. RT-qPCR

2.10.2.1. For each test, prepare serial dilutions of the virus to a specific concentration (such as 1,000 $CCID_{50}$/mL), extract viral RNA by nucleic acid extraction method. Then the concentration of viral nucleic acid is determined by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR).

2.10.2.2. The average, standard deviation and other relevant statistical data of the viral nucleic acid concentration were calculated based on at least 3 independent tests.

2.10.2.3. Nucleic acid concentration is calibrated by WHO SARS-CoV-2 international standard (20/146) or TFDA SARS-CoV-2 national standard (TFDA Lot COV 111-05-01).

2.10.2.4. After the virus is diluted to a specific concentration (such as 1,000 $CCID_{50}$/mL), the concentration of viral nucleic acid is determined by RT-qPCR. The test result should be within 2 standard deviation of the average.

2.11. Test procedure

2.11.1. Control line testing group: Apply the buffer provided by the SARS-CoV-2 antigen diagnostic device to 2 test pieces (2 replications) and follow the instructions to complete the test. Interpret and record the results within the defined time frame.

2.11.2. Negative test sample group (blank testing group): Mix 150 μL of PBS or DMEM with the buffer provided by the SARS-CoV-2 antigen diagnostic device, apply the mixture to 2 test pieces (2 replications) and follow the instructions to complete the test. Interpret and record the results within the defined time frame.

2.11.3. Positive test sample group:
Prepare 150 μL of serial diluted SARS-CoV-2 as described in section 2.10. and mix it with the buffer provided by the SARS-CoV-2 antigen diagnostic device. Apply the mixture to 3 test pieces (3 replications) for each dilution and follow the instructions to complete the test. Interpret and record the results within the defined time frame.

2.12. Validity and results presentation

2.12.1. The results of the test pieces should meet the valid conditions as described on the product manual and do not meet the invalid conditions.

2.12.2. Test validity

2.12.2.1. The C line should be found in the control line testing group, the negative test sample group and the positive test sample group.

2.12.2.2. The T line should not be found in the control line testing group and the negative test sample group.

2.12.3. Result presentation

2.12.3.1. Diagnostic device analytical reactivity test

2.12.3.1.1. Positive reactivity test: The T line of the 3 test pieces in the positive test sample group all showed

reactivity at an appropriate virus concentration, for example, if they all show reactivity at a concentration of 1,000 $CCID_{50}$/mL in a diluted virus solution, then record virus concentration of 1,000 $CCID_{50}$/mL as positive reaction.

2.12.3.1.2. Negative reactivity test: Only the C line of the 2 test pieces in the control line testing group and the negative test sample group showed reactivity.

2.12.3.2. The lowest detectable concentration of diagnostic device

The lowest detectable concentration is the lowest concentration of virus in the positive test sample group for which all 3 test pieces present the T line showing reactivity.

Notes

1. The method and result are solely used for confirming the lowest detectable concentration of the product, not for application documents of product registration or product emergency use authorization.
2. 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.
3. 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.

References

1. Lei, C., Yang, J., Hu, J. and Sun, X. 2021. On the calculation of $TCID_{50}$ for quantitation of virus infectivity. Virol Sin. 36: 141-144.
2. World Health Organization. 2021. Technical specifications for selection of essential *in vitro* diagnostics for SARS-CoV-2. [https://www.who.int/publications/m/item/technical-specifications-for-selection-of-essential-in-vitro-diagnostics-for-sars-cov-2.]
3. U.S. Food & Drug Administration. 2021. Coronavirus disease 2019 (COVID-19) emergency use authorizations for medical devices. [https://www.fda.gov/medical-devices/emergency-use-authorizations-medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices.]
4. Australia Therapeutic Goods Administration. 2022. Test to verify the analytical sensitivity of COVID-19 rapid antigen test kits. [https://www.tga.gov.au/resources/resource/guidance/test-verify-analytical-sensitivity-covid-19-rapid-antigen-test-kits.]
5. Ministry Health and Welfare Taiwan Food and Drug Administration. 2020. SARS-CoV-2 Neutralization Assay (RA05I001.001). Publish, Dec 10, 2020.