



Laboratory Procedures

Serological detection of avian influenza A(H7N9) virus infections by turkey haemagglutination- inhibition 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) virus infections by turkey haemagglutination-inhibition assay.

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SEROLOGICAL DETECTION OF AVIAN INFLUENZA A(H7N9) VIRUS INFECTIONS BY TURKEY HAEMAGGLUTINATION-INHIBITION ASSAY

INTRODUCTION

Serological diagnosis is an important approach when clinical specimens are unobtainable or when a laboratory does not have the resources required for virus isolation. The haemagglutination-inhibition (HAI) assay is a traditional method for assessing immune responses to influenza virus haemagglutinin (HA) and for identifying influenza virus field isolates. 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 haemagglutination and is the basis for the HAI. In general, a standardized quantity of HA antigen (4 HA units) is mixed with serially diluted anti-sera, and red blood cells are added to determine specific binding of antibody to the HA molecule. This assay is extremely reliable, relatively simple and inexpensive technique. Disadvantages of the HAI test include the need to remove nonspecific inhibitors which naturally occur in sera, to standardize antigen each time a test is performed, and the need for specialized expertise in reading the results of the test.

The receptor specificity of influenza virus HA correlates with the ability to agglutinate RBC from different animal species. Most avian influenza viruses preferentially bind to sialic acid receptors that contain N-acetylneuraminic acid α 2,3-galactose (α 2,3-Gal) linkages, while human influenza viruses preferentially bind to those containing N-acetylneuraminic acid α 2,6-galactose (α 2,6-Gal) linkages. We discovered that the novel H7N9 virus could bind to both avian-type (sialic acid α 2, 3) and human-type (sialic acid α 2, 6) receptors. The HAI assay, using turkey RBC that express a mixture of α 2,3-Gal and α 2,6-Gal linkages is a sensitive and relatively specific assay for detecting antibody response to current avian influenza A(H7N9) viruses in human sera following natural infection and vaccination and potentially, for detecting antibody to other avian subtypes. However, it is important to continuously monitor the specificity of this assay when new viruses are used, as there may be strain to strain variation in the detection of non-specific HAI antibody responses.

I. Materials Required

1. Virus strains

Live or beta-propiolactone (BPL)-inactivated viruses in allantoic fluid. Aliquot and store at -70°C.

2. Serum samples:

If serum is to be tested repeatedly, it is best to make several aliquots of the serum. Sera should not be repeatedly freeze-thawed and can be stored at -20 to -70°C. Both human and animal sera must be treated with receptor-destroying enzyme (RDE) to remove nonspecific inhibitors before use.

3. Buffers and Reagents

3.1. TRBC (Turkey red blood cell) in Acid Citrate Dextrose (ACD) solution

Preparation of ACD solution for collection of TRBC:

- 3.1.1. 22.0g/L sodium Citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)
- 3.1.2. 8.0g/L Citric acid ($\text{C}_6\text{H}_8\text{O}_7$)
- 3.1.3. 24.5 g/L Dextrose
- 3.1.4. Sterile distilled water to 1L

3.2. Phosphate-buffered saline (PBS), 0.01M, pH 7.2.

3.3. Receptor destroying enzyme, RDE (II) "Seiken", (Denka Seiken Co., Ltd, cat # 370013)

Note: Reconstitute each vial of RDE with 20 ml of PBS. Use immediately or freeze in single use aliquots at -20°C or colder.

4. Equipment

- 4.1. 37°C water bath
- 4.2. 56°C water bath
- 4.3. Bench centrifuge
- 4.4. Class II Biological Safety Cabinet (BSC)
 - 4.5. 4°C refrigerator
- 4.6. Freezer, - 70°C (for long term virus storage) or - 20°C (for serum storage)

5. Supplies

- 5.1. Centrifuge tubes (graduated conical 50ml and 2ml)
 - 5.2. 96-well V bottom microtiter plates
 - 5.3. Disposable reservoirs for multi-channel pipettes.
 - 5.4. Assorted sterile pipettes and pipetting device; assorted microliter pipettes and disposable tips
 - 5.5. 96-well PCR plate
- Note** that 96 sera can be treated in 1 plate.

II. Quality Control

- A. Serum controls** - make multiple aliquots of RDE-treated control sera and store at -20 to -70°C. Include both animal and if possible human negative and positive serum controls.

1. Positive serum control

- 1.1 For animal sera, use sera from infected ferrets or mice, or other (rabbit, sheep, goat) immunized animals.
- 1.2 For human sera, an optimal positive control would be acute and convalescent serum samples from a laboratory-confirmed H7N9 case that shows a > 4-fold rise in titer. Alternatively, a single high titered convalescent serum sample may also be used.

2. Negative serum control

- 2.1 For animal sera, use non-immune serum from same animal species used for negative control.
- 2.2 For human sera, use age-matched sera from individuals that have not been exposed to the particular virus strain in question to estimate the levels of non-specific antibody responses for a particular virus. Screening of 50-100 sera from non-exposed anti-H7N9 antibody negative people is recommended to determine the specificity of the assay, each time a new virus is used.

B. Virus back titration

In each assay, include a virus back-titration of the working solution of the virus. Add 50µl of PBS to wells B-H of well 1 and 2 (duplicate). Add 100µl of the working solution of virus (= 8 HAU/50µl) to the first well (A1 and A2). Serially transfer 50µl down from well-A to H). Add 50µl of 1% TRBC and incubate 30min at RT.

C. RBC control

RBC controls allow adjustments in incubation times. There should be a RBC control on each plate, if possible.

III. PROCEDURE FOR HAI ASSAY

1. Generation of Stock Virus

Grow up virus to high titer in the allantoic cavity of 9 or 10-day old embryonated hens' eggs.

Note: Any work with infectious highly pathogenic H7N9 virus must be performed in a BSL3 laboratory with enhancements according to CDC guidelines

- 1.1. Dilute virus sample (usually 1:100 to 1:50,000) in PBS + antibiotics (100 U/ml penicillin, 100 µg /ml streptomycin, and 100µg/ml gentamycin).
- 1.2. Pierce hole in the tops of eggs.
- 1.3. Inoculate eggs with diluted virus (100-200µl/egg) using a syringe fitted with a 22 gauge/1" needle. Seal hole in eggs with glue or paraffin wax.
- 1.4. Incubate eggs for 48 hr at 37°C.
- 1.5. Chill eggs (overnight at 4°C or 30-40 min at -20°C).
- 1.6. Remove tops of eggs and harvest allantoic fluid with pipette.
- 1.7. Clarify allantoic fluid by low-speed centrifugation (600 x g or 2000 rpm, 10 min).
- 1.8. Determine the HAU of the virus and aliquot virus in multiple ampoules (≈1.5ml/ampoules) and freeze at -70°C.

H7N9 viruses should be inactivated by BPL. Complete inactivation should be confirmed before being brought into the BSL-2 laboratory for the HAI assay.

2. Treatment of reference anti-sera and human sera for inactivation of non-specific inhibitors

Non-antibody molecules present in serum are capable of binding to the influenza virus HA, resulting in nonspecific inhibition and leading to false interpretations. This effect is believed to occur because these serum components contain sialic acid residues that mimic the receptors of RBC, and compete with RBC receptors for the influenza HA. To perform a valid HAI test, one must ensure that the serum does not contain nonspecific inhibitors reactive with the virus antigen being tested. The inhibitors exhibit different levels of activity against the HA of different influenza strains. Several methods exist for inactivating nonspecific inhibitors in sera of different species. Treatment with RDE is a commonly used method. When nonspecific inhibitors of RDE-treated serum create a problem with interpretation in HAI test, alternative treatment methods need to be investigated.

- 2.1. Reconstitute the lyophilized RDE with 20 ml PBS, aliquot, and store at -20°C .
- 2.2. Add 3 volumes of RDE to 1 volume serum and incubate in 37°C water-bath for 16-18 hours.
- 2.3. Heat in a 56°C water-bath for 30 min to inactivate remaining RDE.
- 2.4. Add 6 volumes of PBS. The final dilution of sera is 1:10.

3. Detection of nonspecific agglutinins in treated sera and adsorption of Serum with RBCs to Remove Nonspecific Agglutinins

Nonspecific agglutinins must be removed from sera to prevent false negatives in the HAI test.

- 3.1. Add 25 μl PBS to each well except the first row.
- 3.2. Add 50 μl of each RDE-treated serum to the first wells.
- 3.3. Prepare serial 2-fold dilutions of the sera by transferring 25 μl from the first well to the successive wells in each column. Discard the final 25 μl after row H.
- 3.4. Add 25 μl PBS to all wells (instead of antigen) in all wells.
- 3.5. Add 50 μl 1% TRBC to all wells.
- 3.6. Mix using a laboratory shaker or by manually agitating the plates thoroughly.
- 3.7. Incubate the plates at room temperature for the appropriate time by checking the RBC control for complete settling of the cells. A total of 30 minutes is usually TRBCs.
- 3.8. Record and interpret the results. If the RBCs settle completely in the wells in a column containing diluted serum, that serum is acceptable for use in the HAI test. The presence of nonspecific agglutinins will be evident by any haemagglutination of the RBCs by the serum. In this case, the serum must be adsorbed with RBCs as follows:
 - 3.8.1. To one volume of packed RBCs in a centrifuge tube add 20 volumes of RDE-treated serum.
 - 3.8.2. Mix thoroughly and incubate at RT or 4°C for 1 hour, mixing at intervals to resuspend the cells.
 - 3.8.3. Centrifuge in a microfuge at $600 \times g$ (2000 rpm) for 5 minutes.

- 3.8.4. 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.
- 3.8.5. The final serum dilution after adsorption is still a 1:10. The removal of nonspecific agglutinins must be confirmed if the initial haemagglutination titer was ≥ 80 by combining the adsorbed serum with 1% TRBC as above and observing for the absence of agglutination. If the titer is still ≥ 20 then re-adsorb the serum with packed RBCs.

4. Collection of turkey blood and standardized RBC to 1%.

- 4.1. Collect turkey blood into a syringe with ACD solution (1 cc for 1 cc blood). Put the blood with ACD solution into a bottle and gently agitate it. Turkey blood should be store at 4°C and used as fresh as possible.
- 4.2. Add 5ml of blood into a 50ml conical centrifuge tube.
- 4.3. Fill tube with cold PBS to 50ml, mix gently.
- 4.4. Centrifuge at 2000 rpm for 5 min.
- 4.5. Remove supernatant and wash the partially packed cells with PBS two more times, discarding the final supernatant from the partially packed cells.
- 4.6. To determine the TRBC concentration, add 1.0 ml of partially packed previously washed TRBC into a 1.5 ml centrifuge tube, and centrifuge at 8000 rpm for 10 min. Estimate the volume of completely packed TRBC which should be appropriately at 60% to 75% of the original volume of partially packed TRBC. Discard the complete packed TRBC.
- 4.7. Dilute partially packed TRBC to final concentration of 1% in PBS. (1ml RBCs+99ml PBS)

5. HA titration of virus strains

- 5.1. Mark the V bottom plates with the names of tested viruses (duplicate)
- 5.2. Add 50 μ l of PBS to wells 2 through 12.
- 5.3. Add 100 μ l of each tested virus to the first well (duplicate, well-1A and well -1B).
- 5.4. Make serial 2-fold dilutions by transferring 50 μ l from the first well to successive well-11, discard the final 50 μ l from well-11. Well-12 contains only PBS as RBC control.

- 5.5. Add 50 μ l of 1% TRBC suspension to each well on the plate.
- 5.6. Gently mix the plates and incubate the plates at room temperature (RT) for 30min.
- 5.7. Record the titers of viruses after 30 min by tipping plates and reading RBC buttons that stream. The highest dilution of virus that causes complete haemagglutination is considered the HA titration end point. The HA titer is the reciprocal of the dilution of virus in the last well with complete haemagglutination.
- 5.8. Dilute virus in cold PBS to make a working solution containing 8HAU/50 μ l. Calculate the antigen dilution by dividing the HA titer (which is based on 50 μ l) by 8 because you wish to have 8 HAU/50 μ l. For example, an HA titer of 160 divided by 8 is 20. Mix 1 part of virus with 19 parts cold PBS to obtain the desired volume of standardized antigen. Keep a record of the dilution for the next HAI assay.
- 5.9. Perform a "back titration" to verify HA units (=8 HAU/50 μ l) by performing a second HA test. Store this working solution on ice and use within the same day (see Figure. 1).

Interpretation

Haemagglutination occurs when the RBC control has settled completely (well-12). This is recorded using a "+" symbol. When a portion of the RBC is partially agglutinated or partially settled, a "+/-" symbol is used. In the absence of haemagglutination, tear-shaped streaming of erythrocytes which flow at the same rate as RBC controls is observed.

Standardized working solution must have an HA titer of 8 HAU/50 μ l (= 4 HAU/25 μ l). This titer will haemagglutinate the first four wells of the back titration plate. If the working solution does not have an HA titer of 8 in 50 μ l, it must be adjusted accordingly by adding more antigen to increase units or by diluting to decrease units. For example, if complete haemagglutination is present in the fifth well, the virus now has a titer of 16 HAU/50 μ l and the test antigen should be diluted 2-fold. Conversely, if haemagglutination is only present to the third dilution, the antigen has a titer of 4 HAU/50 μ l and an equal volume of virus must be added to the test antigen as was used when the antigen was initially diluted. This will double the concentration of virus in the working solution to give a titer of 8 HAU/50 μ l. Continue adjusting the concentration of antigen until 4 HAU/25 μ l (= 8 HAU/50 μ l) is obtained.

6. HAI Assay

- 6.1. Label appropriate V bottom microtiter plates with serum numbers, antigens used, and plate numbers.
- 6.2. Add 25 µl cold PBS to wells B through H (B1-11 to H1-11) of each numbered column.
- 6.3. Add 50µl of each RDE-treated serum (1:10) to the first well (A1-11) of the appropriate numbered column.
- 6.4. Prepare serial 2-fold dilutions by transferring 25µl serum from the first well of numbered columns A1-11 to successive wells. Discard the final 25µl after row H.
- 6.5. Add 25µl of standardized virus containing 4 HAU to 2-fold diluted sera.
- 6.6. Gently tap the plates and incubate at RT (22° to 25°C) for 30 min.
- 6.7. Add 50µl of PBS to well-12 as a RBC control.
- 6.8. Add 50µl of standardized 1% TRBC to all wells. Mix as before.
- 6.9. Cover the plates and allow the RBC to settle at RT for the appropriate 30min.
- 6.10. Record HAI titers.

Interpretation

If an antigen/antibody reaction occurs, haemagglutination of the RBC will be inhibited. Symbols of "+" for complete haemagglutination, "+/-" for partial haemagglutination, and "-" for inhibition of haemagglutination are used. The HAI titer is the reciprocal of the last dilution of serum that completely inhibits haemagglutination.

To ensure optimal HAI results when diagnosing infections serologically, it is essential that test procedures be followed exactly. Occasionally, the HAI assay may be difficult to interpret, in such cases, consider the factors presented below.

1. Selecting virus isolated from same outbreak for optimal antigenic match or an antigenically equivalent strains used in HAI assay for maximum sensitivity.
2. Standardized virus working solution must contain 4 HAU/25µl. The antigen dilutions must be prepared and back titrated in **each** assay.
3. Incubation times must be strictly observed. Plates must be read promptly when the RBC control has completely settled. Elution of the RBC from the virus can occur with some virus strains. When this happens, the plates may be read earlier or placed at 4°C.

4. RBC suspension must be standardized in a consistent manner each time.
5. Test viruses must be handled and stored in the prescribed manner. To minimize freeze-thaws and to avoid bacterial contamination, dispense reagents in small volumes using sterile techniques.
6. All tested sera must be treated by RDE to remove nonspecific inhibitors.
7. The positive control antisera must be included in each diagnostic serologic assay, and should give consistent results when compared with previous test.
8. The turkey blood in ACD buffer should be used as fresh as possible.
9. Some human sera may contain nonspecific agglutinins and cause nonspecific haemagglutination of the TRBC, resulting in false diagnosis in sera containing low levels of HAI antibody. In this case, the sera must be adsorbed with RBC to remove the nonspecific agglutinins.

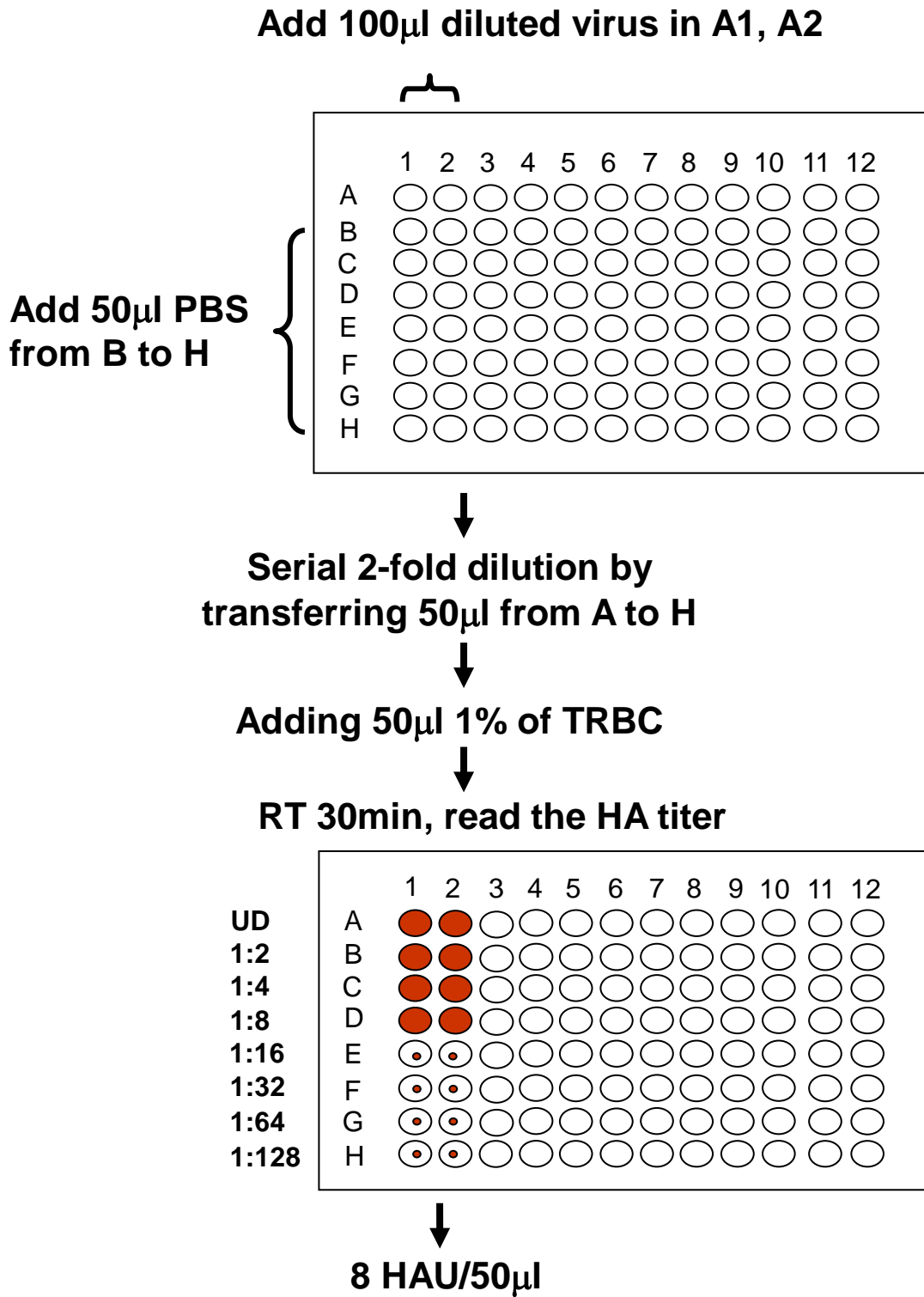


Figure 1. HA assay

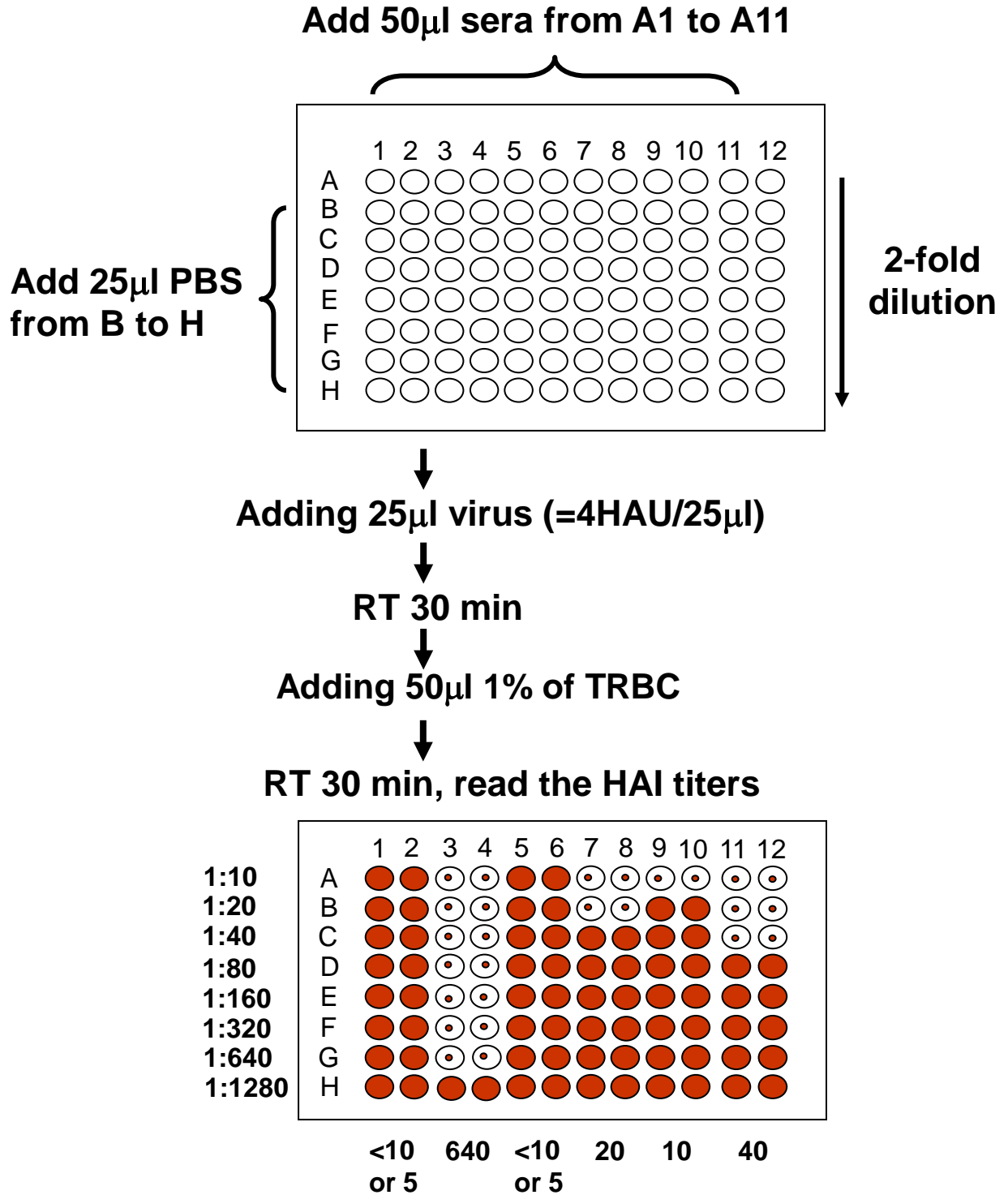


Figure 2. HAI assay set up