MODIFIED HEMAGGLUTINATION INHIBITION (HI) ASSAY USING HORSE RBCS FOR SEROLOGIC DETECTION OF ANTIBODIES TO H7 SUBTYPE AVIAN INFLUENZA VIRUS IN HUMAN SERA

Pandemic Preparedness Team
Immunology and Pathogenesis Branch
Influenza Division
Centers for Disease Control and Prevention
USA

VERSION 1

INTRODUCTION

The hemagglutination-inhibition (HI) assay is a traditional method for assessing immune responses to influenza virus hemagglutinin (HA). The HA protein on the surface of influenza virus agglutinates erythrocytes. Specific attachment of antibody to the antigenic sites on the HA molecule interferes with the binding between the viral HA and receptors on the erythrocytes. This effect inhibits hemagglutination and is the basis for the HI assay. In general, a standardized quantity of HA antigen (4 HA units) is mixed with serially diluted serum samples and red blood cells (RBCs) are added to detect specific binding of antibody to the HA molecule. The presence of specific anti-HA antibodies will inhibit the agglutination which would otherwise occur between the virus and the RBCs.

The choice of the erythrocytes to use in the HI assay is dependent on the virus being tested. Human influenza viruses bind preferentially to SA receptors containing α-2, 6-gal when avian influenza viruses preferentially bind to those containing α-2, 3-gal. Horse RBCs express a high proportion of SA α-2, 3 -gal linkages compared with turkey RBCs. With H7 influenza subtype viruses, an improved sensitivity has been observed using horse RBCs for detecting HI antibodies than using turkey RBCs. However, the below factors need to be considered when conducting HI assay using horse RBCs:

1. Higher levels of non-specific agglutinins can be detected in sera samples using horse RBCs. When nonspecific agglutinins are present in serum, agglutination occurs due to the interaction of the nonspecific agglutinins with the RBC. This is independent from the hemagglutination that occurs through the interaction of the influenza virus and the RBC. Specific antibodies to the virus can inhibit the virus-RBC interaction, but may have no impact on the nonspecific agglutinin-RBC interaction resulting in a false negative result in the HI assay. The presence of nonspecific agglutinins can be observed when diluted serum and a solution of RBC are combined and hemagglutination occurs. The serum must be adsorbed with RBC before testing in the HI assay.

2. During adsorption with horse RBCs, non-specific virus inhibitors may be introduced into serum, which will cause a false positive result in HI assay with horse RBC. We have observed that these non-specific inhibitors can be eliminated by RDE treatment. Sera need to be RDE treated after horse RBC hemadsorption prior to the horse HI assay.

Here we describe an improved horse HI assay with modified order of hemadsorption and RDE-treatment procedures that are necessary to remove non-specific agglutinins to horse RBC as well as non-specific inhibitors after hemadsorption.

MATERIALS, SUPPLIES AND EQUIPMENTS

A: MATERIALS and SUPPLIES

1. Influenza virus.
   - H7 subtype viruses
   - BPL inactivated virus can be used in place of the live virus if:
     1). It is demonstrated that BPL inactivated virus will yield equivalent HI results compared with live virus.
     2). BPL inactivation of the virus is confirmed prior to lowering the biocontainment level of laboratory testing.

   Note: Refer to national and international guidance documents to establish the appropriate biocontainment level required to perform the assay with live H7 subtype viruses.

2. Serum samples.
   Note: Serum samples should not be repeatedly freeze-thawed. Ideally, aliquot and store sera at -20 to -70°C.

3. Horse RBCs in Alsever's Solution
   Note:

   1). **Horse RBCs** in Alsever's solution can be obtained from Lampire Biological or equivalent source, and used at a concentration of **1.0% in PBS + 0.5% BSA**.
   2). It is preferred that the horse blood cells in Alsever's solution should be as fresh as possible.

4. Phosphate buffered saline (PBS) (0.01M PBS, pH 7.2), must be cold. Store at 4°C and keep on ice during use.

   8.00 g sodium chloride (NaCl)
   0.20 g potassium chloride (KCl)
   1.15 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)
   0.21 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)
   Adjust pH to 7.2 with HCl, and bring volume up to 1 liter with HPLC grade dH_2O
   Sterilize by autoclaving

5. Bovine serum albumin, fraction V, protease-free, Roche, cat. # 03117332001 or equivalent
6. 70% EtOH in deionized water
7. 0.85% saline, sterile
8. Receptor destroying enzyme, RDE (II) "Seiken". (Denka Seiken Co., Ltd, cat # 370013 or equivalent)
   RDE is supplied as a filter-sterilized, lyophilized culture supernatant of *Vibrio cholerae Ogawa type 558*.
   Note: Reconstitute each vial of RDE with 20 ml of 0.85% saline. Use immediately or freeze in single use aliquots at -20°C or colder.

9. Sterile cotton gauze pads (Fisher, cat. # 22-415-469 or equivalent)
10. Conical centrifuge tubes
11. Assorted pipettes and tips for appropriate volumes.
12. Disposable reservoirs for multi-channel pipettes.
13. Titertube Micro test tubes (Bio-Rad, cat. # 223-9390 or equivalent) and Titertube Plugs (Bio-Rad, cat. # 223-9393 or equivalent)
14. 96-well, V-bottom, polystyrene, microtiter plates (Nunc, cat. # 249570)

B. Equipment

1. Class II Biological Safety Cabinet (BSC)
2. Hemocytometer
3. Low speed, bench-top centrifuge
4. Standard microscope with 10X ocular and 10X or 20X objectives lenses
5. 37°C and 56°C water bath

METHODS

Horse HI assay can be performed with below steps:

- **I. Preparation of 1.0% horse RBCs**
- **II. Testing for Nonspecific Agglutinins**
- **III. Adsorption of Serum with Horse RBCs to Remove Nonspecific Agglutinins**
- **IV. RDE-treatment of Serum to Remove Nonspecific Virus Inhibitors**
- **V. Determination of HA titer of influenza virus**
- **VI. HI assay with horse RBCs**

I. Preparation of 1.0% horse RBCs

Notes:
1). Horse RBC is prepared at 1.0% (v/v). To standardize the RBC concentrations, cells can be counted by hemocytometer. In our laboratory, the target concentration of a 1.0% (v/v) horse RBC solution has been empirically determined to be approximately **1.5×10^8 cells / ml.**
2). The 1.0% RBCs are prepared the day that sera are tested for nonspecific agglutinins and may be stored at 4°C for use on the second day in the HI assay. Discard at the end of the second day.

1. To start **preparation of packed RBCs**, carefully collect, using a 10 ml pipette, 5-7 ml of horse RBCs from the bottom of the bottle. Remove horse RBCs from the bottom of the container to minimize contamination with cell fragments. Filter through a sterile cotton gauze pad into a 50 ml conical centrifuge tube.
2. Gently fill the conical tube with cold **PBS +0.5% BSA** and mix gently by inversion.
3. Centrifuge at **800 x g** (for example: 2000 rpm in Sorvall Heraeus 75006445 rotor, Sorvall Legend RT centrifuge) for **5 minutes at 4°C.**
4. Aspirate the supernatant using a 10 ml pipette. Be careful to not disturb the pellet of RBCs.
5. Gently fill the conical tube with cold PBS +0.5% BSA and mix gently by inversion.
6. Centrifuge at 800 x g (for example: 2000 rpm in Sorvall Heraeus 75006445 rotor, Sorvall Legend RT centrifuge) for 5 minutes at 4°C.
7. Aspirate the supernatant using a 10 ml pipette. Be careful to not disturb the pellet of RBCs.
8. **Carefully repeat the cold PBS +0.5% BSA wash** (steps 5-7) one more time for a total of three PBS washes to prevent hemolysis, always handle the RBCs gently, keep the PBS on ice or at 4°C, and do not wash more than 3 times.
9. Aspirate the remaining supernatant with a P1000 microliter pipette for **final packed RBCs.** Keep packed RBCs on ice.
10. **Prepare a 1.0% v/v suspension of RBCs**.
    For example, add 2.5 ml of the packed RBCs from step 9 to 247.5 ml cold PBS +0.5% BSA in a 500 ml glass bottle (rinse with PBS before use). Mix gently by swirling.

11. To count the RBCs, prepare a 1:200 dilution of the RBC suspension by adding 50 µl of the suspended RBCs (from step 10) to 10 ml cold PBS in a 15 ml conical, polystyrene tube. Mix gently by inversion.
12. Clean the hemocytometer thoroughly with 70% ethanol and dry with lens tissue or a soft lint-free cloth. Clean and dry the cover slip in the same way and place it gently onto the hemocytometer so that it covers the counting area.
13. Transfer 10 µl of the 1:200 diluted RBC (from step 11) onto the hemocytometer loading channel and allow the cells to spread throughout the unit, being careful not to overfill the channel.

15. Count the RBCs in each of the 4 large corner squares of the hemocytometer as shown in Figure 1

    Concentration of the cells are calculated as below:

    Cells per ml = the average count per square x the dilution factor x 10^4

    The cell count for a 1.0% (v/v) of horse RBC has been empirically determined to be approximately **1.5 x 10^8 cells / ml.** Based on this concentration, the expected number of RBCs counted from the 4 large corner squares is 300 from the 1:200 dilution prepared in step 11.

$$\textbf{Cells per ml} = \frac{300}{4} \times 200 \times 10^4$$

15. After counting the RBCs, rinse the chamber and cover slip with 70% ethanol and dry.
16. Based on the RBC count, add cold PBS +0.5% BSA, or more packed RBC to obtain final concentration of **1.5 X 10^8 cells / ml (1.0% horse RBC).**

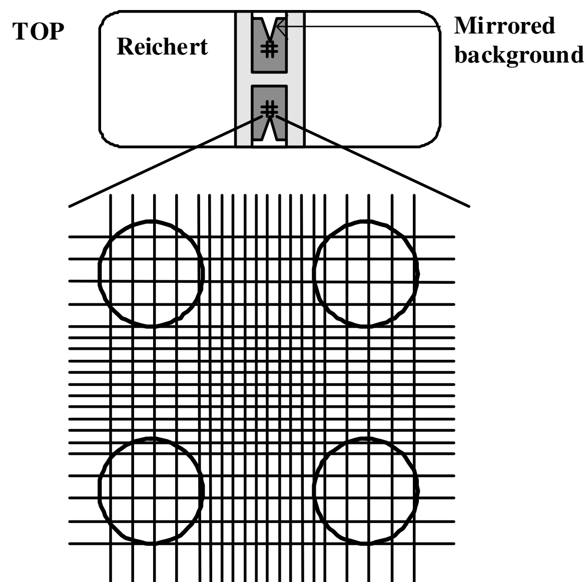

Figure 1. Diagram of hemocytometer

II. Testing for Nonspecific Agglutinins

Notes:

Higher level of non-specific hemagglutinin can be detected in sera when horse RBCs are used.

1. Heat-inactivate aliquoted serum samples at 56°C for 30 minutes, then cool down at room temperature.
2. **Add 4 part of cold PBS to pre-dilute each serum sample in 1:5**.
3. Add 25 µl of cold PBS to V bottom plate in rows A through H (A1-A11 to H1-H11). Row A (A1-A11) will serve as the sera loading wells.
4. Add 25 µl of each 1:5 pre-diluted serum to the wells A1-A11 to get **initial dilution of 1:10**.
5. Prepare serial 2-fold dilutions by transferring 25 µl of serum from the first row A to H. Discard 25 µl after row H.
6. Add 25 µl of cold PBS to wells containing sera, columns 1-11 for a total volume of 50ul.
7. Add 50 µl of cold PBS to column 12 on the plate as a RBC control.
8. Gently tap the plates to make sure the sera mix with PBS.
9. Add **50 µl of 1.0% of horse RBCs** to all wells.
10. Gently tap the plates to mix. Stack plates and cover with an empty plate.
11. Incubate at room temperature, **22°C to 25°C, for 60 minutes** to allow the RBCs to settle.
12. Tilt the plate at 45° to 60° angle. The settled RBCs in column 12 should start "running" and forming a tear-shape due to gravity. Wait until these RBCs finish "running" and then record samples where agglutination of RBCs occurred. Agglutinated RBCs do not "run" or cause a tear-shaped spot to form.
13. Serum samples with RBC agglutination in wells with a serum dilution of 1:20 or higher need to be adsorbed with horse RBCs prior to testing in HI assay. If agglutination is only observed in the first well, the 1:10 serum dilution, then adsorption is not necessary.

III. Adsorption of Serum with Horse RBCs to Remove Nonspecific Agglutinins

1. Combine **1 part packed horse RBCs (from step I-9) with 9 parts 1:5 pre-diluted serum** in a microfuge tube (1:10 v/v).
2. Gently invert to mix and incubate for 15 minutes at 4°C for adsorption.
3. Gently invert again and incubate an additional 15 minutes at 4°C for adsorption.
4. Centrifuge in a microfuge at 300 x g (for example, 2000 rpm in Sorvall Heraeus 3328 rotor, Sorvall fresco centrifuge) for 5 minutes at 4°C to pellet down RBCs.
5. Carefully remove the adsorbed serum without disturbing the packed cells. It is expected that the total amount of diluted serum recovered will be similar to the volume of diluted serum added. Monitor the recovered volume of sera.
6. The final serum dilution after adsorption is still approximately 1:5.

IV. RDE-treatment of Serum to Remove Nonspecific Virus Inhibitors

Notes:

Adsorption of sera with horse RBCs can potentially introduce non-specific virus inhibitors to cause false positive HI result. Horse RBC pre-adsorbed sera must be RDE treated prior to the HI assay.

1. Add **RDE** that is equal to **3 parts of the original sera volume** in the serum recovered from hemadsorption.

   For example, if 20 µl of original serum was pre-diluted into 1:5 to make a total volume of 100 µl, in step II above, 5ul of original vol (25 µl of 1:5) is used for testing of non-specific agglutinin. Therefore, there were 15 µl of original serum in the remaining 75 µl of 1:5 sera recovered from hemadosorption. Add 45ul RDE (equals to 3 parts of original vol in the sera after hemadsorption).

2. Incubate **RDE-serum mixture at 37°C for 18-20 hrs**.
3. Heat serum samples at **56°C for 30 minutes to inactivate RDE**.
4. Add appropriate volume of cold PBS to bring the final dilution to 1:10. Volume of PBS is calculated based on the total volume from step IV-1.
   In the example above, adding 3 parts of RDE brings the total volume to 120 µl (75 µl + 45 µl). The target volume to make 1:10 dilution of 15 µl original serum equals to 150 µl. So, the volume of PBS needed after RDE-treatment will be 30 µl (150 µl - 120 µl).

5. The removal of nonspecific agglutinins needs to be confirmed by combining the treated serum with 1.0% horse RBCs as above and observing for the absence of agglutination.
6. Treated sera may store at 4°C overnight. If longer storage is needed, freeze at -20°C or colder.

V. Determination of HA Titer of Influenza Virus (Work in BSC)

1. Mark the V bottom plates with the names of the viruses to be tested. Viruses are tested in duplicate. See figure 2.
2. Add **50 µl of cold PBS** to wells 2 through 12 in rows A and B. If more than 1 virus, use the rest of rows as needed.
3. Add 50 µl of cold PBS to the entire H row. This row will serve as the RBC control.
4. Immediately prior to removing virus from vial, gently vortex the vial of virus using three quick pulses. Then add **100 µl of the virus** to be tested to wells A1 and B1.
5. Make **serial 2-fold dilutions** by transferring 50 µl from well 1 successively through well 12. Discard 50 µl from well 12.
6. Add 50 µl of **1.0% horse RBC** suspension to all wells in rows A, B (or other rows if more than 1 virus), and H on the plate.
7. Gently tap the plates to mix. Stack plates and cover with an empty plate.
8. Incubate at room temperature for **60 minutes**.
9. Read the viruses HA titers by tilting the plate at a 45 to 60° angle. The settled RBCs in row H should start "**running" and form a teardrop-shape** due to gravity. Wait until these RBCs finish "running" and then note the RBC buttons in the virus titrations that "run". These RBCs do not exhibit hemagglutination. The highest dilution of virus that causes complete hemagglutination is considered the HA titration end-point. **The HA titer is the reciprocal of the dilution of virus in the last well with complete hemagglutination.**
10. Dilute virus in cold PBS to make a working solution containing **8HAU/50 µl**.
11. Verify that the diluted virus contains 8 HAU per 50 µl by performing a second HA test as described above. The titer of the virus should be 8. If not 8, then adjust the virus concentration by adding virus if < 8 HAU or cold PBS if > 8 HAU. Do not use until the virus concentration is 8 HAU per 50 µl.
12. Store the working dilution of virus on ice and use within the same day.

Note:
Working dilution of virus for HI assay. The standardized working dilution of virus must have an HA titer of **8 HAU per 50 µl. This is the same as 4 HAU per 25 µl**. This titer will hemagglutinate the first four wells in the virus back titration in the HA assay. If the working solution does not have an HA titer of 8 in 50 µl, it must be adjusted accordingly by adding more virus to increase the number of HAU or by diluting the virus with PBS to decrease the number of HAU.

VI. HI Assay with Horse RBCs

A. Quality Control

1. Serum controls - Prepare multiple, single use aliquots of RDE-treated control sera and store at -20 °C to -70°C. Include animal and, if possible, human positive and negative serum controls.

   For each virus tested, include at least one positive control serum, and one negative control serum.

2. RDE treatment control – Ideally, each assay should include a normal ferret serum known to contain non-specific inhibitors of hemagglutination that is RDE treated at the same time as the human serum samples being tested.

3. Virus back titration - In each assay, include a virus back titration of the working dilution of the virus.

4. Horse RBC control - The RBC control contains 50 µl PBS and 50 µl of 1.0% horse RBC suspension. A horse RBC control must be added to every plate.

B. HI Assay with Horse RBCs (Work in BSC.)

1. Thaw at room temperature if treated sera are frozen, then keep on ice during use.

2. Mark the V bottom plates with the plate number and the names of the viruses accordingly based on experiment design.

3. Column 12 of all plates can be reserved for the RBC control. Positive and negative control sera, and back titration can be run in a separate plate or incorporated in available columns of plates.

4. If Titertubes are used, for duplicate test with one virus, make a serial 2-fold dilution of treated sera by adding 110 µl of treated sera (1:10) to Titertubes in rows A, columns 1-11.

5. Add 55 µl of cold PBS to Titertubes in rows B-H, columns 1-11.

6. Transfer 55 µl of RDE-treated sera from row to row (A -> B -> C…..H) using a P200 multichannel pipette to make serial 2-fold dilutions.

7. Discard 55 µl from row H after mixing.

8. Positive and negative control with appropriate initial dilution should be serially diluted following the same procedure above.

9. Transfer **25 µl of each diluted serum sample** from Titertubes into V-bottom plates starting with row H and going to row A. No need to change tips if transferring from the highest dilution (row H) to the lowest dilution (row A). It is critical that the tips must be changed before beginning to pipet the next set of serum samples.

10. If Titertubes are not available, serial dilution of sera samples can be done directly on plates (Figure 3). For each replicate test with one virus, first, add 25 µl of cold PBS to V-bottom plate in rows B-H, columns 1-11. Second, add 50 µl of RDE-treated sera to row A, columns 1-11. Then, transfer 25 µl RDE-treated sera from row to row (A -> B -> C…..H) to make serial 2-fold dilutions. Discard 25 µl from row H after mixing.

11. Add **25 µl of standardized virus containing 4 HAU** to wells containing sera. Note this is the same as 50 µl containing 8 HAU.

12. Gently tap the plates to mix. Stack plates and cover with an empty plate.

13. **Incubate virus and sera at room temperature (22° to 25°C) for 30 minutes.**

14. Add 50 µl of PBS to column 12. This will serve as the RBC control.

15. Add **50 µl of 1.0% horse RBC** suspension to each well.

16. Gently tap the plates to mix. Stack plates and cover with an empty plate.

17. Incubate at **room temperature for 60 minutes.**

18. Record the HI titers of sera after the 60 minute incubation by tilting the plates at a 45 to 60° angle. The settled RBCs in column 12 should start "pulling" or "running" and form a "teardrop-shape" due to gravity. Wait until these RBC's finish "pulling" and then read the RBC buttons that "run" or "stream" in the same way. **A well with complete hemagglutination inhibition will look the same as the RBC controls. The serum HI titer is the reciprocal of the serum dilution in the last well with complete hemagglutination inhibition**. See figure 3.

19. The dilution of serum if starting from 1:10, for the purpose of determining 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 1:1280 (Figure 3).

Notes:

1. All sera tested with horse HI must be tested for non-specific agglutinin, pre-adsorb as needed, and then treated with RDE prior to HI assay.

2. In order to ensure optimum results and assay reproducibility, it is essential that test procedures be followed exactly. Occasionally, the HI assay may be difficult to interpret, in such cases, consider the factors presented below:

   The virus dilutions must be freshly prepared, the HA titer of the virus determined for each HI assay, and the concentration of virus in the working virus solution confirmed in each HI assay.

   Select virus isolated from same outbreak for optimal antigenic match or use an antigenically equivalent strain.

   Incubation times must be strictly observed. Plates must be read promptly when the RBC control has completely settled.

The quality of RBC may vary. Lot to lot variation may occur.

RBC suspension must be standardized in a consistent manner each time as described in step I.

Virus stocks must be stored at -70℃. Virus, for use in the HA and HI assays must be diluted in cold PBS and kept on ice until added to the micro titer plates.

REFERENCE:

Stephenson I et al., Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J Medical Virology 70:391-398 (2003)

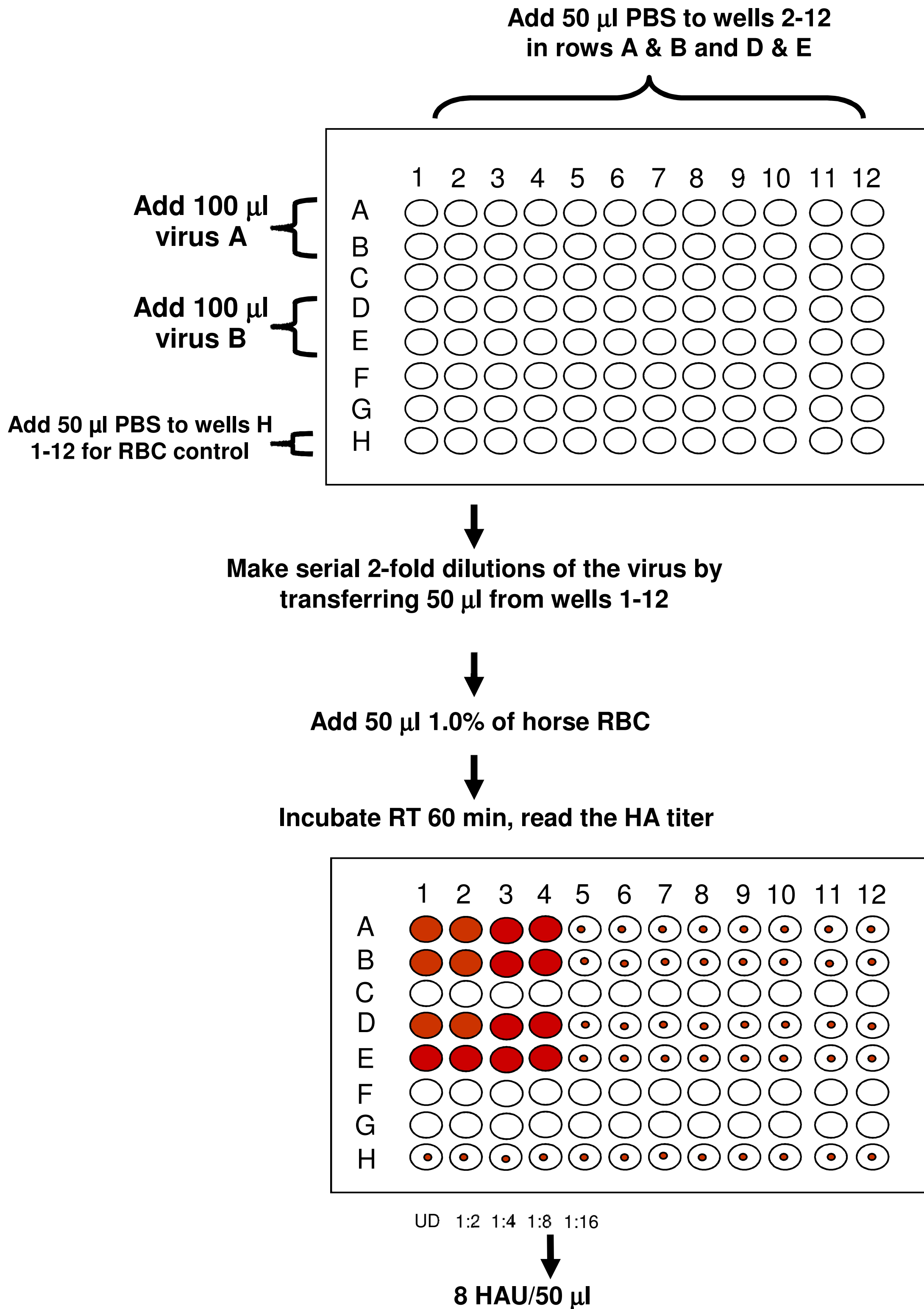

Figure 2. HA assay

Add 50 µl sera to wells A1 - A11

Add 25 µl PBS to wells B - H

1 2 3 4 5 6 7 8 9 10 11 12

A B C D E F G H

2-fold dilutions

Add 25 µl virus (=4HAU)

Incubate at RT 30 min

Add 50 µl 1.0% horse RBC

Incubate RT 60 min, read the HI titers

1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280

1 2 3 4 5 6 7 8 9 10 11 12

A B C D E F G H

<10 or 5 | 640 | <10 or 5 | 20 | 10 | 40

For illustration, duplicate serum samples are shown on the same plate.

Figure 3. HI assay

Supplementary data

Serologic Detection of H7N9 Influenza Virus Antibody Response by Hemagglutination Inhibition Assay using Horse RBCs

1. HI assay using horse RBCs provides better sensitivity than HI using turkey RBCs to detect serological responses against several H7 viruses, including wild type H7N9 virus. Below are comparison of horse and turkey RBCs in HI assay using human sera from a H7 vaccine study with A/NL/219/2003 live attenuated vaccine prime and A/Mallard/NL/12/2000 inactivated vaccine boost (Figure 4). The use of horse RBC in HI assay results in titers that are 2-8 folds or more higher than titers detected using turkey RBCs.

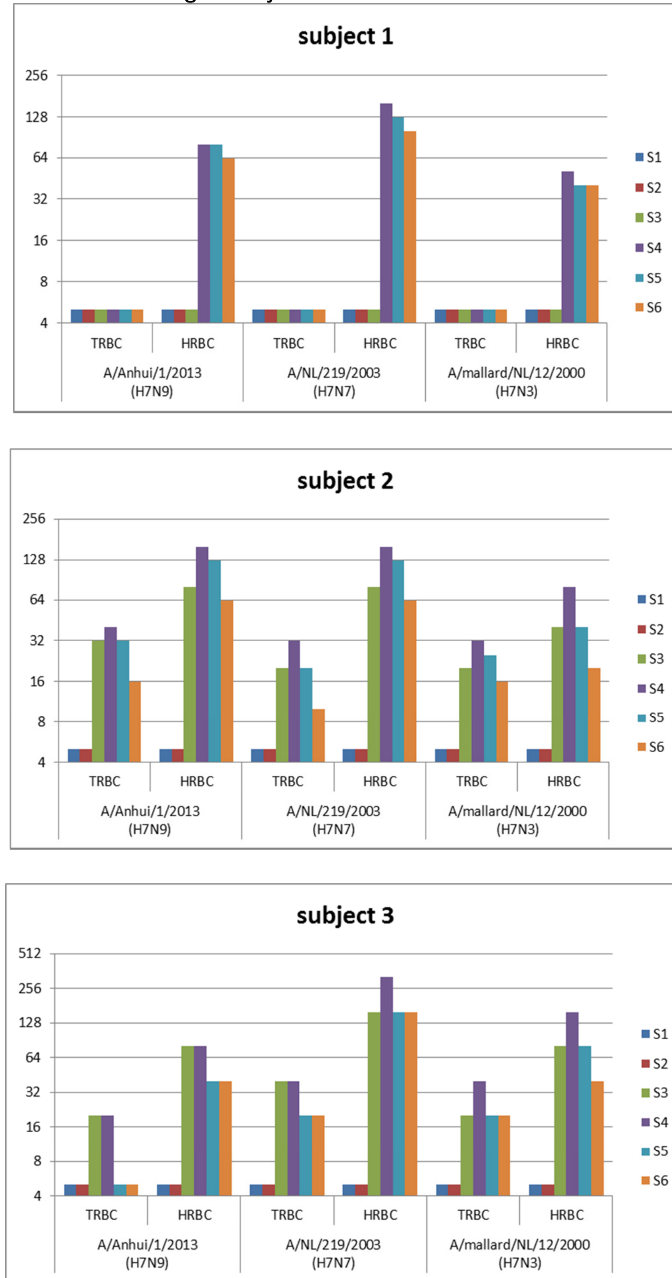

Figure 4: Improved sensitivity of HI assay using horse RBC vs turkey RBC for testing of human H7 vaccine sera.

2. Comparison of antibody titers achieved by horse RBC HI assay vs microneutralization (MN) assay using human sera from a H7 vaccine study with A/NL/219/2003 live attenuated vaccine prime and A/Mallard/NL/12/2000 inactivated vaccine boost (Figure 5). HI assay using horse RBC yielded generally comparable titers as the microneutralization assay.

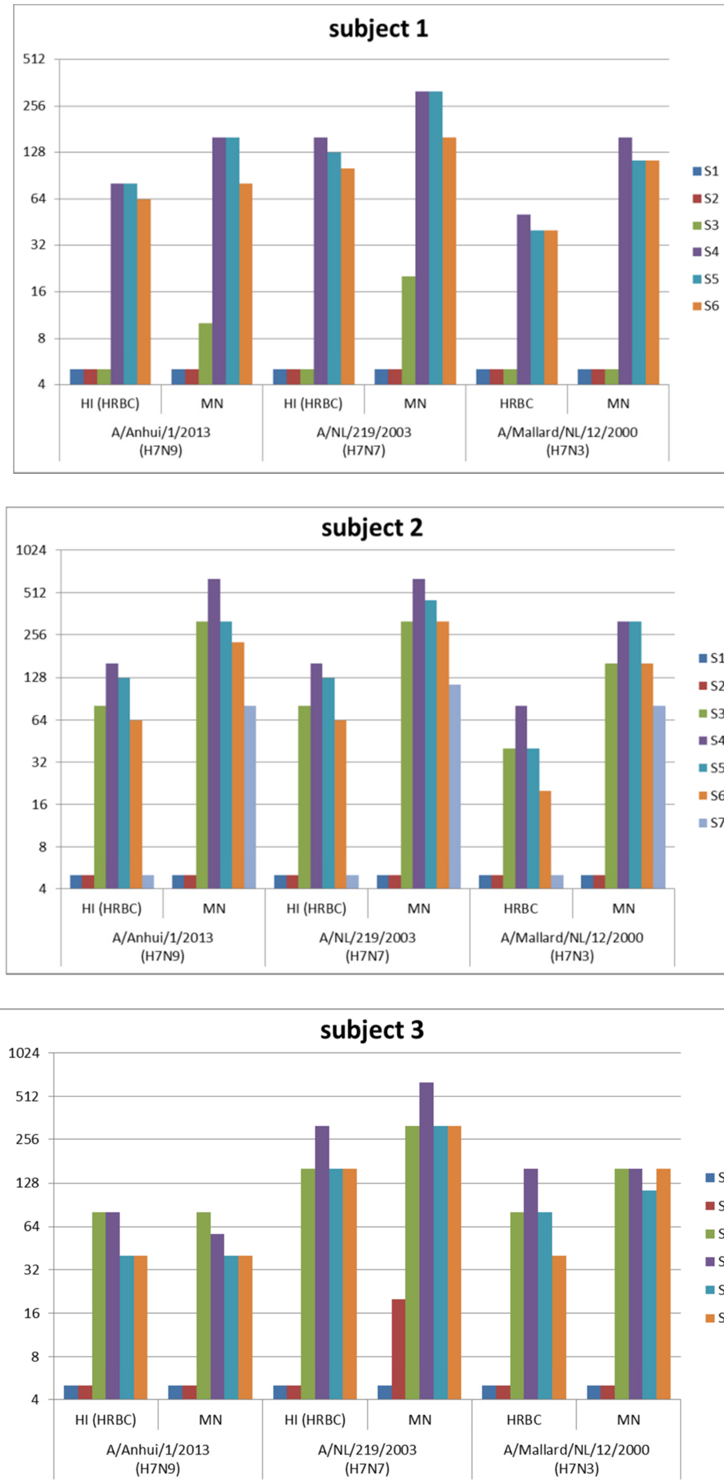

Figure 5: Comparison of HI assay using horse RBC vs microneutralization (MN) assay for testing of human H7 vaccine sera.