

Video Article

# Measuring Influenza Neutralizing Antibody Responses to A(H3N2) Viruses in Human Sera by Microneutralization Assays Using MDCK-SIAT1 Cells

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## Abstract

Neutralizing antibodies against hemagglutinin (HA) of influenza viruses are considered the main immune mechanism that correlates with protection for influenza infections. Microneutralization (MN) assays are often used to measure neutralizing antibody responses in human sera after influenza vaccination or infection. Madine Darby Canine Kidney (MDCK) cells are the commonly used cell substrate for MN assays. However, currently circulating 3C.2a and 3C.3a A(H3N2) influenza viruses have acquired altered receptor binding specificity. The MDCK-SIAT1 cell line with increased  $\alpha$ -2,6 sialic galactose moieties on the surface has proven to provide improved infectivity and more faithful replications than conventional MDCK cells for these contemporary A(H3N2) viruses. Here, we describe a MN assay using MDCK-SIAT1 cells that has been optimized to quantify neutralizing antibody titers to these contemporary A(H3N2) viruses. In this protocol, heat inactivated sera containing neutralizing antibodies are first serially diluted, then incubated with 100 TCID<sub>50</sub>/well of influenza A(H3N2) viruses to allow antibodies in the sera to bind to the viruses. MDCK-SIAT1 cells are then added to the virus-antibody mixture, and incubated for 18 - 20 h at 37 °C, 5% CO<sub>2</sub> to allow A(H3N2) viruses to infect MDCK-SIAT1 cells. After overnight incubation, plates are fixed and the amount of virus in each well is quantified by an enzyme-linked immunosorbent assay (ELISA) using anti-influenza A nucleoprotein (NP) monoclonal antibodies. Neutralizing antibody titer is defined as the reciprocal of the highest serum dilution that provides  $\geq 50\%$  inhibition of virus infectivity.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/56448/>

## Introduction

Influenza viruses continue to cause morbidity and mortality in the human population each year. HA is the major surface glycoprotein of influenza viruses. Neutralizing antibodies targeting HA are the main immune mechanism that correlates with protection of influenza infection<sup>1,2</sup>. Hemagglutination inhibition (HI) assays and MN assays are two methods widely used to measure antibody responses in human sera after influenza infection or vaccination<sup>3</sup>. The HI assay measures antibody inhibition of virus hemagglutination of red blood cells and is considered a surrogate assay. Unlike HI, the MN assay can directly measure the levels of antibodies in human sera that neutralize influenza infection in cell cultures. MDCK cells are often used in influenza virus isolation and MN assays<sup>4</sup>.

Influenza viruses constantly undergo antigenic drift and shift, acquiring mutations on HA proteins that can alter the receptor binding specificity of viruses. Since 2014, new clusters of A(H3N2) viruses emerged and continued to circulate until the current season. The majority of these viruses belong to the genetic group 3C.2a and 3C.3a based on phylogenetic analysis of the HA proteins. Many of the circulating 3C.2a viruses had reduced ability to hemagglutinate red blood cells and therefore cannot be characterized by HI assays<sup>5</sup>. Neutralization assays must be used to measure the antibody responses to these viruses that do not hemagglutinate<sup>6</sup>. Furthermore, studies have shown that these contemporary A(H3N2) viruses have altered receptor binding properties compared with earlier A(H3N2) viruses and tend to accumulate culture adapted mutations and polymorphism when passaged in *in vitro* cell cultures<sup>7,8,9</sup>. Compared with conventional MDCK cells, MDCK-SIAT1 is a cell line developed by Matrosovich *et al.* through stable transfection of MDCK cells with cDNA of human  $\alpha$ 2,6-sialtransferase (SIAT1). This cell line expresses increased amounts of  $\alpha$ 2,6-sialic galactose moieties and decreased amounts of  $\alpha$ 2,3-sialic acid moieties than the parent MDCK cells<sup>10</sup>. MDCK-SIAT1 cells have shown to improve isolation rates for A(H3N2) viruses compared to MDCK cells<sup>11</sup>. Recently, Lin *et al.* reported that for newly emerged 3C.2a and 3C.3a human A(H3N2) influenza viruses, more faithful virus replications and better virus infectivity were achieved when viruses were cultured in MDCK-SIAT1 cell lines compared with MDCK cells<sup>7</sup>. Thus, the MDCK-SIAT1 cells are better suited in MN assays to characterize antibody responses to the recent clusters of A(H3N2) viruses.

Here we describe a MN assay using MDCK-SIAT1 cells to measure antibody responses to contemporary 3C.2a and 3C.3a A(H3N2) viruses in human sera. Viruses grown in either eggs or cells can be used in this assay. Heat inactivated sera containing neutralizing antibodies are first serially diluted, then incubated with 100 TCID<sub>50</sub>/well of influenza A(H3N2) virus to allow antibodies in the sera to bind to the virus. MDCK-SIAT1 cells are then added to the virus-antibody mixture, and incubated for 18 - 20 h at 37 °C, 5% CO<sub>2</sub> to allow the A(H3N2) virus to infect MDCK-SIAT1 cells and replicate. After overnight incubation, the plates are fixed and the amount of virus in each well is quantified by an ELISA

using anti-influenza A NP monoclonal antibodies. The detection of NP indicates the presence of virus infection and the absence of neutralizing antibodies. Neutralizing antibody titer is defined as the reciprocal of the highest serum dilution that provides  $\geq 50\%$  inhibition of virus infectivity.

## Protocol

All influenza viruses should be handled according to appropriate biosafety level requirements (BSL-2 or higher) as defined in the Biosafety on Microbiological and Biomedical Laboratories (BMBL)<sup>12</sup>.

## 1. Preparation of Reagents and Starting Material

### 1. Prepare MDCK-SIAT1 cells and sterile cell culture medium

1. Prepare the **MDCK-SIAT1 cell culture medium** using 500 mL of Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 10% v/v heat inactivated fetal bovine serum (FBS), 2 mM L- glutamine, 1 mM sodium pyruvate, 1 mg/mL G418 sulphate (e.g., geneticin), and 100 U/ml penicillin with 100 µg/mL streptomycin (optional). Sterilize by filtration through a 0.2 µm pore-size membrane. G418 sulphate is added to ensure plasmid stability in the MDCK-SIAT1 cells.
2. Prepare the **MDCK-SIAT1** cell line. In a 162 cm<sup>2</sup>- tissue culture flask(s) containing 30 mL of sterile cell culture media, seed flasks with  $2 - 2.5 \times 10^6$  MDCK-SIAT1 cells and incubate at 37 °C in 5% CO<sub>2</sub> for 2 days; this will be used for tissue-culture infectious dose (TCID) determination and MN assays.

NOTE: MDCK-SIAT1 cells were kindly provided by Dr. M. Matrosovich, Marburg, Germany<sup>10</sup>. This cell line can also be obtained commercially (see the **Table of Materials**).

2. Prepare the **sterile virus propagation media** using 500 mL of DMEM high glucose, 0.3% bovine serum albumin (BSA) fraction V, 100 U/mL penicillin, 100 µg/mL streptomycin, and 20 mM HEPES. Sterilize by filtration through a 0.2 µm pore-size membrane.
  3. Prepare 0.75% (v/v) **guinea pig red blood cells (gpRBCs)** in phosphate buffered saline (PBS) containing 0.01 M PBS, at pH 7.2.
  4. Prepare the **sterile virus diluent** using DMEM high glucose, 1% Bovine Albumin Fraction V (BSA), 100 U/mL penicillin, 100 µg/mL streptomycin, and 20 mM HEPES. Sterilize by filtration with a 0.2 µm pore-size membrane, and prepare fresh for each assay.
  5. Prepare the **cold cell fixative** as 80% cold acetone in PBS (0.01 M PBS, pH 7.2).
  6. Prepare the **wash buffer** using PBS (0.01 M PBS pH 7.2) and 0.3% (v/v) tween-20.
  7. Prepare the **antibody diluent** using PBS (0.01 M PBS, pH 7.2), 0.3% (v/v) tween-20, and 5% non-fat dry milk.
  8. Use anti-influenza A NP mouse monoclonal antibody clone A1 and A3 pool as the **primary antibody**. Dilute in the antibody diluent at the optimal concentration as determined by titration.
  9. Use goat anti-mouse IgG conjugated to horse radish peroxidase (HRP) as the **secondary antibody**. Dilute in the antibody diluent at the optimal concentration as determined by titration.
  10. Prepare the **peroxidase substrate** using o-phenylenediamine dihydrochloride (OPD) in 0.05 M phosphate citrate buffer at pH 5. Prepare 0.05 M phosphate citrate buffer by dissolving 1 capsule/100 mL deionized H<sub>2</sub>O. Dissolve 1 OPD tablet (10 mg)/20 mL of phosphate citrate buffer immediately before use.
  11. Use 0.5 M of sulfuric acid as the **OPD stop solution**. Add 28 mL of 18 M sulfuric acid stock to 972 mL deionized H<sub>2</sub>O in a chemical hood.
  12. **Treatment of human and animal sera**
    1. Heat inactivate the **human sera** used in the MN assays in a water bath at 56 °C for 30 min prior to the assay. Use immediately or if needed, store thawed sera at 4 °C for no more than 24 h; if longer storage period is needed, store sera frozen at -20 °C or colder.
    2. Treat the **animal sera** used in the MN assays with Receptor Destroying Enzyme (RDE) and heat inactivate prior to the assay as follows.
      1. Thaw the sera in a 37 °C water bath then place on ice. Mix 1 volume of animal serum sample with 3 volumes of RDE. Incubate at 37 °C for 18 - 20 h.
      2. Heat inactivate the RDE treated sera at 56 °C for 30 min. Add 6 volumes of PBS, pH 7.2 to each sample for a final pre-dilution of 1:10. If needed, store thawed sera at 4 °C for no more than 24 h; if longer storage period is needed, store sera frozen at -20 °C or colder.
- NOTE: Animal sera may contain various sialic acid glycans which may bind to the HAs of influenza viruses and inhibit the binding of influenza-specific anti-HA antibodies. Therefore, it is necessary to RDE treat all animal sera prior to the MN assays to remove these non-specific viral HA binders in the serum samples.

## 2. Passage of MDCK-SIAT1 Cell Culture

Note: All cell cultures must be performed in a biological safety cabinet to prevent contamination.

1. Decant the cell culture medium from the cell monolayer in 162 cm<sup>2</sup> flasks. Trypsinize the cells by rinsing the monolayer with trypsin-EDTA. Add 5 mL trypsin-EDTA to cover the cell monolayer. Incubate at 37 °C, 5% CO<sub>2</sub> until the monolayer detaches (5-10 min). Add 15 mL of MDCK-SIAT1 cell culture media to each flask containing trypsinized cells, pipette up and down to separate cells.
  2. Use a hemocytometer and trypan blue to determine the cell counts and viability. Seed the cells in new 162 cm<sup>2</sup> tissue culture flasks containing 30 mL of cell culture media with  $2 - 2.5 \times 10^6$  cells /flask and incubate at 37 °C with 5% CO<sub>2</sub> for 2 days for use in TCID and MN assays. Seed cells at  $4 - 5 \times 10^6$  cells/flask and incubate at 37°C with 5% CO<sub>2</sub> for 2 days for use in virus propagation.
- NOTE: The seeding density can be adjusted if shorter or longer culture periods are desired. For virus propagation, cells should reach over 100% confluency. For Tissue Culture Infection Dose (TCID) and MN assays, the cells should be at 75 - 95% confluency (**Figure 1**).

### 3. Propagation of A(H3N2) Viruses in MDCK-SIAT1 Cells

NOTE: A(H3N2) virus can be propagated either in 10 - 11 day old embryonated hen's eggs according to standard protocol<sup>3</sup>, or MDCK-SIAT1 cell cultures. MDCK-SIAT1 cells should reach over 100% confluency for virus inoculation. Virus seed stocks can be inoculated with multiple dilutions of inoculum. The inoculum dilutions with the best harvest HA and infectivity can be used for further MN assays.

1. Remove the cell culture medium from the cell monolayer (>100% confluency) in 162 cm<sup>2</sup> flasks. Wash the monolayer twice with 15 mL of sterile 0.01 M PBS, pH 7.2, and decant.
2. Label 1 flask as control and add 20 mL of virus propagation media. Incubate flask at 37 °C with 5% CO<sub>2</sub>. Leave this flask untouched and use for comparison after day 1 of virus propagation.
3. Add 10 mL of sterile virus propagation media to the flask(s) and incubate all flask(s) at 37 °C, 5% CO<sub>2</sub>. Thaw a vial of influenza virus stock at room temperature then place on ice. Dilute with the virus propagation media to the target dilutions.  
NOTE: Target dilutions can be adjusted based on HA titers of virus stocks. To prepare a 1:1000 diluted inoculum, add 100 µL of virus to 9.9 mL of sterile virus propagation media. Invert gently, remove 1 mL of 1:100 dilution to 9 mL of sterile virus propagation media, invert gently for a 1:1000 diluted inoculum. Place on ice.
4. Remove all flasks except control flask from the incubator. Remove the virus propagation media from flask(s) of MDCK-SIAT1 cells and inoculate each flask with 10 mL of diluted virus. Incubate the flasks at 37 °C, 5% CO<sub>2</sub> for 1 h, rotating the flask(s) every 15 min.
5. Remove 5 mL of inoculum from the flask(s) and replace with 15 mL virus propagation media containing 1 µg/mL TPCK-trypsin. Incubate the flask(s) at 37 °C, 5% CO<sub>2</sub> for 16-18 h.
6. After the overnight incubation, observe the flask(s) under 100X microscope magnification and look for cytopathic effects (CPE) on the cells. Remove 15-17 mL of virus supernatant from the flask(s), and replace with 15 - 17 mL of freshly prepared virus propagation media containing 2 µg/mL TPCK-trypsin.
7. Incubate the flasks at 37 °C, 5% CO<sub>2</sub>. Monitor the CPE of the monolayer (by comparison with the cell control flask) and check the HA periodically (every 4 h) with 0.75% gpRBC until harvest.
  1. Set up an HA 96-well microtiter plate. Add 50 µL of 0.01 M PBS, pH 7.2 to wells A2 - A12 and B2 - B12 (duplicates), and wells H1 - H12 for control.
  2. Add 100 µL of virus supernatant to A1 and A2, perform a 2-fold serial dilution from A1 and B1 through A12 and B12. Discard 50 µL after mixing in A12 and B12. Add 50 µL of 0.75% gpRBCs to Rows A, B, and H.
  3. Tap the plate and incubate at room temperature for 1 h.
    1. Determine HA of virus supernatant by tilting the 96-well microtiter plate at a 45° to 60° angle.
    2. Read plates for hemagglutination; the highest dilution of virus that achieves complete hemagglutination is considered the HA titration end point for the specific virus. Record the reciprocal of the highest virus dilution with complete hemagglutination as the HA titer of the virus.

NOTE: The settled RBCs in row H (controls) should start "running" and form a small teardrop-shape due to gravity. Wait till RBCs in control wells finish running, then read the RBC buttons in the virus titration wells. Those that exhibit RBC buttons and "run", do not achieve hemagglutination. Locate the highest dilution of virus that completely inhibit hemagglutination as the end point HA titer of the virus.
8. Harvest the virus when the HA of the virus culture plateaus or the virus culture reaches the target HA (for example, >16 HAU), but before the cell monolayer starts to display significant CPE.
  1. Centrifuge the virus culture supernatant at 300 x g for 10 min at 4 °C to pellet the cellular debris. Transfer the clarified supernatant containing the virus harvest to a clean tube. Aliquot the supernatant containing the virus harvest into single-use sterile cryogenic vials and freeze immediately at -70 °C or colder.

NOTE: The virus stocks used for the MN assays should have high infectious titer and minimal defective particles. The minimum dilution of virus stocks to achieve 100 TCID<sub>50</sub>/50 µL for MN assay is 1:100.

NOTE: The virus stocks used in the MN assays should not be thawed and refrozen.

### 4. Determination of TCID of the Virus

1. **Day 1: Virus titration**
  1. Thaw a vial of virus at room temperature and immediately place on ice.
  2. Test the virus at two different starting dilutions: 10<sup>-2</sup> and 10<sup>-3</sup>. Add 100 µL of virus to 9.9 mL of virus diluent for 10<sup>-2</sup> dilution. Add 1 mL of 10<sup>-2</sup> dilution to 9.0 mL of virus diluent for 10<sup>-3</sup> dilution.
  3. Using two microtiter plates (plate 1 for 10<sup>-2</sup> dilution and plate 2 for 10<sup>-3</sup> dilution), add 100 µL of virus diluent to all wells except column 1 of the 96-well microtiter plate.
  4. Perform ½log<sub>10</sub> dilutions (10<sup>-2</sup>, 10<sup>-2.5</sup>, 10<sup>-3</sup>, etc.). Add 146 µL of the virus starting dilution to all wells in column 1 and transfer 46 µL serially from column 1 through column 11 (**Figure 2**). Change pipette tips between wells. After mixing column 11, discard the tips with the 46 µL dilution.
  5. Use column 12 as the Cell Control (CC); it only contains virus diluent. Incubate for 1 h at 37 °C, 5% CO<sub>2</sub>.
2. **Day 1: Preparation of MDCK-SIAT1 cells**  
Note: The MDCK-SIAT1 cell monolayer should reach 75 - 95% confluency for TCID and MN assays. One 162-cm<sup>2</sup> flask at 95% confluency should yield enough cells to seed ~ 4 - 5 microtiter plates.
  1. Wash the 75 - 95% confluent monolayer with 20 mL PBS to remove the FBS in the culture media. Trypsinize the cells as follows.

1. Rinse the monolayer with sterile PBS, add 7 mL trypsin-EDTA to cover the cell monolayer. Lay the flask flat and incubate at 37 °C, 5% CO<sub>2</sub> until the monolayer detaches (approximately 5 - 10 min). Add 7 mL of virus diluent to each flask containing the trypsinized cells.
  2. Wash the cells with virus diluent to remove the FBS.
    1. Gently pipette up and down to separate the cells. Transfer the cells to a 50-mL conical tube; fill the tube with virus diluent.
    2. Centrifuge at 485 x g for 5 min. Decant the virus diluent, replace with fresh 50 mL virus diluent, and centrifuge at 485 x g for 5 min. Decant the virus diluent and replace with fresh virus diluent (10 mL/flask) to resuspend the pellet.
  3. Use a hemocytometer and trypan blue to determine the cell count and viability. Adjust the cell concentration with the virus diluent to 1.5 x 10<sup>5</sup> cells/mL.
  4. Add 100 µL of diluted MDCK-SIAT1 cells to each well of the microtiter plates (1.5 x 10<sup>4</sup> cells/well) and cover the plates. Incubate at 37 °C, 5% CO<sub>2</sub> for 18 - 20 h.
- 3. Day 2: ELISA**
1. **Fixation of cells**
    1. Remove the medium from the microtiter plates. Wash each well with 200 µL of PBS. Add 300 µL cold 80% acetone to each well and incubate at room temperature for 10 min. Remove the fixative and allow the plates to air dry.
  2. **ELISA**
    1. **Primary antibody addition**

**Note:** Anti-influenza A NP monoclonal antibody should be used in excess as the primary antibody in ELISA. Determine the optimal antibody dilution for each lot of primary antibodies by performing antibody titrations in MN. Select the primary antibody concentration that is in excess and with the best signal to background ratio.

      1. Dilute the anti-influenza A NP monoclonal antibody (primary antibody) to the target concentration in the antibody diluent (e.g. add 30 µL of primary antibody to 30 mL of antibody diluent for a target dilution of 1:1000).
      2. Wash plates 3 times with 300 µL of wash buffer. Add 100 µL diluted primary antibody to each well. Incubate at room temperature for 1 h.
    2. **Secondary antibody addition**

**Note:** Goat anti mouse IgG conjugated to horse radish peroxidase (HRP) should be used in excess as the secondary antibody in ELISA. Determine the optimal antibody dilution for each lot of secondary antibodies by performing antibody titrations. Select the secondary antibody concentration in excess and with the best signal to background ratio.

      1. Dilute the goat anti-mouse IgG conjugated to HRP antibody (secondary antibody) to the target concentration in the antibody diluent (e.g. Add 7.5 µL of secondary antibody to 30 mL of antibody diluent for a target 1:4000 dilution).
      2. Wash the plates 3 times with 300 µL wash buffer. Add 100 µL diluted secondary antibody to each well. Incubate at room temperature for 1 h.
    3. **Substrate addition and plate reading**
      1. Wash the plates 5 times with 300 µL wash buffer and tap on a lint-free wipe.
      2. Add 100 µL of freshly prepared substrate to each well and incubate at room temperature until the color development saturates and the optical density (OD) of cell control wells <0.2.
      3. Add 100 µL of stop solution to all wells. Read the OD of wells at 490 nm using a microplate spectrophotometer.
- 4. TCID<sub>50</sub> calculation**
1. Calculate the median OD<sub>490</sub> of the cell controls (column 12).
  2. Consider any test well with an OD<sub>490</sub> greater than twice the median OD<sub>490</sub> of the CC wells as "positive"; otherwise, it is considered "negative".
  3. Calculate the TCID<sub>50</sub> of the virus using the Reed-Muench method<sup>13</sup>.
    1. Determine the number of positives and negatives at each dilution.
    2. Calculate the "cumulative positive", "Cumulative negative", "Ratio", and "% positive" as illustrated in **Table 1**.
    3. Calculate the "proportional distance" between the dilution showing >50% positives and the dilution showing <50% positives using the following:
 

**Proportional distance**

$$= ((\% \text{ positive value above } 50\%) - 50) / (\% \text{ positive value above } 50\% - \% \text{ positive value below } 50\%) \times 0.5 (\text{correction factor for } 1/2 \text{ log dilution})$$

**Note:** The correction factor for 1/2 log dilution is 0.5. For example, in **Table 1**: (80 - 50)/(80 - 20) x 0.5 = 0.25
    4. Calculate the virus TCID<sub>50</sub> by adding the proportional distance to the dilution showing > 50% positive.
 

**NOTE:** For example, in **Table 2**, TCID<sub>50</sub> is 10<sup>-5+(-0.25)</sup> = 10<sup>-5.25</sup>. Note this is the TCID<sub>50</sub> of the virus per 100 µL (or 10<sup>-5.25</sup>/100 µL).
  4. Calculate the virus dilution. For MN assays, dilute the virus to 200 TCID<sub>50</sub>/100 µL (equivalent to 100 TCID<sub>50</sub>/50 µL per well).
 

**NOTE:** In the example in **Table 1**, 1 TCID<sub>50</sub> is 10<sup>-5.25</sup> in 100 µL, and the dilution to achieve 200 TCID<sub>50</sub>/100 µL is 1:891 based on the calculations: 200 x 10<sup>-5.25</sup> = 10<sup>-2.95</sup> = 1/10<sup>2.95</sup> = 1/891

## 5. MN Assay Using MDCK-SIAT1 Cells

1. **Day 1: Test and control sera preparation and plate layout**

1. Thaw the sera in 37 °C water bath and remove immediately after thawing. Aliquot the amount of sera that needs to be tested; a minimum of 10 µL of original sera is needed to test with one virus in singlet. Test sera in duplicates if possible.
  2. Heat inactivate the human sera for 30 min in a 56 °C water bath as in step 1.12.1. Place sera on ice post heat inactivation, add the virus diluent to sera to achieve a 1:10 pre-dilution.
  3. RDE treat and pre-dilute **animal sera** to 1:10 to use for controls per 1.12.2.  
NOTE: Thawed and treated human and animal sera can be stored at 4 °C for no longer than 24 h. Sera should be stored frozen at -20 °C or colder if longer storage period is needed prior to the assay.
  4. To test with one virus, add 100 µL 1:10 diluted sera to columns A1 to A10 (**Figure 4**). Add 50 µL of virus diluent to rows B through H, except columns 11 and 12 (**Figure 4**). Perform a 2-fold serial dilution from rows A through H, and discard the last 50 µL at row H (**Figure 4**).  
NOTE: When multiple viruses are to be tested, sera can be diluted in titer tubes. The dilution of serum, for the purpose of determining the serum titer, in well A is 1:10, well B 1:20, well C 1:40, well D 1:80, well E 1:160, well F 1:320, well G 1:640, well H 1:1,280 (**Figure 4**).
  5. For the virus control, add 50 µL of virus diluent to wells A12, B12, C12, and D12 (no sera). For the cell control, add 100 µL of virus diluent to wells E12, F12, G12, and H12 (no virus, no sera).
  6. For the control sera (for example, ferret sera controls), add 100 µL of diluted control sera to well A column 11 and add 50 µL of virus diluent to wells B11 - H11. Serial dilute down.
  7. Cover the plates, incubate at 37 °C, 5% CO<sub>2</sub> until ready for the virus addition.
- 2. Day 1: Virus addition**
1. Dilute the virus to 100 TCID<sub>50</sub>/50 µL with virus diluent.
  2. Add 50 µL diluted virus to all wells, except for column 11 on the back titration (BT) plates and the cell control wells E12, F12, G12, and H12 on all plates (**Figure 4**). For those plates with control sera at column 11, add virus to column 11.
  3. Back titration (BT)
    1. Include a BT in column 11 of one set of duplicate plates (for example, plate 1A and 1B). Add 50 µL of virus diluent to all wells in column 11. Add 50 µL of the virus at 100 TCID<sub>50</sub>/50 µL to the first well (A11). Mix by pipetting up and down.
    2. Mix and transfer 50 µL to successive wells to perform 2-fold serial dilutions. Change the pipette tips between wells to avoid virus carry-over. Discard 50 µL from well H11.
    3. Add 50 µL of virus diluent to column 11 to bring the final volume to 100 µL. Tap the plates to mix.
  4. Incubate plates at 37 °C, 5% CO<sub>2</sub> for 1 h.
  5. Perform MDCK-SIAT1 cell addition on day 1 and ELISA on day 2 as described in steps 4.2 and 4.3 (also described in 5.3 and 5.4)
- 3. Day 1: MDCK-SIAT1 cell addition**
1. Prepare the MDCK-SIAT1 cells as described in step 4.2. Add 100 µL MDCK-SIAT1 cells at 1.5 x 10<sup>5</sup> cells/mL to each well (1.5 x 10<sup>4</sup> cells/well). Incubate the plates at 37 °C, 5% CO<sub>2</sub> for 18 - 20 h.
- 4. Day 2: ELISA**
1. After overnight incubation, on the second day, fix the cells with 80% cold acetone as described in step 4.3.
  2. **Primary antibody addition**
    1. Wash the plates 3 times with 300 µL wash buffer. Dilute the anti-influenza A NP monoclonal antibody (primary antibody) to the optimal concentration as determined by titration in antibody diluent (e.g. add 30 µL of primary antibody to 30 mL of antibody diluent for a target dilution of 1:1000).  
NOTE: 100 µL of primary antibody is required per well. ~10 mL is needed per plate.
    2. Add 100 µL diluted primary antibody to each well. Incubate at room temperature for 1 h.
  3. **Secondary antibody addition**
    1. Wash plates 3 times with 300 µL wash buffer. Dilute the goat anti mouse IgG conjugated to HRP antibody (secondary antibody) to the target concentration in antibody diluent (e.g. Add 7.5 µL of secondary antibody to 30 mL of antibody diluent for a target 1:4000).  
NOTE: 100 µL of secondary antibody is required per 96 well. ~10 mL is needed per plate.
    2. Add 100 µL diluted secondary antibody to each well. Incubate at room temperature for 1 h.
  4. **Substrate addition and plate reading**
    1. Wash the plates 5 times with 300 µL wash buffer and tap on lint-free wipe.
    2. Add 100 µL of freshly prepared substrate to each well and incubate at room temperature until the virus control wells reach an OD<sub>490</sub> = 0.8 - 3, with the cell control at a low background OD<sub>490</sub> <0.2.
    3. Add 100 µL of the stop solution to all wells. Read the OD of wells at 490 nm using a microplate spectrophotometer.
- 5. Data analysis**
- NOTE: The MN calculations are determined for each plate individually.
1. Determine the neutralizing antibody titer of each serum sample.
    1. Calculate the OD<sub>490</sub> cut-off of 50% virus neutralization for each plate using the following equation:
 
$$x = \frac{\text{median OD}_{490} \text{ of VC wells} + \text{median OD}_{490} \text{ of CC wells}}{2}$$

NOTE: Here x = 50% of the neutralization cut-off. The reciprocal serum dilution corresponding to the highest dilution with OD<sub>490</sub> less than 50% of the cut-off (≥50% inhibition) is considered the neutralization antibody titer for that serum sample (**Figure 5**).
  2. Check that the cell control wells have an OD<sub>490</sub> <0.2 and the virus control wells have an OD<sub>490</sub> = 0.8 - 3.



3. Verify the virus infectivity in each assay (100x TCID<sub>50</sub>) by virus BT. The acceptable range of BT is 50, 100, or 200 TCID. If using the same cut-off as defined in step 4.4.2, the highest virus dilution in the BT column with OD above the cut-off should be in wells E11, F11, G11.

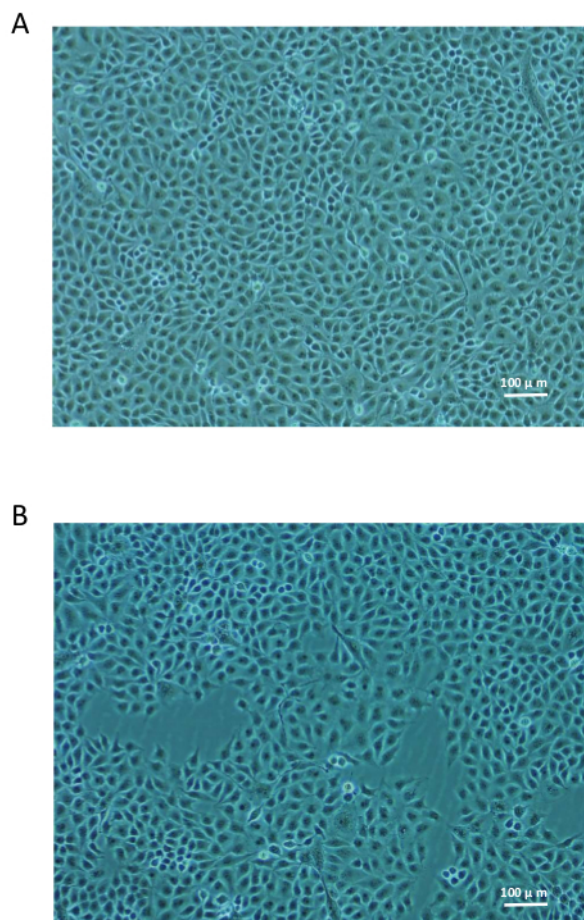
Note: The serum positive controls should give titers within 2-fold of the values obtained in the previous tests. The OD<sub>490</sub> of the negative serum control should be similar to that observed for the virus control.

## Representative Results

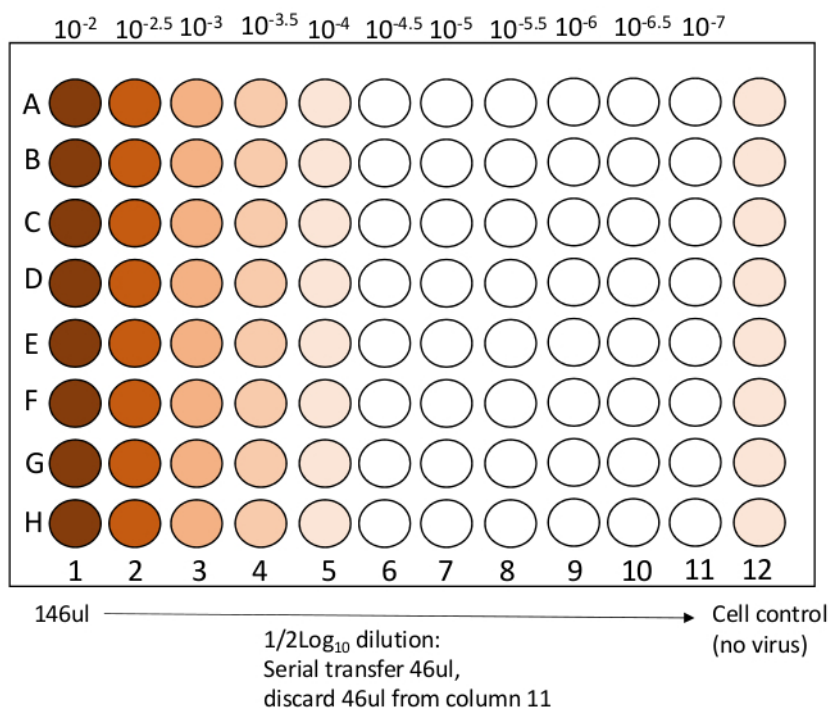
Determination of the infectivity of the virus stocks is the first step in the MN assay. **Figure 2** illustrates the plate layout to determine the TCID<sub>50</sub> of the virus stocks. For virus stocks with unknown infectivity, the virus can be titrated from multiple pre-dilutions, for example both 10<sup>-2</sup> and 10<sup>-3</sup>, in order to capture the best titration curve to calculate infectivity of the virus. The virus amount used in the MN assay should be standardized to 100 TCID<sub>50</sub>/well (50 µL). The minimum dilution of virus stocks to achieve a 100 TCID<sub>50</sub>/well is 1:100.

**Figure 3** describes the critical steps of a MN assay using MDCK-SIAT1 cells. Heat-inactivated sera are serial-diluted in 96-well plates as illustrated in **Figure 4**. 100 TCID<sub>50</sub>/50 µL virus is then added to each well. After 1 h incubation to allow the antibody in the sera bind to the virus, 100 µL of 1.5 x 10<sup>5</sup> cells/mL of MDCK-SIAT1 cells are added to each well. For optimal results, MDCK-SIAT1 cells should be at 75 - 95% confluency (**Figure 1**) for both the TCID<sub>50</sub> and MN assays. The plates are then incubated for 18 - 20 h at 37 °C, 5% CO<sub>2</sub>. After overnight incubation, the amount of virus in each well is detected and quantified by an anti-influenza A NP ELISA (**Figure 3**).

The OD in each well represents the amount of virus infection and replication in MDCK-SIAT1 cells in the presence of serially diluted sera containing neutralizing antibodies. It can be plotted against the sera dilutions (**Figure 5**). The reciprocal of the highest serum dilution that achieved ≥50% neutralization is considered the antibody titer for the serum sample. **Figure 5** contains examples of results from 2 patients with paired sera tested against A/HongKong/4801/2014 A(H3N2) 3C.2a virus pre- and post-influenza vaccination. With patient 1 pre-vaccination sera, none of the sera dilutions inhibited the virus infection (**Figure 5**, light blue curve) and therefore is considered negative (<10). In contrast, post-vaccination sera from this patient inhibited virus replication, indicating vaccination induced neutralizing antibodies. The third dilution of this sera is the highest dilution below the 50% cut-off, thus this patient has a post-vaccination titer of 40 (**Figure 5**, dark blue curve). In comparison, the serum from the second patient pre-vaccination contains pre-existing neutralizing antibodies at a titer of 320. Following vaccination, the titer increased to >1,280. In this case, at a 1:10 pre-dilution of serum, no antibody end titer was achieved. The serum should be re-tested at a higher pre-dilution in order to achieve end titer.

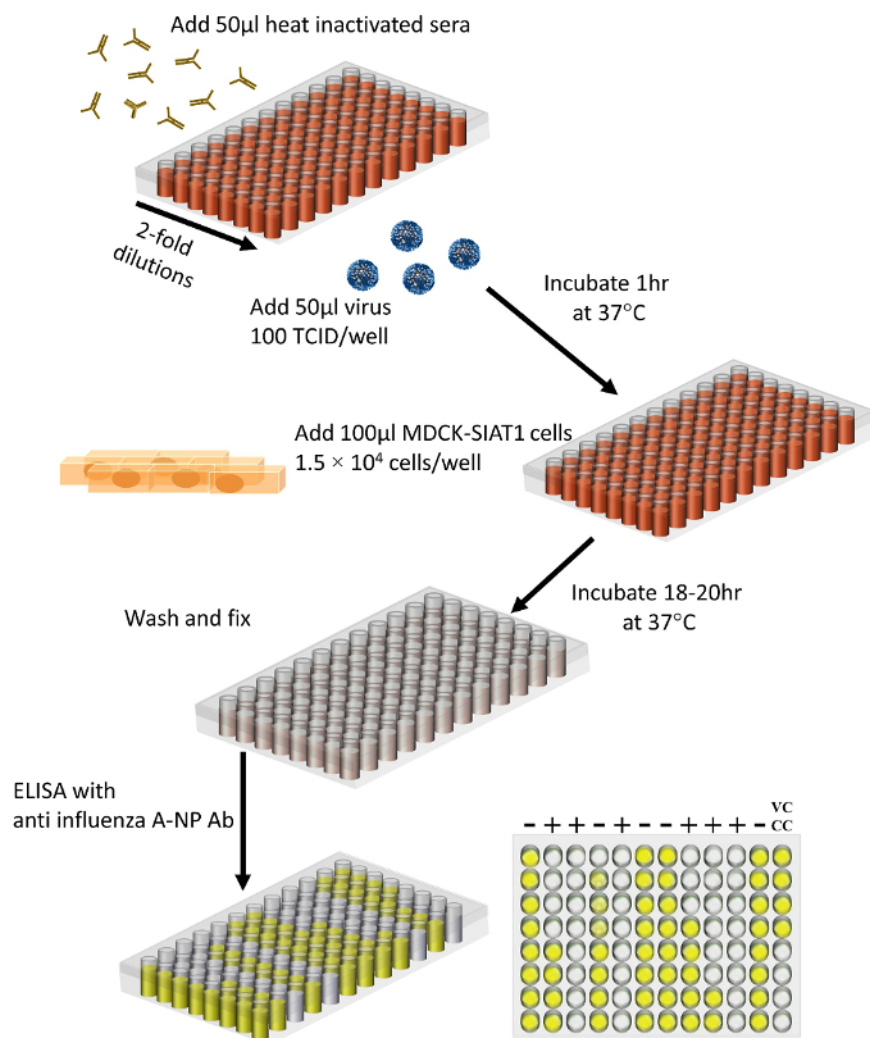


**Figure 1. MDCK-SIAT1 cell culture.** MDCK-SIAT1 cells. (A) MDCK-SIAT1 confluent monolayer (>100% confluency) for virus inoculation; (B) MDCK-SIAT1 cells at log phase with 75 - 95% confluency for TCID<sub>50</sub> and MN assays.

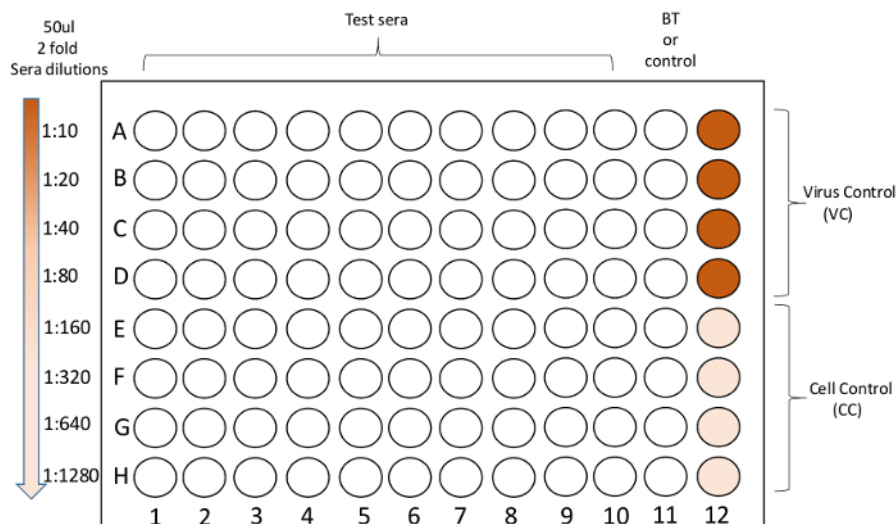


**Figure 2. TCID plate layout.** Virus infectivity is determined by TCID<sub>50</sub>. Virus stock is pre-diluted to 10<sup>-2</sup>, and then serially diluted at a ½ log per dilution to 10<sup>-7</sup> (column 1 - 11), at 8 replicates per dilution (row A -H). Column 12 is used as the cell only control. TCID<sub>50</sub> of the virus stock can be calculated using the Reed-Muench method.

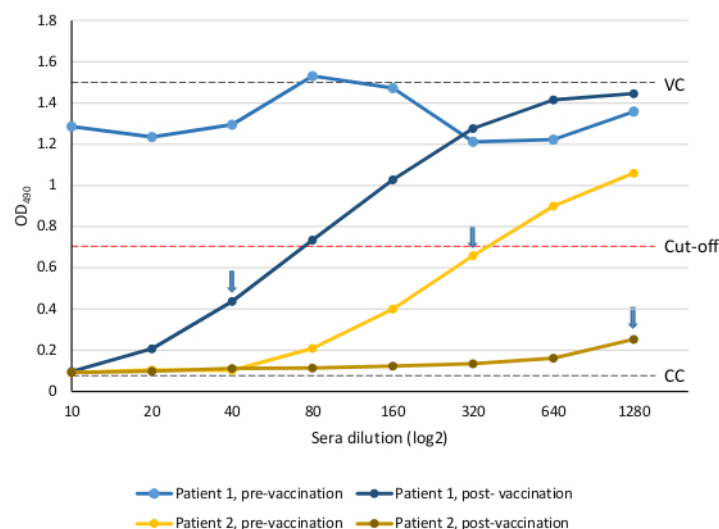




**Figure 3. Schematic of the MN assay using MDCK-SIAT1 cells.** 50  $\mu$ L of heat-inactivated sera are serially 2-fold diluted into 96-wells plates. 50  $\mu$ L of 100 TCID<sub>50</sub> of A(H3N2) virus are then added to each well. Plates are incubated at 37 °C for 1 h to allow the binding of virus and antibody. Then  $1.5 \times 10^4$  MDCK-SIAT1 cells are added to each well. Plates are incubated for 18 - 20 h at 37 °C, 5% CO<sub>2</sub>. After overnight incubation, plates are washed and fixed, the amount of virus in each well is quantified by an ELISA using anti-influenza A NP monoclonal antibodies. Sera antibody titers are calculated based on cut-offs defined by virus controls and cell controls. [Please click here to view a larger version of this figure.](#)



**Figure 4. The MN assay plate layout.** Heat-inactivated sera are pre-diluted at 1:10, then serially 2-fold diluted in 96-well plates in column 1 - 10. Virus controls (virus and cells only, no sera) and cell controls (cells only, no virus and no sera) are in column 12. Column 11 is used for back titration or control sera. [Please click here to view a larger version of this figure.](#)



**Figure 5. The MN assay results of human sera tested against A/HongKong/4801/2014 A(H3N2) virus using MDCK-SIAT1 cells.** Sera from two patients pre- and post-2016-17 seasonal influenza vaccination were tested against A/HongKong/4801/2014 A(H3N2) virus using MDCK-SIAT1 cells. Neutralization titers are the reciprocal of the highest serum dilution that achieves ≥50% virus neutralization, as indicated by arrows on each curve. Patient 1: pre-vaccination titer <10, post-vaccination titer 40; patient 2: pre-vaccination titer 320, post-vaccination titer >1,280.

## Discussion

The MN assay is one of the main assays used for influenza serology to detect antibody responses following influenza infection or vaccination. Titers generated from MN assays are often used as the primary outcome of many influenza seroepidemiology studies. MN assays are also widely used for sero-diagnosis, and the evaluation of vaccine immunogenicity. International inter-lab studies have been conducted to compare MN assays performed in multiple laboratories<sup>14</sup>.

In contrast to HI, the MN assay is designed to directly measure functional antibodies that can neutralize virus infection in cell culture. There are various forms of MN assays used in field laboratories around the world. The read-out of the assays (*i.e.*, reduction of virus infectivity in the presence of neutralizing antibodies) can be based on ELISA quantification of virus NP antibodies, HA quantification of virus, or immunostaining of virus plaques in each well following virus infection in the presence of antibodies and incubation in cell cultures<sup>14,15</sup>. Various forms of fluorescent-based plaque reduction neutralization assays (*e.g.*, focus reduction assays and ViroSpot assays) are often used for antigenic characterization of large numbers of influenza virus field isolates, partly due to the low virus amount required in these assays<sup>15,16</sup>. For the characterization of antibody responses to influenza in human serology, the 2-day ELISA based MN assay is recommended by World Health Organization (WHO) global influenza surveillance network for serological diagnosis of influenza<sup>3</sup>. This method is also widely used in sero-epidemiology studies and the evaluation of antibody responses following influenza vaccination. It primarily detects antibodies to influenza HA surface protein and therefore can detect functional strain-specific antibodies.

Here, we describe a 2-day ELISA-based MN assay that utilizes MDCK-SIAT1 cells. This assay is optimized to detect neutralizing antibodies to contemporary A(H3N2) viruses in human sera. Several critical steps should be considered when performing MN assays using MDCK-SIAT1 cells. First, MDCK-SIAT1 cells should be passaged in culture media containing G418 sulphate to maintain the stability of the human  $\alpha$ SIAT1 cDNA in the cell line. G418 sulphate (e.g., geneticin) is not required in the media during virus propagation and MN assays using MDCK-SIAT1 cells. Cells used in MN assays should be at log growth phase with 75 - 95% confluency. Second, good virus stocks are essential in order to carry out successful MN assays. Virus stocks should have high infectivity as measured by TCID<sub>50</sub> titration and a minimum amount of defective particles. The minimum dilution of virus stocks to achieve 100 TCID<sub>50</sub>/well virus should be equal or greater than 1:100. Although both egg and cell passaged viruses can be used in MN assays, MDCK-SIAT1 cell line maintains better genetic stability for propagating 3C.2a and 3C.3a A(H3N2) viruses<sup>7</sup>. After virus propagation, HA and NA genes of the virus stocks should be sequenced to ensure that no egg or cell culture adapted mutations are introduced at key antigenic sites that may cause changes in the antigenicity of the virus used in the assay. Third, the amount of infectious virus used in each assay should be carefully titrated, and verified with the inclusion of a back titration for each virus in each assay. Too much virus used in the assay may lower the antibody titers detected and reduce the sensitivity of the assay. Likewise, too little virus used in the assay will cause weak VC signals and high backgrounds (CC), and false positive results. Thus, it is important to include appropriate positive and negative control sera in the assays to monitor performance. When comparing antibody responses to multiple viruses using the same sera, it is critical to ensure that back titrations of all viruses are within an acceptable range.

The MN assay using MDCK-SIAT1 cells is sensitive and specific in detecting antibody responses to contemporary 3C.2a and 3C.3a A(H3N2) influenza viruses in human sera, including antigenically drifted A(H3N2) viruses<sup>6</sup>. This assay has been used to evaluate vaccinated human sera panels to 3C.2a and 3C.3a A(H3N2) viruses following inactivated influenza vaccination<sup>5,17,18</sup>. However, as HAs of influenza viruses continue to acquire mutations that can alter antigenicity and receptor binding properties of viruses and cause antigenic drift, continuous efforts are needed to optimize existing influenza serology assays in order to maintain the sensitivity and specificity required to characterize new emerging influenza viruses.

## Disclosures

The authors report no conflict of interest. The findings and conclusions in this publication are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention and the funding agency.

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