World Health Organization

SEROLOGICAL DIAGNOSIS OF INFLUENZA BY MICRONEUTRALIZATION ASSAY

6 December 2010

Serological methods rarely yield an early diagnosis of acute influenza virus infection. However, the demonstration of a significant increase in antibody titres (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 microtitre plates in combination with an ELISA to detect virus-infected cells can yield results within two days. The protocol here was provided by WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, USA.

On day 1, the following two-step procedure is performed:

1. a virus-antibody reaction step, in which the virus is mixed with dilutions of serum and time allowed for any antibodies to react; and
2. an inoculation step, in which the mixture is inoculated into the appropriate host system – MDCK cells in the case of the following assay.

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On day 2, an ELISA is then performed to detect virus-infected cells. 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 titre demonstrates a seroconversion and is considered to be diagnostic. With single-serum samples, care must be taken in interpreting low titres such as 20 and 40. Generally, knowledge of the antibody titres in an age-matched control population is needed to determine the minimum titre that is indicative of a specific antibody response to the virus used in the assay.

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 nucleoprotein (NP) protein in infected cells is detected by ELISA. The microneutralization protocol is therefore divided into three parts:

Part I: Determination of the tissue culture infectious dose (TCID).
Part II: Virus microneutralization assay.
Part III: ELISA.

An overview of the microneutralization assay is shown in **FIGURE 1** and an assay process sheet is provided in **ANNEX I**.

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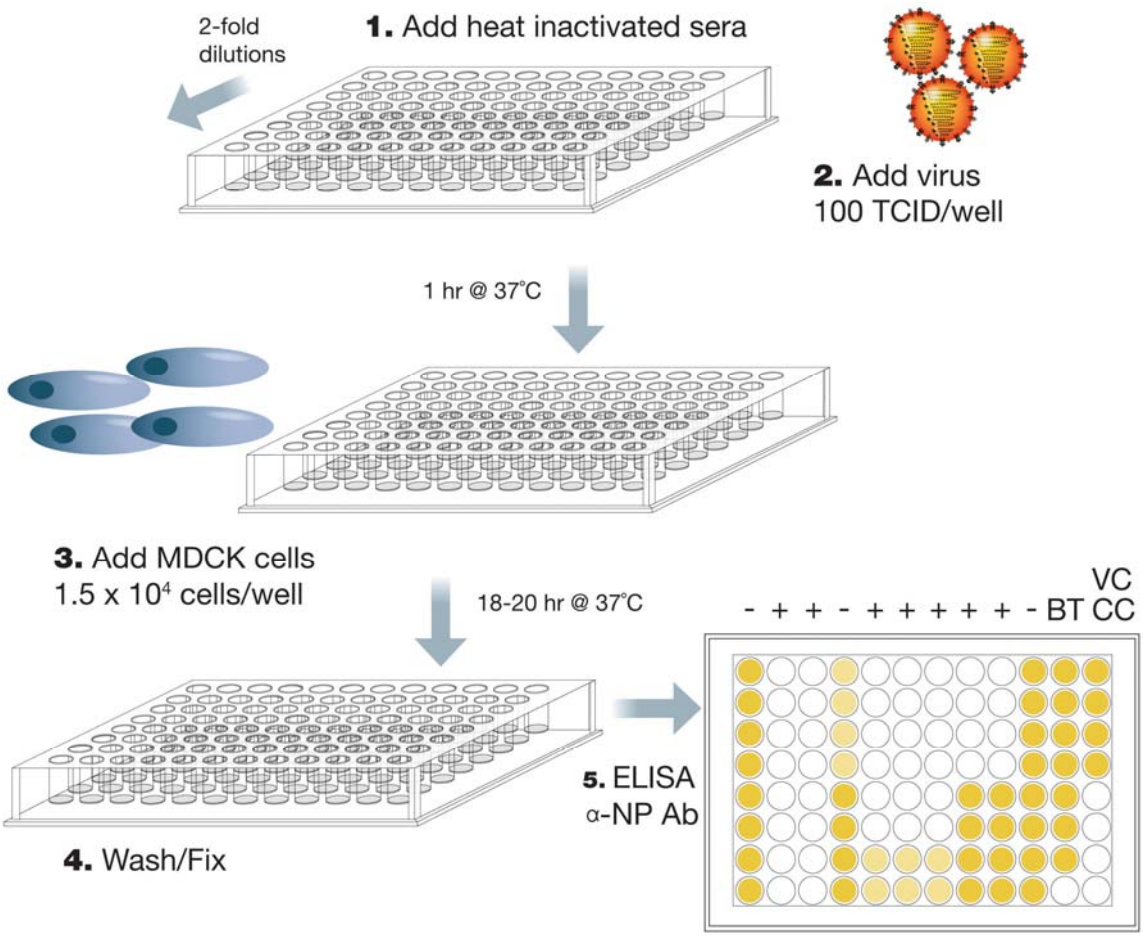
FIGURE 1: Overview of the microneutralization assay



Materials required

Equipment

Water-bath (37 °C)	Water-bath (56 °C)
Automatic ELISA reader with 490 nm filter	Incubator (humidified, 37 °C; 5% CO_2)
Automatic plate washer (not essential but would be optimal)	Microscope (inverted or standard)
Centrifuge (low speed; benchtop; preferably with refrigeration)	

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Sorvall – cat. no. 75006434	

Supplies

Cell culture flasks (162 cm^2, sterile, vented) **Corning Life Sciences – cat. no. 3151**	Cryovials (2 ml, sterile) **Wheaton Science – cat. no. 985731**
96-well microtitre plates (flat-bottom, Immulon-2HB plates) **Thermo – cat. no. 3455**	Pipettes (assorted sizes, sterile)
Haemacytometer (double rule "bright line") **Reichert – cat. no. 1490**	Haemacytometer coverslips **Reichert** – cat. no. 1492
Cell counter (2-unit counter) **Fisher Scientific – cat. no. 02-670-12**	Tips for Pipetman (sterile) **Rainin – cat. no. RT-20**
Multichannel pipetter **Rainin – cat. no. L12-200**	Tips for multichannel pipetter **Rainin – cat. no. RT-L200F**
Pipetman (1–200 µl) **Rainin – cat. no. P-200**	

Cells, media and buffers

MDCK cell culture monolayer – low passage (<25–30 passages) at low crowding (70–95% confluence)	MDCK sterile cell culture maintenance medium (see below)
D-MEM high glucose (1x) liquid, with L-glutamine and without sodium pyruvate **Invitrogen – cat. no. 11965-092**	HEPES buffer (1 M stock solution) **Invitrogen – cat. no. 15630-080**
Antibody diluents (see below)	Wash buffer (see below)
0.01 M PBS (pH 7.2) **Invitrogen – cat. no. 20012-043**	Citrate buffer capsules (optional) **Sigma – cat. no. P4922**
Water (distilled and deionized)	

Reagents

Penicillin-streptomycin (stock solution contains 10 000 U/ml penicillin; and 10 000 µg/ml streptomycin sulfate) **Invitrogen – cat. no. 15140-122**	Fetal bovine serum (FBS) **Hyclone – cat. no. SH30070.03**

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200 mM L-glutamine **Invitrogen – cat. no. 25030-081**	Bovine albumin fraction V (prepared as a 10% solution in water) **Roche – cat. no. 03117332001**
Trypsin-EDTA (0.05% trypsin; 0.53 mM EDTA · 4Na) **Invitrogen – cat. no. 25300-054**	Non-fat dry milk **Fisher Scientific – cat. no. 15260-037**
Tween 20 **Sigma – cat. no. P1379**	Ethanol (70%) **Fisher Scientific – cat. no. S71822**
Trypsin – TPCK-treated (type XIII from bovine pancreas) **Sigma – cat. no. T1426**	Trypan blue stain (0.4%) **Invitrogen – cat. no. 15250-061**
o-phenylenediamine dihydrochloride (OPD) **Sigma – cat. no. P8287**	Acetone **Fisher Scientific – cat. no. A18-500**
Virus diluent (see below)	Fixative (see below)
Stop solution (see below)	

Antibodies

Anti-influenza A NP mouse monoclonal antibody **United States Centers for Disease Control and Prevention – cat. no. VS2208**	Goat anti-mouse IgG conjugated to horseradish peroxidase (HRP), lyophilized **Kirkegaard and Perry Laboratories Inc. – cat. no. 074-1802**

Preparation of media and solutions

MDCK sterile cell culture maintenance medium

a. To 500 ml D-MEM, add 5.5 ml 100x antibiotics.
b. Add 5.5 ml 200 mM L-glutamine.
c. Add 50 ml FBS that has been heat-inactivated at 56 °C for 30 minutes.

Virus diluent (make fresh)

a. To 500 ml D-MEM, add 58 ml of bovine albumin fraction V (10%).
b. Add 6 ml 100x antibiotics.
c. Add 12.5 ml of 1 M HEPES.

Fixative (make fresh and chill to -20 °C before use)

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a. To 100 ml 0.01 M PBS (pH 7.2) add 400 ml acetone.
b. Store at -20 °C until just before use.

PBS (0.01 M, pH 7.2)

a. In 800 ml of distilled deionized water, dissolve:
- 8.0 g sodium chloride (NaCl);
- 0.20 g potassium chloride (KCl);
- 1.15 g dibasic anhydrous sodium phosphate (Na_2HPO_4); and
- 0.21 g monobasic anhydrous potassium phosphate (KH_2PO_4).

b. Adjust pH to 7.2 with HCl and bring volume up to 1 litre with distilled deionized water.
c. Sterilize by autoclaving.

Wash buffer

To 1 litre PBS add 3 ml Tween 20 using a 3 ml or 5 ml syringe with an attached wide-bore blunt-end needle. Wipe the outside of the needle, submerge the tip of the needle, and dispense directly into the PBS while it is vigorously being stirred with a stir bar. Prepare fresh each day.

Antibody diluent

To 1 litre of wash buffer add 50 g non-fat dry milk. Mix using a stir bar for at least 30 minutes before use.

Substrate

a. Prepare a citrate buffer by adding 1 citrate buffer capsule to 100 ml of distilled water.
b. Alternatively, prepare a citrate buffer by adding 29.41 g trisodium citric acid dihydrate (formula weight = 294.10) to 1 litre of distilled water (final concentration = 0.1 M). Adjust pH to 5.0 with HCl. Add 10 µl 30% hydrogen peroxide (0.015% H_2O_2) to each 20 ml of substrate ***just before use***.
c. To 20 ml of either of the above citrate buffers, add 1 OPD tablet (10 mg) ***just before use***.

Stop solution

To 972 ml of distilled water, add 28 ml of stock sulphuric acid (95–98%).

Dye (for determination of cell viability)

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Use 0.4% trypan blue stain.

Preparation of antibodies

Primary antibodies

Anti-influenza A NP mouse monoclonal antibody – dilute 1:1000 (or at an optimal concentration determined through testing by the user) in antibody diluent.

Secondary antibody

Goat anti-mouse IgG conjugated to HRP – dilute 1:2000 (or at an optimal concentration determined through testing by the user) in antibody diluent.

Preparation of negative and positive serum controls

If control sera are to be tested repeatedly, it is better to make several aliquots and store them at -20 °C to -70 °C. Both animal and human negative and positive serum controls should be included for each virus used in the assay. Sera should not be repeatedly freeze–thawed. Human sera need to be heat inactivated at 56 °C for 30 minutes and animal sera require treatment with receptor destroying enzyme (RDE) before use.

Negative (normal) serum control

This is included to determine whether the virus is nonspecifically inactivated by serum components. The negative serum control must be used at the same dilutions as the matching viral antiserum.

- For animal sera, wherever possible use normal serum from the same animal species that is being tested. The best results will be obtained if animal control sera are treated with RDE before use in the assay.
- For human sera, use age-matched normal serum from a population not exposed to the particular virus subtype in question. Human sera must be inactivated at 56 °C for 30 minutes before use in the assay.

Positive (infected or immunized) serum controls

Include antisera to known viruses as positive controls.

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- For animal sera, use sera raised in infected ferrets or other immunized animals (sheep, goat, rabbit or mouse). The best results will be obtained if animal control sera are treated with RDE before use in the assay.
- For human sera, optimal positive controls would be acute-phase and convalescent-phase serum samples. Human sera must be inactivated at 56 °C for 30 minutes before use in the assay.

Preparation of virus and cell controls

Viruses in allantoic fluid need to be stored at -70 °C. Determine the virus working dilution before use. Never use any freeze–thawed virus other than the initial freeze–thawed aliquot required to prepare the assay. Include a virus back titration, virus controls (VCs) and cell controls (CCs) with each assay as follows.

Virus titration check (back titration)

1. Add 50 µl of virus diluent to each of the wells A11–H11.
2. Add 50 µl of the working dilution of virus, containing 100x TCID, to well A11. Titrate in 2-fold serial dilutions down the plate (i.e. the 8 wells A11– H11) discarding the last 50 µl from H11. To avoid virus carry-over, change pipette tips between each well.
3. Add an additional 50 µl of virus diluent to the virus titration wells (A11–H11).
4. Incubate for 1 hour at 37 °C in 5% CO_2.
5. Add 100 µl MDCK cells (1.5 x 10^4/well) and then incubate for 18–20 hours (at 37 °C in 5% CO_2) with the rest of the assay.

Positive virus controls (VCs) and negative cell controls (CCs)

Set up 4 wells as positive VCs (50 µl medium + 50 µl working dilution of virus + 100 µl MDCK cells) and 4 wells as negative CCs (100 µl virus diluent + 100 µl MDCK cells) and assay in parallel with the neutralization test. These controls must be included on each plate for analysis of the data on that plate.