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Hemagglutination Inhibition (HI) Assay of Influenza Viruses with Monoclonal Antibodies

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[Abstract] Haemagglutination is inhibited when antibodies are present because antibodies to the influenza virus will prevent attachment of the virus to red blood cells. The highest dilution of antibody that prevents hemagglutination is called the HI titer. Human monoclonal antibodies generated from single human B cells were tested to characterize their ability to inhibit hemagglutination against virus A/California/07/2009 (H1N1) and A/Brisbane/10/2007 (H3N2).

Materials and Reagents

A. Materials
   1. 96 well microtiter plates (V bottom) (Corning, catalog number: 3897)
   2. Tips for multichannel pipette (Gilson, model: D10, D200 and D1000)
   3. Centrifuge tubes (SARSTEDT AG & Co, catalog number: 72.690)

B. Reagents
   1. Viral antigen
      a. H1N1
         A/California/07/2009

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b. H3N2
A/Brisbane/10/2007

Note: Viruses were amplified in embryonated eggs. Refer to Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza, WHO.

2. Erythrocytes
Turkey red blood cells (RBCs)
Note: Both in-house RBCs and commercial RBCs are usable.
3. Antibody
Human monoclonal antibodies CT146, CT147, CT149, CT164 and CT166
Notes:
a. Maximum concentration of antibody is 20 µg/ml.
b. Expressed in CHO cells.
c. Purified according to manufacturer's instruction [HiTrap™ MabSelect SuRe (GE Healthcare, catalog number: 11-0034-93)].
4. Other reagents
a. Receptor destroying enzyme (RDE) (DENKA SEIKEN CO., catalog number: 370013)
b. Phosphate buffered saline (1x PBS) (pH 7.2) (Sigma-Aldrich, catalog number: P4417)
c. Physiological saline (0.85% NaCl) (Sigma-Aldrich, catalog number: S9888)

Equipment

1. Hemocytometer (Hausser Scientific, catalog number: 1492)
2. Multichannel pipette (Gilson, model: PIPETMAN Neo® Multichannel)
3. Centrifuge (Beckman Coulter, model: Allegra X-15R)
4. Water bath (JULABO GmbH, model: MB13)
5. Inverted routine microscopy (Nikon Instruments Inc., model: TS-100)

Procedure

A. Preparation of erythrocytes
1. Prepare the diluted RBC for washing step: 5 ml of RBC + 45 ml of 1x PBS (room temperature).
2. Washing step (just one time): Centrifuge the diluted RBC, 2,000 *x g*, 10 min, 25 °C.
3. Discard supernatant and then suspend the RBC pellet with 20 ml (initial volume) of 1x PBS.
4. 1:100 dilution (0.5 ml of RBC suspension from step A3 + 49.5 ml of 1x PBS).

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5. Transfer 10 µl of RBC from step d onto a hemocytometer and count the RBC in each of the 4 squares to calculate the final volume of RBC.
6. Calculate the final volume of RBC by formula below (from Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza, WHO).
   a. Formula

      Final volume = total number of cells counted X initial volume /160
   b. Example of calculation

      Initial volume =20

      Total number of RBC = 320

      Final volume = 320 x 20/160 (160 is used as constant when use avian red blood cells; Chicken or turkey) = 40

      Then, add 20 ml of 1x PBS to 20 ml of RBC to make 40 ml and use it for HI assay.

B. Hemagglutination titration of viral antigen (Serial dilution is described in Figure 1)
   1. Add 50 µl of PBS to the V bottom 96 wells in column 2 to 12.
   2. Add 100 µl of each antigen to the first wells of rows.
   3. Make serial 2-fold dilutions by transferring 50 µl from the first to successive wells of each row using multi-channel pipette.
   4. Discard the final 50 µl.
   5. Add 50 µl of prepared RBCs to each well.
   6. Incubate the plate at room temperature for 30 min to 1 h.
   7. Record the HAU results (refer to Example of HAU titers as shown in Figure 2).

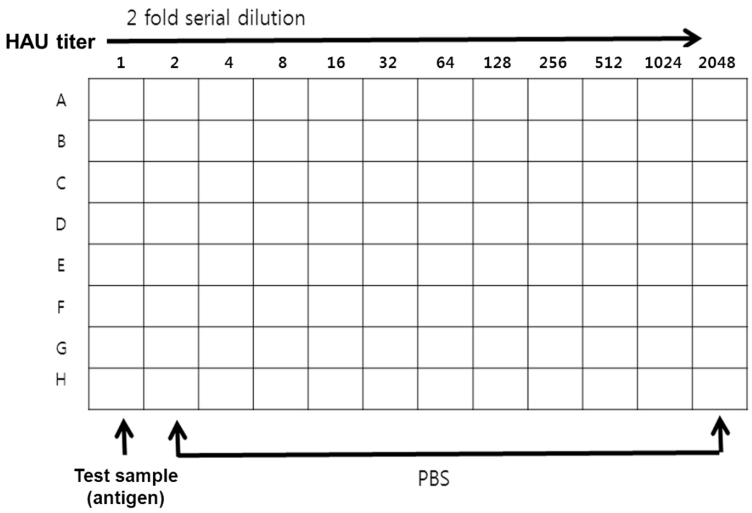

Figure 1. Serial dilution of test sample for HAU titration. Add 50 µl of PBS to all wells in column 2 to 12 of a v-bottom 96-well plate and then add 100 µl of test sample to the first well of each row. Transfer 50 µl of sample to the next wells using a multi-channel pipette to make a 2-fold dilution. Dilution factor is 1 to 2,048.

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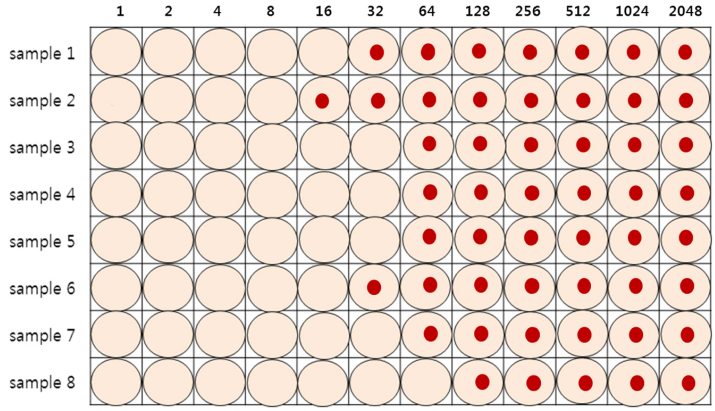

HAU titer			
Sample1	16	Sample5	32
Sample2	8	Sample6	16
Sample3	32	Sample7	32
Sample4	32	Sample8	64

Figure 2. Example of HAU titers. Hemagglutinin on the surface of influenza virus binds to the sialic acid receptors of red blood cells and creates a lattice structure. The agglutinated lattice maintains the red blood cells in a suspended state and shows as a reddish solution. HAU titer is the dilution factor of the last well showing reddish solution. HAU titer of sample 1 to sample 8 is 16, 8, 32, 32, 32, 16, 32 and 64.

C. Preparation of standardized antigen for HI test

1. Prepare 4 hemagglutination units (HAU) of antigen by dilution of antigen.
   Example of dilution: If antigen HAU is 16, 4 fold dilution with 1x PBS for 4 HAU.

D. Hemagglutination inhibition (HI) test (Serial dilution is described in Figure 3)

1. Add 25 µl of PBS to the V bottom 96 wells in column 2 to 12.
2. Add 50 µl of the antibody (diluted with PBS, 1:10) to the well in row A1 to H1.
3. Transfer 25 µl of the antibody from column 1 to 12 for 2 fold serial dilution.
4. Add 25 µl of standardized antigen to all wells of plates.
5. Mix the plates using a laboratory shaker for 10 sec or by manually agitating the plates thoroughly.
6. Cover the plates and incubate at room temperature for 30 min.
7. Add 50 µl of prepared RBC to all wells and incubate at room temperature for 30 min.
8. Observe the hemagglutination inhibition. If sample inhibits hemagglutination, RBCs appear as a dot after falling to the bottom of a well. Record the results by refering to Example of HI titers as shown in Figure 4.

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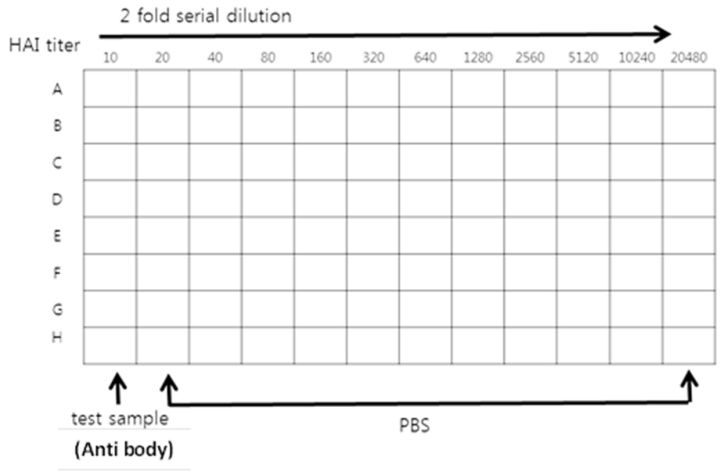
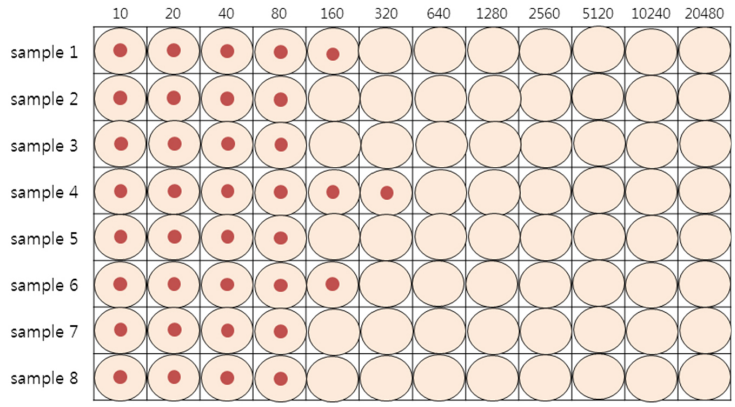


Figure 3. Serial dilution of test sample for HI assay. Add 25 µl of PBS to all wells in column 2 to 12 of a V-bottom 96-well plate and then add 50 µl of test sample (pre diluted 1:10) to the first well of each row. Transfer 25 µl of sample to the next wells using a multi-channel pipette to make a 2-fold dilution. Dilution factor is 10 to 20,480.



HI titer			
sample 1	160	sample 5	80
sample 2	80	sample 6	160
sample 3	80	sample 7	80
sample 4	320	sample 8	80

Figure 4. Example of HI titers. If antibodies bind to the viral particles, the influenza virus is effectively blocked from causing hemagglutination. The HI titer value is the last dilution factor of antibody showing completely inhibited hemagglutination. HI titer of sample 1 to sample 8 is 160, 80, 80, 320, 80, 160, 80 and 80.

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Notes

1. Inactivation of nonspecific inhibitors (in case of using serum)
   a. Reconstitute the RDE with the volume of physiological saline (0.85% NaCl).
   b. Add 3 volumes of RDE to 1 volume of antibody.
   c. Incubate overnight in 37 °C water bath.
   d. Heat in a 56 °C water bath for 30 min to inactivate any remaining RDE.
   e. Cool at room temperature and add 6 volumes of physiological saline. The final dilution of antibody is 1:10.

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