Laboratory Procedures

Serological detection of avian influenza A(H7N9) infections by microneutralization assay

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The WHO Collaborating Center for Reference and Research on Influenza at the Chinese National Influenza Center, Beijing, China, has made available attached laboratory procedures for serological detection of avian influenza A(H7N9) infections by microneutralization assay.

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These procedures were adapted from the WHO Manual for the laboratory diagnosis and virological surveillance of influenza (Chapter 2G on page 63)[1].

INTRODUCTION

Serological methods rarely yield an early diagnosis of acute influenza virus infection. However, the demonstration of a significant increase in antibody titers (greater than or equal to 4-fold) between acute-phase and convalescent-phase sera may establish the diagnosis of a recent influenza infection even when attempts to detect the virus are negative. Apart from their retrospective diagnostic value, serological methods such as virus neutralization and haemagglutination inhibition are the fundamental tools in epidemiological and immunological studies, as well as in the evaluation of vaccine immunogenicity.

The microneutralization assay is a highly sensitive and specific assay for detecting virus-specific neutralizing antibodies to influenza viruses in human and animal sera, potentially including the detection of human antibodies to avian subtypes. Virus neutralization gives the most precise answer to the question of whether or not an individual has antibodies that can neutralize the infectivity of a given virus strain. The assay has several additional advantages in detecting antibodies to influenza virus. First, it primarily detects antibodies to the influenza viral HA protein and thus can identify functional strain-specific antibodies in human and animal sera. Second, since infectious virus is used, the assay can be carried out quickly once the emergence of a novel virus is recognized. Although conventional neutralization tests for influenza viruses (based on the inhibition of cytopathogenic effect formation in MDCK cell culture) are laborious and rather slow, a microneutralization assay using microtiter plates in combination with an ELISA to detect virus-infected cells can yield results within two days. The influenza virus microneutralization assay presented below is based on the assumption that serum-neutralizing antibodies to influenza viral HA will inhibit the infection of MDCK cells with virus. Serially diluted sera should be pre-incubated with a standardized amount of virus before the addition of MDCK cells. After overnight incubation, the cells are fixed and the presence of influenza A virus

[1] http://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/, accessed 23 May 2013

nucleoprotein (NP) protein in infected cells is detected by ELISA.

The absence of infectivity constitutes a positive neutralization reaction and indicates the presence of virus specific antibodies in the serum sample. In cases of influenza-like illness, paired acute and convalescent serum samples are preferred. An acute sample should be collected within seven days of symptom onset and the convalescent sample collected at least 14 days after the acute sample, and ideally within 1–2 months of the onset of illness. A 4-fold or great rise in antibody titer demonstrates a seroconversion and is considered to be diagnostic. With single-serum samples, care must be taken in interpreting low titers such as 20 and 40. Generally, knowledge of the antibody titers in an age-matched control population is needed to determine the minimum titer that is indicative of a specific antibody response to the virus used in the assay.

The microneutralization protocol is therefore divided into three parts:

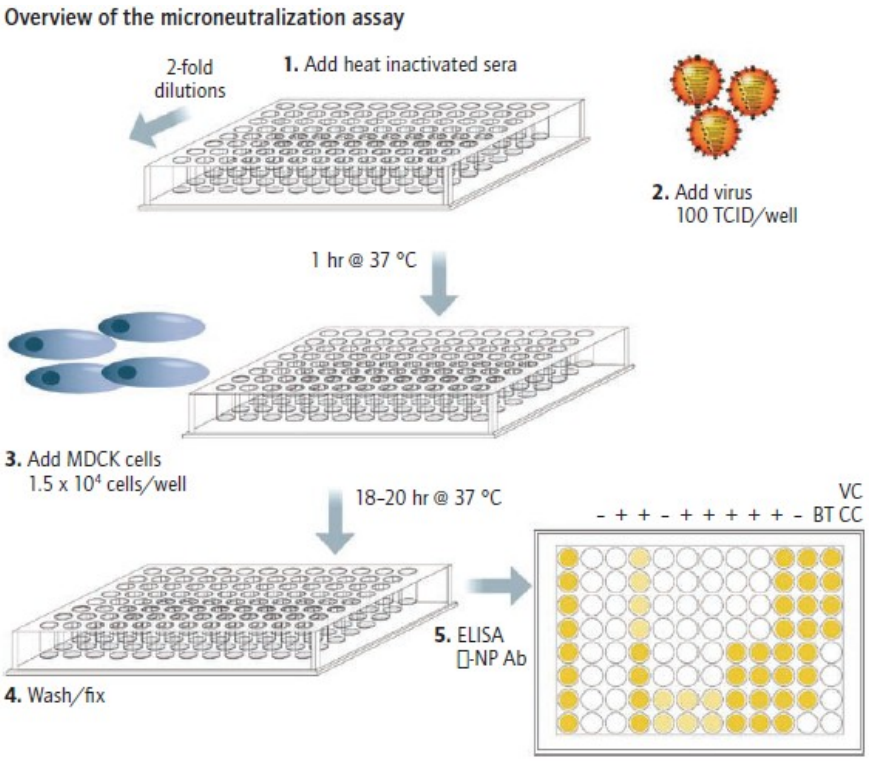
Part I: Determination of the tissue culture infectious dose ($TCID_{50}$).

Part II: Virus microneutralization assay.

Part III: ELISA.

An overview of the microneutralization assay is showed in FIGURE 1 as below.

FIGURE 1.



1. Materials required

1.1 Equipments

Class II biological safety cabinet.
Water baths, 37°C and 56°C.
Incubator, 37°C, 5% CO_2.
Inverted microscope or standard microscope for the observation of cells.
Automatic ELISA reader with 492 nm filter.
Automatic plate washer (not essential but would be optimal).
Low speed, bench top centrifuge.
4°C refrigerator.
Freezer, - 70°C (for long term virus storage) or - 20°C (for serum storage).

1.2 Supplies

Cell culture flasks
96-well microtiter plates(flat-bottom)
Haemacytometer and haemacytometer coverslips
Cell counter
Multichannel pipette and tips
Pipettes
Tubes

1.3 Cell, media and buffers

MDCK cell culture monolayer – low passage (<25–30 passages) at low crowding (70‑95% confluence)
D-MEM high glucose (1x) liquid, with L-glutamine and without sodium pyruvate (Invitrogen-cat.no 11965-092)
0.01 M PBS (pH 7.2)(Invitrogen-cat.no 20012-43)
HEPES buffer (1 M stock solution)(Invitrogen-cat.no 15630-080)
Citrate buffer capsules(Sigma cat.no P4922)
Water (distilled and deionized)
MDCK sterile cell culture maintenance medium(see below)
Virus diluent(see below)
Wash buffer(PBST)
Fixative solution(see below)
Stop solution(see below)

1.4 Reagents

Penicillin-streptomycin (stock solution contains 10000 U/ml penicillin; and 10000µg/ml
streptomycin sulfate) Invitrogen-cat.no 15140-122
200 mM L-glutamine Invitrogen-cat.no 25030-081
Trypsin-EDTA (0.05% trypsin; 0.53 mM EDTA 4Na) Invitrogen-cat.no 25300-054
Trypsin – TPCK-treated (type XIII from bovinepancreas) Sigma-cat.no T1426
o-phenylenediamine dihydrochloride (OPD)Sigma-cat.no.P8287
Fetal bovine serum (FBS) Invitrogen cat. no. 10099-141
Bovine albumin fraction V (prepared as a 7.5% solution in water) Roche,70250224
Trypan blue stain (0.4%) Invitrogen-cat.no 15250-061
PBST
Acetone

1.5 Antibodies

1º-antibody: Anti-Influenza A NP mouse monoclonal antibody (millipore, mixed A1,A3), dilute 1:4000 in blocking buffer or at optimal concentration

2º-antibody: Goat anti-mouse IgG conjugated to HRP(KPL), dilute 1:2000 in blocking buffer or at optimal concentration

2. Preparation of media and solutions

2.1 MDCK sterile cell culture maintenance medium:

DMEM, 10% FBS, P/S
440 mL Dulbecco's Modified Eagles Medium (DMEM)
5 mL 100 X P/S (100µ/mL penicillin, 100g/mL streptomycin)
5 mL 200 mM L-glutamine
50 mL FBS
FBS need to be heat-inactivated at 56°C for 30 min before use.

2.2 Virus diluent:

DMEM, 1% Bovine serum albumin (Roche,70250224), P/S – freshly prepared
415.4 mL DMEM
5 mL 100 X Antibiotics
67 mL 7.5% BSA; or 5 g BSA fraction V powder
12.5 mL HEPES buffer solution (1 M)
100ul Trypsin-TPCK-treated(2ug/ml) (type XIII from bovine pancreas)

2.3 Fixative: Cold 80% Acetone in PBS – freshly made

80 mL Acetone

20 mL PBS

2.4 Blocking Buffer: PBST, 1% BSA

1000 mL PBST

144 mL 7.5% BSA or 10 g BSA fraction V powder

2.5 Substrate: o-phenylenediamine dihydrochloride (OPD); For 20 mL of Citrate buffer, add 1 tablet of OPD (10 mg) just before use.

For citrate buffer, prepare as follows:

Prepare with Sigma Citrate Buffer capsules. Add1 capsule into 100 mL dH_2O.

2.6 Stopping Solution: 1N Sulfuric acid. Add 28mL stock sulfuric acid [18M] into 1L dH_2O

3.Quality Control

The procedure of Virus microneutralization assay is complicated. The changes of any factors involved in this assay such as virus, cell or sera may affect the final result. Thus quality control is necessary. Setting Positive and negative serum control and cell control in every test plates is requested, virus titer should be determined before virus microneutraliazation assay.

3.1 Serum controls:

Include serum samples, positive serum control and negative serum control. If samples are to be tested repeatedly, it is better to make aliquots. Sera should not be repeatedly freeze-thaw. Sera can be stored at -20 to -70℃. Human sera needs to be heat-inactivated at 56°C for 30 min and animal sera need be treated by RDE before use.

3.1.1 Positive (column1,2,infected or vaccinated) serum controls:

Include anti-sera to test viruses as positive control. For human sera, an optimal positive control would be acute and convalescent serum samples.

3.2.2 Negative (normal) serum control (column3,4): (FIGURE 2)

Include a normal serum to determine whether the virus is nonspecifically inactivated by serum components.

1) For human sera, use normal serum from a population not exposed to the particular virus subtype in question.

2) Use the normal serum at the same dilution as the matching viral antiserum.

Virus and cell controls include a virus back-titration and positive and negative cell controls with each assay.

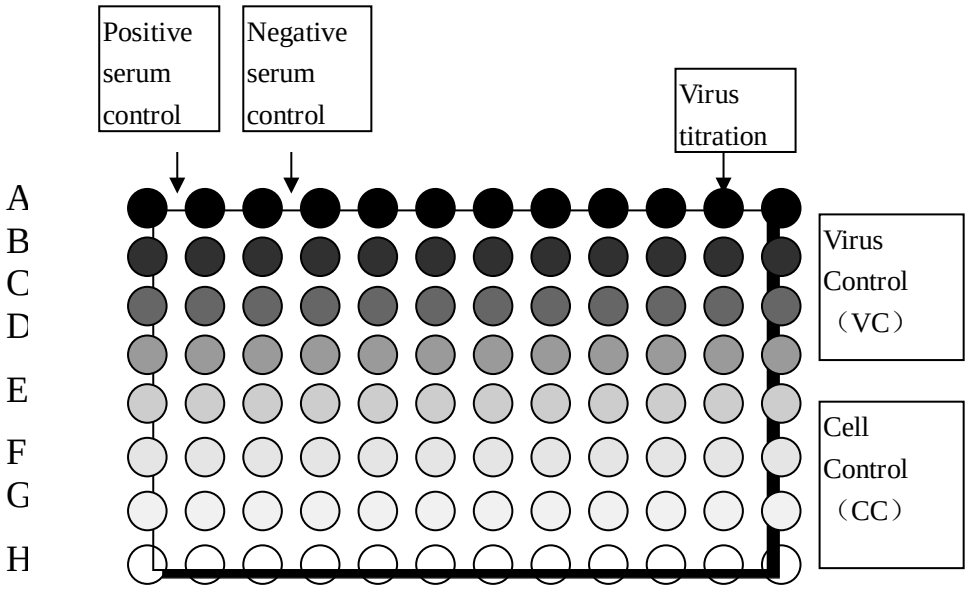
3.2 Negative and positive cell controls:

Set up four wells as positive cell controls in column 12 (wells A-D) - VC (50μl medium + 50μl test dilution of virus + 100 μl of MDCK cells) and four wells as negative cell controls in column 12 (wells E-H) - CC (100μl medium + 100μl of MDCK cells) and assay in parallel with the microneutralization test. The cell controls should be included on each plate to control for plate to plate variation.

3.3 Virus titration check:

In each assay, include a back-titration of the test dilution of virus. Add 50μl of medium to wells A-H of column 11. Add 50μl of the test dilution of virus to the first well (A11). Serially transfer 50μl down the column 11 (7 wells, B to H). Add an additional 50μl of virus diluent to column 11. Add 100μl of MDCK cells ($1.5x10^4$/well) to column 11.

FIGURE 2.



Part I: Virus titration and determination of tissue culture infectious dose ($TCID_{50}$) for microneutralization assay

For safety reasons, seasonal and low pathogenic avian viruses, and human serum samples, should be handled in a class-II biosafety cabinet in BSL-2 laboratories, while highly pathogenic avian influenza (including the novel H7N9 virus) ***microneutralization assays should additionally be performed only in BSL-3+ laboratories***

1. Virus dilution

Virus should be stored at –70℃; Determine $TCID_{50}$ before use; never using freeze-thawed virus. Procedure of virus $TCID_{50}$ assay was described as below:

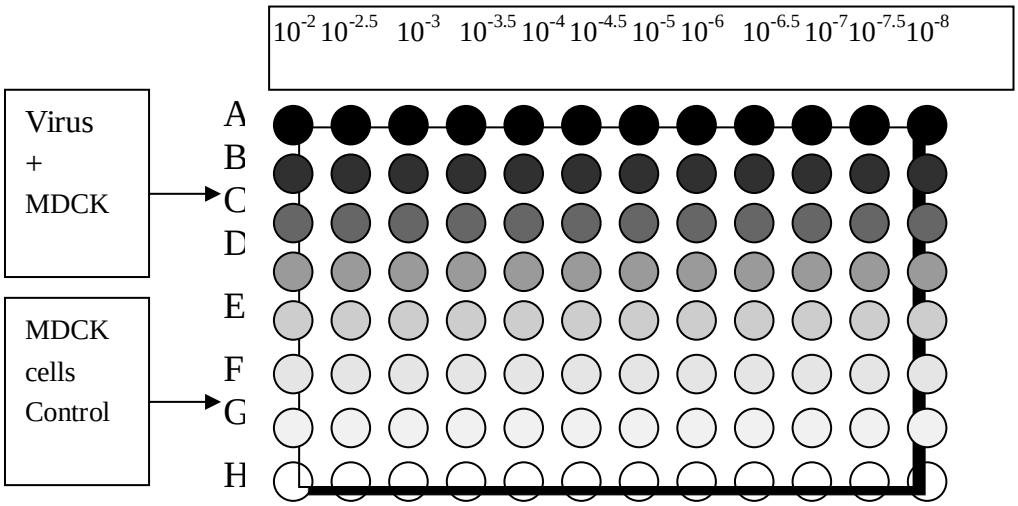
Virus can be diluted by log_{10} or ½ log_{10} dilution, ½ log_{10} dilution was introduced as below（FIGURE 3）:

1.1 Dilute virus 1/100 in dilution buffer (100 µl virus + 9.9 mL dilution buffer) as working stock.

1.2 Add 100 µl of *virus diluent* to all wells, except column A1-D1, of a 96-well tissue culture plate.

1.3 Add 146 µl virus of 1/100 working stock to column A1-D1. Transfer 46 µl serially from column 1→2→3→…11 (½ log_{10} dilutions). Change tips in each dilution. Dilutions will be 10^{-2}, $10^{-2.5}$, 10^{-3}…10^{-7}

FIGURE 3.



2. Preparation of MDCK cells

2.1 Check the MDCK cell monolayer (which should be 70–95% confluent).
Do not allow the cells to overgrow. Typically, a confluent T75 flask (approximately 2×10^7 cells/flask) should yield enough cells to seed 4–6 96-well microtiter plates.
2,2 When ready to perform the assay, wash 70–95% confluent cells with PBS to remove FBS.
2.3 Add 7 ml trypsin-EDTA to cover the cell monolayer.
2.4 Lie flask flat and incubate at 37°C until monolayer detaches (approximately 8–10 minutes).
2.5 Add 7 ml of virus diluent to each flask.
2.6 Wash cells twice with virus diluent to remove FBS.
— gently mix to resuspend and break up clumps of cells;
— fill tube to 50 ml with virus diluent;
— pellet cells by centrifugation at 1000rpm for 10 minutes;
— decant supernatant;
— perform one repeat of the previous 4 steps.
2.7 Resuspend cells in virus diluent (10 ml per trypsinized flask) and count cells with a haemacytometer as described below in FIGURE4, Determination of cell count and viability.
2.8 Adjust cell concentration to 1.5×10^5 cells/ml with virus diluents.
2.9 Add 100 µl diluted cells to each well of the microtiter plate.
2.10 Incubate cells for 18–20 hours at 37°C in 5% CO_2.

FIGURE 4.

Example of determining the percentage cell viability in a cell suspension diluted 1:10 in PBS and 1:2 in trypan blue stain

1. Count the number of viable and nonviable cells in each corner quadrant as in the example below:

Corner quadrant	No. of viable cells	No. of nonviable cells (stained with trypan blue)
1	106	0
2	88	2
3	99	0
4	115	1
Total	408	3

2. The average viable cell count (since 4 quadrants were counted) is 102 (408 divided by 4).

3. The volume of cell suspension counted is 0.1 mm^3. Therefore, the concentration of cells in the suspension counted is:

$$\frac{102}{0.1\ mm^3} = \frac{102}{10^{-4}\ ml} = (102 \times 10^4)/ml = 1.02 \times 10^6\ cells/ml$$

4. As the suspension was diluted from the original material 10-fold and diluted 2-fold in dye, the above result must be multiplied by 20 (i.e. 101 and 2) as follows:

$1.02 \times 10^6 \times 101 \times 2 = 2.04 \times 10^7$ cells/ml

5. The condensed version of the derivation of the equation is:

$$\frac{\text{viable cell count}}{4} \times 10^1 \times 2 \times (1 \times 10^4) = \text{number of viable cells/ml}$$

4 = the number of quadrants counted
10^1 = the dilution factor for PBS – if multiple 10-fold dilutions were made adjust accordingly
2 = the dilution factor for stain
1×10^4 = the volume counted per quadrant (per ml)

6. Percentage cell viability is then determined by the following equation:

$$\text{cell viability (\%)} = \frac{\text{number of viable cells counted}}{\text{total number of cells counted}} \times 100\%$$

$$\text{In this example, cell viability} = \frac{408}{411} \times 100\% = 99.2\%$$

3.Fixation of cells

3.1 Remove medium from microtiter plate.
3.2 Wash each well with 200 µl PBS.
3.3 Remove PBS (do not allow wells to dry out) and add 100 µl/well of *cold* fixative.
3.4 Cover with lid and incubate at room temperature for 10–12 minutes.
3.5 Remove fixative and let the plate air dry.

4.Determination of $TCID_{50}$ for microneutralization assay

4.1 Perform ELISA (see protocol below).
4.2 Calculate the mean absorbance (OD492) of the CCs.
4.3 Any test well with an OD492 greater than twice the average of OD492 of the CC wells is scored positive for virus growth.
4.4 Once all test wells have been scored positive or negative for virus growth, the $TCID_{50}$ of the virus can be calculated as shown below by the Reed-Muench method (Reed & Muench, 1938).

Calculation (TABLE 2)
1. Record the number of positive values observed in column (**1**) and negative values in column (**2**) wells of the microtiter plates at each dilution.
2. Calculate the cumulative numbers of positive values in column (**3**) of TABLE 2. and negative values in column (**4**) of TABLE 2:
— column (**3**) – obtained by adding the numbers in column (**1**) starting at the ***bottom***.
— column (**4**) – obtained by adding the numbers in column (**2**) starting at the ***top***.
3. Calculate the ratios at each dilution in column (**5**) by dividing the number of positives in column (**3**) by the number of positives plus negatives in columns (**3**) + (**4**).
4. Calculate the percentage of positive wells in column (**6**) by converting each of the ratios in column (**5**) to percentages.
5. Calculate the proportional distance between the dilution showing >50% positives in column (**6**) and the dilution showing <50% positives in column (**6**) as follows:

$$\frac{\text{\% positive value above 50\% – 50 x 0.5 (}\textbf{\textit{correction factor}}\text{)}}{\text{\% positive value above 50\% – \% positive value below 50\%}}$$

$$= \frac{89 - 50}{89 - 11} \text{ x } 0.5 = 0.5 \text{ x } 0.5 = 0.25$$

6. The virus working dilution is 200 times the log10 virus dilution at the cut-off point determined by the Reed-Muench method. 200x times the virus dilution at the cut-off point yields a virus working dilution that contains 100x $TCID_{50}$ in 50 µl.

7. Calculate the microneutralization $TCID_{50}$ by adding the proportional distance to the dilution showing >50% positive. In the above example, add 0.25 to 4.5 to obtain $10^{-4.75}$.The virus working dilution that is 200x the cut-off dilution is $10^{-4.75}$ x 200 = $10^{-4.75}$ +$10^{2.30}$ = $10^{-2.45}$ = 1/$10^{2.45}$ = 1:282. This dilution will give 100x TCID per 50 µl.

If other dilution series are used, other ***correction factors*** must be used. For example, in this case, the correction factor for a 2-fold dilution series would be 0.3; for a .log10 dilution series it would be 0.5; for a 5-fold dilution series it would be 0.7; and for a 10-fold dilution series it would be 1.0.

TABLE 2.

Calculation of tissue culture infectious dose by the Reed-Muench method

	Observed value (optical density)		Cumulative value			
	(1)	(2)	(3)	(4)	(5)	(6)
Dilution	No. positive	No. negative	No. positive	No. negative	Ratio	Positive (%)
10^{-4}	8	0	16	0	16/16	100
$10^{-4.5}$	7	1	8	1	8/9	89
10^{-5}	1	7	1	8	1/9	11
$10^{-5.5}$	0	8	0	16	0/16	0

Part II: Virus microneutralization assay

1.Preparation of test sera

1.1 10 µl of sera are needed for each virus to be tested once. Sera should be tested in duplicate (requires 20 µl when possible).

1.2 Heat-inactivate sera for 30 min at 56°C.

1.3 Add 50 µl of diluent to each well of the plates.

1.4 Add an additional 40 µl of diluent to Row A (wells A1-A11).

1.5 Add 10µl of heat-inactivated sera including positive serum, negative serum and sera samples to Row A (1 serum/well except A12).

1.6 Perform 2-fold serial dilutions by transferring 50µl from row to row (A→B→C…H).

2. Addition of virus

2.1 Determine virus titer by $TCID_{50}$.

2.2 Dilute virus to 100 $TCID_{50}$ per 50 µl (200 $TCID_{50}$ /100 µl) in virus diluent (5 mL/plate).

2.3 Add 50 µl diluted virus to all wells except CC (wells E12, F12, G12, and H12).

2.4 Add 50 µl diluent without virus to CC wells for negative cell control.

2.5 Set-up back-titration, start with the virus test dilution (100 $TCID_{50}$), and prepare additional serial 2-fold dilutions with diluent.

2.6 Gently agitate the virus-serum mixtures, and incubate them and the virus back-titration for 1 hr at 37°C, 5% CO_2.

3. Addition of MDCK cells:

3.1 Prepare MDCK cells as described above.

3.2 Add 100 µl cells to each well of plate ($1.5x10^4$ cells /well).

3.3 Incubate plates overnight at 37°C, 5% CO_2 (18-20 hrs).

Note: To ensure even distribution of heat and CO_2, stack plates only 4 to 5 high in incubator. Media color should always maintain an orange color (this indicates the desired pH best for sera, virus, and cells). To avoid pH change, work with fewer plates at one time.

4. Fixation of the plates:

4.1 Remove medium from plate.

4.2 Wash each well with 200 µl PBS.

4.3 Remove PBS (Do not let wells dry out) and add 100 µl/well of cold *fixative*.

4.4 Cover with lid and incubate at RT for 10 min.

4.5 Remove *fixative* and let plate air-dry.

Part III: ELISA

Based on the principle of antigen-antibody interaction, this test allows for easy visualization of results. In this assay, when the secondary antibody which is linked to an enzyme binding to the antigen-antibody complex formed in cell, a visible signal will be produced by adding an enzymatic substrate.

1. Wash plate(s) 3X with wash buffer. Fill wells completely with wash buffer for each wash.
2. Dilute 1°-antibody (anti-Influenza A pool; NP monoclonal) 1:4000 or at optimal concentration in blocking buffer.
3. Add diluted 1°-antibody to each well (100 µl / well).
4. Cover plate(s) and incubate for 1 h at RT.
5. Wash plate(s) 3X with wash buffer.
6. Dilute 2°-antibody (goat anti-mouse IgG; HRP conjugated) 1:2000 or at optimal concentration in blocking buffer.
7. Add diluted *2*°-antibody to each well (100µl/well).
8. Cover plate(s) and incubate for 1 hr at RT.
9. Wash plate(s) 5X with wash buffer.
10. Add freshly prepared substrate (10 mg OPD to each 20 mL citrate buffer) to each well (100µl/well).
11. Incubate for 3 min (or until color change in VC is intense and before background CC begins to change color) at RT. Incubation time will vary between viruses.
12. Add stop solution (100 µl /well) to all wells.
13. Read absorbance (OD) of wells at 492 nm.
14. Data Analysis:

Calculations are determined for each plate individually.

14.1 Determine virus neutralizing antibody end point titer of each serum utilizing the equation below:

X = (Average OD of VC wells—Average OD of CC wells)/2 + (Average OD of CC wells)

where X = 50% of specific signal (i.e. 50% of the cells are infected). All values <u>below</u> this value are positive for neutralization activity.

14.2 The negative cell control (CC) should show OD < 0.2. The positive cell control (VC) should show OD > 0.8.

14.3 The virus test dose (100 $TCID_{50}$) is confirmed by virus back-titration. In most cases, the test dose of virus is acceptable if the back-titration is positive in 5-7 wells containing the lowest dilution of test virus.

14.4 The serum positive controls should give titers within 2-fold of expected. The value of OD in the normal serum control should be similar to that observed in VC wells.

Cautions:

1. Human sera needs to be heat-inactivated at 56°C for 30 min and animal sera need be treated by RDE before use.
2. Sera should not be repeatedly freeze-thawed. If samples are to be tested repeatedly, it is better to make several aliquots of sera.
3. Virus should not be repeatedly use. In most cases, the test dose of virus is acceptable if the back-titration in positive in 5-7 wells, if not virus titer should be determined again by $TCID_{50}$.
4. Check MDCK cell monolayer should be low passage (< 25-30 passages) at low crowding (70-95% confluent). Liquid nitrogen for cell storage should be performed within 10 passages.
5. Some factors in FBS can neutralize virus infectivity, do not use cell culture maintenance medium as diluent during the assay.
6. Plates should be strictly washed in each time to remove any proteins or antibodies that are not specifically bound in ELISA.
7. Properly control the incubation time after adding substrate in order to avoid a high background.
8. HEPES can stabilize cell culture maintenance medium when a large scale test is needed.

Occasionally, the microneutralization test may be difficult to interpret. In such cases, consider the factors presented in TABLE 3.

TABLE 3.

Problems associated with interpretation of the microneutralization test

Problem	Possible cause(s)	Solution(s)
Weak or no colour in virus control wells	Problem with ELISA:	
	a. Wrong antibodies or substrate used	a. Check antibodies and substrate
	b. Buffer solutions incorrect	b. Prepare fresh buffers
	c. Test dose of virus too weak or no virus added to virus control wells	c. Redetermine virus TCID or adjust the dilution of virus used, ensuring virus is added to virus control cells
	d. Virus inactivated during virus-serum incubation step	d. Check incubator temperature and CO_2 level
	e. MDCK cells not optimal – e.g. too old (>30 passages); not in log-phase growth; or contaminated	e. Thaw a new vial of cells; do not allow cells to enter stationary phase
Weak or no neutralization by positive control sera	a. Test dose of virus too strong	a. Redetermine virus TCID or adjust the dilution of virus used
	b. Serum deteriorated	b. Obtain new antisera; and check storage conditions
	c. Cells not in optimal condition or passage level too high	c. Thaw a new vial of cells; do not allow cells to enter stationary phase
Neutralization by negative control sera	a. Nonspecific reaction or cross-reactivity	a. Heat-inactivate serum (56 °C for 30 minutes); check RDE treatment of animal serum; check samples for cross-reactive antibodies by testing against different subtypes; and try alternative serum treatment such as trypsin-heat-periodate
	b. Test dose of virus too weak	b. Redetermine virus TCID or adjust the dilution of virus used
Nonspecific virus inactivation	a. Serum not heat inactivated or contains nonspecific viral inhibitors	a. Heat-inactivate human serum (56 °C for 30 minutes) – treat animal serum with RDE
Monolayer toxicity	b. Serum not heat inactivated or is toxic to cells	b. Heat-inactivate serum – run serum toxicity check on MDCK cells and check microscopically for toxicity