



THE 2015-2016 WHO INFLUENZA REAGENT KIT FOR IDENTIFICATION OF INFLUENZA ISOLATES

Produced and distributed by:

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**In Collaboration with
Influenza Reagent Resource (IRR) and American Type Culture Collection (ATCC)**
<http://www.influenzareagentresource.org>

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I. Influenza Reagent Kit Content

A. HAI reagents for Identification of Field Strains

The hemagglutination inhibition (HAI) reagent collection consists of reference influenza antigens and antisera for identifying influenza A(H1N1)pdm09, A(H3N2), and B (Yamagata and Victoria lineages) isolates from eggs or tissue culture.

Influenza A reagent list for HAI:

- 1 @ 10ml - Influenza A(H1N1)pdm09 Control Antigen (A/California/07/2009 NYMC X-179A), BPL inactivated
Lot 1516H1AG (Cat. No. FR-1401)
- 1 @ 1ml - Influenza A(H1N1)pdm09 Reference Sheep Antiserum
Lot 1516H1AS (Cat. No. FR-1419)
- 1 @ 10ml - Influenza A(H3) Control Antigen (A/Switzerland/9715293/2013), BPL inactivated
Lot 1516H3AG (Cat. No. FR-1402)
- 1 @ 1ml - Influenza A(H3) Reference Goat Antiserum
Lot 1516H3AS (Cat. No. FR-1408)

Influenza B reagent list for HAI:

- 1 @ 10ml - Influenza B Control Antigen, Yamagata lineage (B/Phuket/3073/2013), BPL inactivated
Lot 1516BYAG (Cat. No. FR-1403)
- 1 @ 1ml - Influenza B Reference Goat Antiserum, B/Yamagata lineage
Lot 1516BYAS (Cat. No. FR-1409)
- 1 @ 10ml - Influenza B Control Antigen, Victoria lineage (B/Brisbane/60/2008), BPL inactivated
Lot 1516BVAG (Cat. No. FR-1404)
- 1 @ 1ml - Influenza B Reference Goat Antiserum, B/Victoria lineage
Lot 1516BVAS (Cat. No. FR-1378)

Other reagents for HAI:

- 1 @ 1ml - Influenza Normal Control Goat Serum, Lot 63461731 (Cat. No. FR-1377)
- 1 @ 25ml - Receptor Destroying Enzyme (DENKA), Lot 475043 (Cat. No. FR-50)

Store antigens at 4°C to 8°C and store reconstituted antisera in aliquots at -20°C to -40°C.

****IMPORTANT NOTICES FOR USE OF THE WHO INFLUENZA REAGENT KIT****

These reagents are neither intended, nor evaluated, for use in test procedures other than HAI testing for identification of field strains. **Antisera from the kits should be used for typing or subtyping of influenza viruses, but should not be used for detailed antigenic characterization.** To identify a viral isolate, the results for each isolate should be compared with those for the antigen controls. A virus isolate is identified as a particular type or subtype if the field isolate reacts with one antiserum having an HAI titer **four-fold or greater** than its titers to the other antisera.

Influenza A(H1N1)pdm09 and H3 antigens can also be used for serologic diagnosis by HAI. Influenza B antigens inactivated by beta-propiolactone (BPL) are to be used for identification of field strains while ether extracted influenza B antigens are recommended for serologic diagnosis (see Serologic Diagnosis, page 3).

B. Serologic Diagnosis Reagents

The BPL-inactivated HAI antigens for influenza A viruses are suitable for use in serologic diagnosis of influenza infections while **ether extracted** influenza B antigens should be used for serologic diagnosis of infection.

Reagent list for serologic diagnosis:

- 1 @ 25ml - Receptor Destroying Enzyme (DENKA). Replacement vials limited. (Cat. No. FR-50)
- 1 @ 10ml - Influenza A(H1N1)pdm09 Control Antigen (A/California/07/2009 NYMC X-179A), BPL inactivated
Lot 1516H1AG (Cat. No. FR-1401)
- 1 @ 10ml - Influenza A(H3) Control Antigen (A/Switzerland/9715293/2013), BPL inactivated
Lot 1516H3AG (Cat. No. FR-1402)
- 1 @ 5ml - Influenza B Control Antigen, Yamagata lineage (B/Phuket/3073/2013), [Ether Extracted](#)
Lot 1516BYEX (Cat. No. FR-1405)
- 1 @ 5ml - Influenza B Control Antigen, Victoria lineage (B/Brisbane/60/2008), [Ether Extracted](#)
Lot 1516BVEX (Cat. No. FR-1406)

Store antigens at 4°C to 8°C.

To overcome the low sensitivity of the HAI test for detection of post-infection influenza B antibodies, ether-extracted influenza B antigens have been supplied. Because of the increased sensitivity of ether extracted antigen, it should be used **ONLY IN SEROLOGIC DIAGNOSIS** and should not be used as a reference antigen when performing the HAI tests to identify isolates.

Previous experience comparing ether-extracted to whole-virus antigen in serologic diagnosis of influenza B infections has indicated that the ether-extracted antigen has approximately an 8-fold higher sensitivity than whole-virus antigen in detecting human serum antibody. This increased sensitivity allows more reliable detection of HAI rises following influenza B infection (Kendal and Cate, 1983).

The antigens for serologic diagnosis are provided for laboratories where only small numbers of acute- and convalescent-phase serum pairs are tested. Large serologic surveys or evaluations of vaccine responses require larger amounts of antigen. Antigens can be prepared locally using either recent laboratory-adapted isolates, or using reference live viruses obtained from a WHO Collaborating Center.

II. The Hemagglutination and Hemagglutination Inhibition Test

A. Principles of HA-HAI

Influenza virus contains on its surface many molecules of hemagglutinin (HA), a protein that binds specifically to sialic acid-containing receptors, such as those found on the plasma membrane of red blood cells (RBCs). When RBCs are mixed with influenza virus in the appropriate ratio, the virus bridges the RBCs and changes their normal settling pattern. This is called hemagglutination. The traditional method for identifying influenza field isolates takes advantage of this property. Specific attachment of antibody to antigenic sites on the HA molecule interferes with the binding between the viral HA protein and the receptors on the RBC membrane. This effect inhibits hemagglutination and is the basis for the hemagglutination inhibition (HAI) test.

The HAI test was developed by Hirst (1941) and later modified by Salk (1944). In general the HAI test is performed by mixing the standardized viral antigen with serial dilutions of serum, adding RBC suspension to determine specific binding of antibody to the HA molecule. Today, the test is commonly performed using microtiter plates.

A general protocol is available from WHO at:

http://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en/

B. WHO Kit Reagents Needed for HAI

Influenza HA Antigens. Influenza A(H3), A(H1N1)pdm09 and B antigens consist of infected allantoic fluid inactivated by BPL. The preparations vary depending upon the strains selected for the vaccine and are derived from either a reassortant made with the vaccine strain or a comparable reference wild type virus strain. Influenza B serologic antigens are ether-extracted to increase sensitivity. **Store the liquid antigen at 4°C to 8°C.** HA titers are determined after packaging and noted on label, but may drop upon storage. Test all antigens before initial use.

Influenza HAI Reference Antisera. Hyperimmune antisera were prepared in sheep or goats by multiple intramuscular injections with purified HA from the influenza vaccine strain or a reference strain. These antisera permit identification of influenza type A(H3N2), A(H1N1)pdm09 and type B (Victoria and Yamagata lineages) viruses in human influenza isolates and are also used as positive control antisera in serologic assays. The product volume is listed on the package label. Reconstitute to that volume with distilled water. **Store the lyophilized sera at 4°C to 8°C but reconstituted sera should be stored at -20°C to -40°C (+/- 5°C).**

Receptor Destroying Enzyme (RDE). The RDE supplied is a commercial preparation and should be used according to the protocol in the procedures. This lot of RDE should be used in treatment of the provided reference antisera and can be used in the treatment of human sera for serologic diagnosis. This kit has been tested only with this lot of RDE and has not been verified with any other products.

Field Isolates. Isolation of influenza may be performed in embryonated eggs or tissue culture. The most frequently used and recommended tissue culture is MDCK cells, however, commercial primary monkey kidney tissue culture is used by many laboratories.

Other Materials (not included in the kit):

1. Red blood cells (RBCs) in Alsever's solution. Chicken (see note #1 under Troubleshooting), turkey, human type "O", or guinea pig RBCs may be used.
2. Phosphate buffered saline (PBS), pH 7.2.
 - a. Prepare stock 25 times concentrated (25X) phosphate buffer containing in 100 mL:
2.74 g dibasic sodium phosphate (Na_2HPO_4) and 0.79 g monobasic sodium phosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$).
 - b. To prepare PBS, mix and dissolve in deionized, distilled water, and q.s. to 1 Liter:
40 mL of 25X phosphate buffer and 8.5 g of sodium chloride (NaCl).
 - c. After thorough mixing, check pH = 7.2 ± 0.1 . Adjust pH with 1 N NaOH or 1 N HCl, if necessary.
 - d. Autoclave or filter to sterilize.
 - e. Store opened PBS, pH 7.2 at 4°C to 8°C for no longer than 3 weeks.
3. Physiological saline, 0.85%.
 - a. Prepare a 20X stock solution by dissolving 170 g of NaCl in deionized water q.s. to 1000 mL.

- b. Sterilize by autoclaving at 121°C.
- c. To prepare physiological saline, 0.85% NaCl, add 50 mL 20X stock solution to 950 mL deionized water.
- d. Sterilize by autoclaving at 121°C.
- e. Store opened physiological saline at 4°C to 8°C for no longer than 3 weeks.

4. Microtitration equipment including plates and covers.

C. Protocol for HAI identification of field isolates

The following protocol is recommended for the identification of influenza field strains by HAI. The procedure is written assuming the use of 96 well microplates. V-shaped 96 well microtiter plates are used with avian, guinea pig, or human type O RBCs. U-shaped microtiter plates may be used with guinea pig or human type "O" RBCs. The wells of the microplate are designated A-H on the 3 inch (8 cm) side and 1-12 on the 5 inch (12 cm) side, so that well #1 is designated as A1 (Figure 1). Basically, the HAI test procedure involves the (1) treatment of sera for removal of nonspecific inhibitors, (2) adsorption of the sera for the removal of nonspecific agglutinins, (3) HA titration of control reference antigens and field strains, (4) preparation of standardized antigen dilution containing the proper units, and finally (5) the HAI test for identification of field strains. The HAI test can also be used for serologic diagnosis or surveys for the detection and quantification of specific antibodies in human and animal sera.

I. STANDARDIZATION OF RBCs

If using chicken or turkey RBCs, the final concentration for HA/HAI should be a 0.5% concentration. A higher concentration of 0.75% for guinea pig and human type "O" RBCs improves reading. Although most laboratories have a procedure for standardization of cells, the following procedure is used at the WHO Collaborating Center as an example.

1. RBCs are usually supplied in Alsever's solution. To wash cells, filter a volume (45 mL) through gauze, and centrifuge 1,200 RPM (about 300 x g) for 5 min. (~500 x g for guinea pig red blood cells).
2. Aspirate Alsever's and add 50 mL PBS (pH 7.2 to 7.4) for wash. Swirl gently to mix and centrifuge at 1,200 RPM (about 300 x g) for 5 min to obtain packed cells. Aspirate supernatant.
3. Repeat wash with PBS two to three times, until supernatant is clear.
4. At final wash, centrifuge at 1,200 RPM (about 300 x g) for 10 min. (~500 x g for guinea pig red blood cells).
5. Dilute the packed cells to appropriate concentration based on packed cell volume. The concentration can be checked and adjusted using a hemacytometer.

Note: It has been observed that most current A(H3) isolates fail to agglutinate chicken RBCs. (See Troubleshooting p. 11)

II. TREATMENT OF SERA

A. Removal of serum nonspecific inhibitors

Sera from many animal species contain non-specific inhibitors to hemagglutination which, unless removed, can lead to false-positive results. Different methods can be used to remove non-specific inhibitors, however, treatment with the supplied receptor destroying enzyme (RDE) is recommended for the reference antisera provided in this kit. If it is suspected that the inhibitors have not been completely removed an increased concentration of RDE can be used. If RDE treatment is unsuccessful, alternative methods may be (1) heat and periodate or (2) trypsin, heat and periodate. Use the following procedure with the RDE supplied in this kit.

RDE treatment procedure

1. Reconstitute the RDE with 25 mL physiological saline.
2. Add 3 vol RDE to 1 vol serum (0.3 mL RDE + 0.1 mL serum). Incubate 12-18 hours at 37°C.
3. Heat 56°C for 30 min. Add 6 volumes physiological saline. (Additional 0.6 ml saline to 0.4 mL of RDE and sera)

B. Removal of nonspecific agglutinins

Treated sera may contain substances that will nonspecifically agglutinate RBCs. To determine the presence of non-specific agglutinins, follow the procedure described in Section V for serum controls (adding PBS instead of antigen). Add RBCs and incubate at room temperature until RBCs have settled completely in the cell control. The presence of hemagglutination in the serum wells indicates the presence of nonspecific agglutinins. These agglutinins can be removed with the following procedure.

1. To one volume of packed RBCs, add 20 volumes of RDE-treated serum.

2. Mix thoroughly and incubate at 4°C to 8°C, mixing at intervals to resuspend cells.
3. After 1 hour, centrifuge at 300 x g for 5 minutes.
4. Carefully remove the adsorbed serum without disturbing the packed cells.
5. Repeat serum controls as described in Section V. Adsorb with RBCs until the serum controls are negative.

III. HA TITRATION OF CONTROL REFERENCE ANTIGENS AND FIELD ISOLATES

The HA antigens should be titrated before every HAI test. [Figure 1](#) is a schematic for HA titration.

1. Add 50 µL of PBS (pH7.2) to wells #2 through 10 of each lettered row on a microtiter plate. (Use a V-bottom plate if using avian RBCs; a U-bottom plate may be used for mammalian RBCs.)
2. Add 100 µL of each antigen to the first well of the lettered row which will be diluted.
3. Two or more RBCs control wells can be prepared in any wells at rows 11 and 12 by adding 50 µL of PBS. These wells serve as indicators of a complete settling pattern without antigen.
4. Make serial two-fold dilutions of each antigen, transferring 50 µL from well to well and discarding the final 50 µL after row 10. The 2-fold dilutions will be 1:1 through 1:512.
5. Add 50 µL of RBC suspension to each well on plate. Mix by using a mechanical vibrator or by manually agitating the plates.
6. Incubate the plates at room temperature (22 to 25°C.) for 30 min if using avian cells, but 60 min for guinea pig RBCs to allow complete settling of cells.
7. Record the results and interpret.

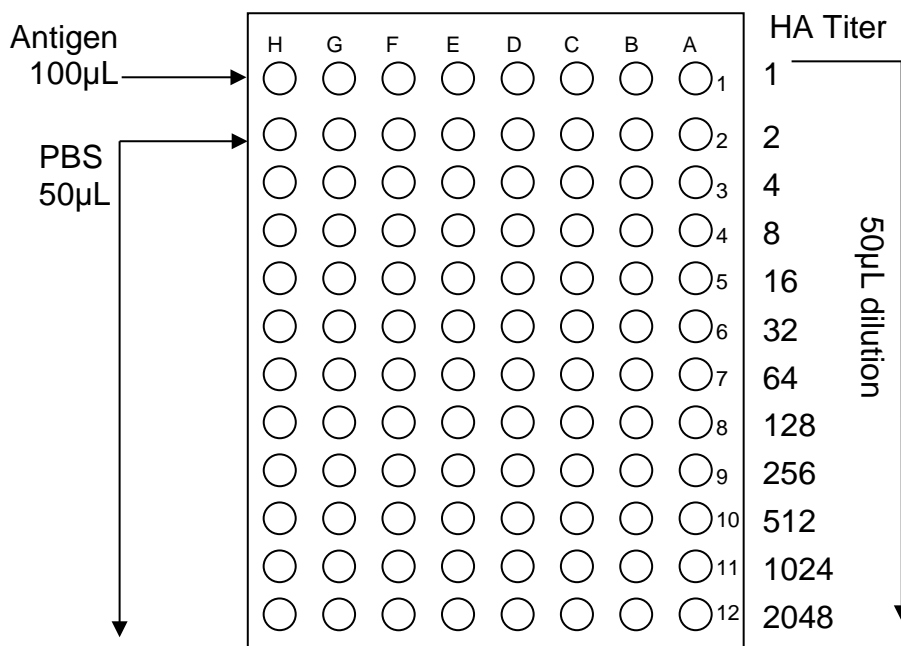
Hemagglutination occurs when the RBCs are in suspension after the RBC control has settled completely. This is usually recorded using a "+" symbol. An "o" symbol is used to record the absence of hemagglutination when a compact button on the bottom of the wells is formed; this button will run if the plate is tilted. This is the case with avian RBCs, however with guinea pig or human type O RBCs, the absence of hemagglutination will appear as a "halo" or circle of settled cells in the bottom of the wells. The RBC control should be completely settled either as a compact button or "halo". The highest dilution of virus that causes complete hemagglutination is considered the HA titration end point. For example, if the last dilution showing complete agglutination is 1:128, then the HA titer is the reciprocal of the dilution which is 128.

IV. PREPARATION OF STANDARDIZED ANTIGEN FOR HAI TEST AND "BACK TITRATION" PROCEDURE

A "unit" of hemagglutination is not a measure of an absolute amount of virus, but is an operational unit dependent on the method used for HA titration. An HA unit is defined as the amount of virus needed to agglutinate an equal volume of a standardized RBC suspension.

1. Determine the volume of standardized antigen needed for the HAI test. For example, 1 mL of antigen will test 5 sera, each of which is diluted in 8 wells, with 25 µL of antigen added to each well (5 sera X 8 wells X 25 µL = 1 mL of standardized antigen). Prepare an additional 1.0 mL additional volume for "back titration" and wastage.
2. The standard for the HAI test is 4 HA units of antigen added to 2-fold serial dilutions of antisera. Since 25 µL of antigen is used in the test, a virus dilution that contains 4 HA units/25 µL or 8 HA units/50 µL is needed. Calculate the antigen dilution by dividing the HA titer, which is based on 50 µL, and dividing by 8. For example, an HA titer of 128 divided by 8 is 16. Mix 1 part of virus with 15 parts PBS to obtain the desired volume of standardized antigen (Ex: add 0.1 mL antigen to 1.5 mL of PBS). Calculate and prepare dilution. Keep a record of the dilution prepared.
3. Perform a "back titration" to verify units by performing a second HA test. Standardized antigens must have an HA titer of 8 HA units/50 µL. This titer will hemagglutinate the first four wells of the back titration plate. If an antigen does not have a titer of 8, it must be adjusted accordingly by adding more antigen to increase units or by diluting with PBS to decrease units. For example, if a virus has a titer of 16 then the test antigen should be diluted twofold. Conversely, if a virus has a titer of 4, 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 test antigen to give a titer of 8. Continue adjusting the concentration of antigen until 4 HA units/ 25 µL (8 units/50 µL) is obtained. **Store the diluted antigen at 4°C to 8°C and use within the same day.**
4. Record results.

Figure 1
Schematic for HA Titrations



I. Dilute antigens and field isolates

| Plate #1 | Plate #2 |
|---|----------------------|
| A - Influenza A(H1N1)pdm09 Control Antigen (A/California/07/2009 X-179A) | A - Field Isolate #1 |
| B - Influenza A(H3) Control Antigen (A/Switzerland/9715293/2013) | B - Field Isolate #2 |
| C - Influenza B Control Antigen (B/Phuket/3073/2013) ¹ | C - Field Isolate #3 |
| D - Influenza B Control Antigen (B/Brisbane/60/2008) ¹ | D - Field Isolate #4 |
| E - Influenza B Ether Extracted Control Antigen (B/Phuket/3073/2013) ² | E - Field Isolate #5 |
| F - Influenza B Ether Extracted Control Antigen (B/Brisbane/60/2008) ² | F - Field Isolate #6 |
| G – Blank | G - Blank |
| H - Cell Control | H - Cell Control |

1. B/Phuket/3073/2013 is a current reference strain for B/Yamagata/16/1988 lineage. B/Brisbane/60/2008 is a current reference strain for B/Victoria/02/1987 lineage.

2. The HA titrations of ether extracted control antigens are performed for use in serologic diagnosis.

II. Add 50 uL standardized RBCs

III. Incubate and record results

V. HEMAGGLUTINATION INHIBITION TEST FOR IDENTIFICATION OF ISOLATES

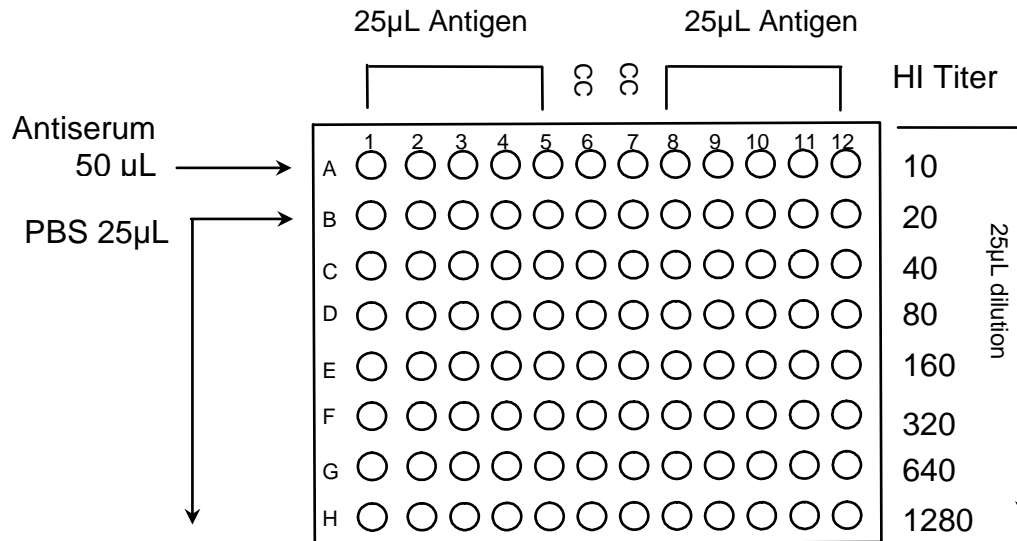
Hemagglutination Inhibition tests for field isolates should be conducted with all of the antisera provided [A(H1N1)pdm09, A(H3), B Yamagata and B Victoria lineage]. At the same time, control antigens provided must be included in the HAI test against each antiserum as shown in Table 1 of Section III. Interpretation of HAI results using the 2015-2016 Kit Reagents

(page 10) for the type/subtype identification is based on the highest HAI titer obtained, which must be at least 4-8 fold or greater than any cross-reaction pattern observed.

At this point, (1) the reference antisera have been treated with RDE and (2) the control reference antigens and field isolate antigens have been standardized for 4 HA units/25 μ L. [Figure 2](#) is a schematic for the HAI test. This scheme can be used for two antigens per plate and a complete set of five reference antisera and a negative control serum. One extra plate is required for 1) serum control in which PBS is added instead of antigen; 2) RBCs control in which only RBCs and PBS are added.

1. Prepare dilutions of RDE treated antisera in the appropriate microtiter plate according to the RBCs being used, by first adding 25 μ L of PBS to wells B through H (B1 - H12) of each numbered column.
2. Using the set of treated sera (6 sera), add 50 μ L of each serum to the first well of the appropriate numbered column. For example, serum #1 should be added to well A1 and well A7; serum #2 to A2 and A10; etc. The starting dilution of the treated serum is 1:10.
3. Prepare serial twofold dilutions of the treated sera by transferring 25 μ L from the first well of numbered columns 1-12 to successive wells. Discard the final 25 μ L after row H.
4. Add 25 μ L of standardized control antigen #1 to all wells of a complete set of diluted treated sera (Ex: A1 - H6). Continue with remaining standardized control antigens and field test antigens.
NOTE: Four HA units are added to the test in 25 μ L because the HA unit calculations were based on a volume of 50 μ L.
5. Add 25 μ L of PBS instead of antigen to the serum control plate.
6. Mix the contents of the plates by shaking on a mechanical vibrator for 10 sec or by agitating the plates manually.
7. Cover the plates and incubate at room temperature (22° to 25°C) for 15 min.
8. Add 50 μ L of standardized RBCs to all wells. Mix as before.
9. Cover the plates and allow the RBCs to settle at room temperature (22° to 25°C) for the appropriate time according to the RBCs being used.
10. Record and interpret the results. Symbols of "+" for HA and "o" for absence of HA or inhibition of HA can be used. The HAI titer is the last dilution of antiserum that completely inhibits HA.

Figure 2
Schematic for HAI Identification of Field Isolates



I. Prepare antisera dilutions.

- 1 - Influenza A(H1N1)pdm09 Reference Sheep Antiserum
- 2- Influenza A(H3) Reference Goat Antiserum
- 3 - Influenza B Reference Goat Antiserum, B/Yamagata lineage
- 4 - Influenza B Reference Goat Antiserum, B/Victoria lineage
- 5 - Negative Control Antiserum
- 6 - Cell Control (50 µL PBS)
- 7 - Cell Control (50 µL PBS)
- 8 - Influenza A(H1N1)pdm09 Reference Sheep Antiserum
- 9 - Influenza A(H3) Reference Goat Antiserum
- 10 - Influenza B Reference Goat Antiserum, B/Yamagata lineage
- 11 - Influenza B Reference Goat Antiserum, B/Victoria lineage
- 12 - Negative Control Antiserum

II. Add 25 µL of standardized control antigens and field test antigens.

Use PBS (25 µL) instead of antigen for serum control.

III. Incubate.

IV. Add 50 µL standardized RBCs.

V. Incubate, record results, and interpret.

III. Interpretation of HAI results using the 2015-2016 WHO kit reagents

To identify a viral isolate, the results for each isolate should be compared with those for the antigen controls. A virus isolate is identified as a particular type or subtype if the field virus reacts with one antiserum giving HAI titer **four-fold or greater** than its titers to the other antisera. As an example, Table 1 provides results from laboratory tests performed by the WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, CDC, Atlanta.

Table 1. Hemagglutination Inhibition Reaction of the 2015-2016 WHO Influenza Reagents

| 2015-2016 WHO KIT REFERENCE ANTIGENS | 2015-2016 WHO KIT REFERENCE ANTISERA | | | | | INTERPRETATION ² |
|---|--------------------------------------|------------|------------|------------|-----------------------|-----------------------------|
| | H3N2 | H1N1pdm09 | B-YAM | B-VIC | NEGATIVE ¹ | |
| FR-1402 INFLUENZA A(H3N2), BPL | 5120 | 40 | 40 | 20 | <10 | A(H3N2) |
| FR-1401 INFLUENZA A(H1N1)pdm09, BPL | 80 | 640 | 40 | 20 | <10 | A(H1N1)pdm09 |
| FR-1403 INFLUENZA B YAMAGATA LINEAGE, BPL | <10 | <10 | 640 | 10 | <10 | B YAMAGATA LINEAGE |
| FR-1404 INFLUENZA B VICTORIA LINEAGE, BPL | 20 | <10 | 40 | 640 | <10 | B VICTORIA LINEAGE |
| TEST ANTIGENS | | | | | | |
| ISOLATE #1 | 5120 | 40 | <10 | <10 | <10 | A(H3N2) |
| ISOLATE #2 | 640 | 10 | <10 | <10 | <10 | A(H3N2) |
| ISOLATE #3 | 320 | 10 | <10 | <10 | <10 | A(H3N2) |
| ISOLATE #4 | 2560 | 20 | 10 | <10 | <10 | A(H3N2) |
| ISOLATE #5 | 80 | <10 | <10 | <10 | <10 | A(H3N2) |
| ISOLATE #6 | <10 | 320 | <10 | <10 | <10 | A(H1N1)pdm09 |
| ISOLATE #7 | <10 | 640 | <10 | <10 | <10 | A(H1N1)pdm09 |
| ISOLATE #8 | <10 | 80 | <10 | <10 | <10 | A(H1N1)pdm09 |
| ISOLATE #9 | <10 | 160 | <10 | <10 | <10 | A(H1N1)pdm09 |
| ISOLATE #10 | <10 | 640 | <10 | <10 | <10 | A(H1N1)pdm09 |
| ISOLATE #11 | <10 | <10 | 160 | <10 | <10 | B YAMAGATA LINEAGE |
| ISOLATE #12 | <10 | <10 | 640 | <10 | <10 | B YAMAGATA LINEAGE |
| ISOLATE #13 | <10 | <10 | 640 | <10 | <10 | B YAMAGATA LINEAGE |
| ISOLATE #14 | <10 | <10 | 640 | <10 | <10 | B YAMAGATA LINEAGE |
| ISOLATE #15 | <10 | <10 | 640 | <10 | <10 | B YAMAGATA LINEAGE |
| ISOLATE #16 | <10 | <10 | <10 | 640 | <10 | B VICTORIA LINEAGE |
| ISOLATE #17 | <10 | <10 | <10 | 640 | <10 | B VICTORIA LINEAGE |
| ISOLATE #18 | <10 | <10 | <10 | 640 | <10 | B VICTORIA LINEAGE |
| ISOLATE #19 | <10 | <10 | <10 | 640 | <10 | B VICTORIA LINEAGE |
| ISOLATE #20 | <10 | <10 | <10 | 320 | <10 | B VICTORIA LINEAGE |

1. The negative is uninfected goat serum. All sera are treated with the WHO Reagent Kit RDE.
2. If cross reactivity between different antisera occurs with some isolates, identification of field strains should be based on 4-fold or greater difference in HAI titer.

Problems in interpretation may arise from the following conditions:

- a. If there is any agglutination in the sera controls, it is due to non-specific agglutinins present in the sera and can be interpreted as false negatives. (See II B. Removal of Nonspecific Agglutinins).
- b. Occasionally isolates are highly sensitive to the non-specific inhibitors present in sera. These are not always completely removed by the RDE treatment. The inhibitors can give false positives and results must be interpreted with care. These isolates will usually give high titers to more than one antiserum present in the test.

A. Troubleshooting

Please contact the WHO Collaborating Center in Atlanta (See page 13) if technical problems arise in the identification of field strains using these reagents.

1. In some cases, guinea pig RBCs or human "O" cells can be more sensitive than avian RBCs for detecting human strains of influenza. **Since the early 1990's, it has been observed that most A(H3) isolates fail to agglutinate chicken RBCs. For this reason, we suggest that you use another species of RBCs, if at all possible.** (See Medeiros et al., 2001: *Virology* 289:74-85). Upon passage in tissue culture most strains will adapt to avian RBC agglutination. This phenomenon has been reported in the literature (see references).
2. Should any antigen react to a significant titer (>20) with more than one reference antiserum, possible explanations may be:
 - a. Use of inadequate titers of virus. Diluted antigens must be titrated immediately before use in the HAI test. This back titration control will verify that the diluted antigen contains the appropriate virus concentration which is four HA units/25 μ L
 - b. Bacterial contamination. Agglutinins of non-influenza origin in contaminated specimens may react nonspecifically with all antisera or may fail to react with any antiserum.
 - c. Incorrect reading of HAI test results. Inhibition should be scored only when hemagglutination activity of virus is completely prevented.
 - d. Artifacts due to anti-host component antibodies. This has been observed at times with influenza B viruses isolated in MDCK cells. Such isolates may be inhibited by normal serum components, and thus react with all sera provided. If this problem is observed, samples of the sera should be absorbed with uninfected MDCK cells. Uninfected sheep serum has been included as a "normal" serum for identifying nonspecific reactions.
 - e. Nonspecific inhibitors. The presence of naturally occurring nonspecific inhibitors in sera from animal species requires treatment before performing the HAI test to avoid false-positive results. More sensitive viruses will react to these inherent nonspecific inhibitors at a titer that can easily lead to misinterpretation. Should this problem occur with the influenza reagents provided, please contact the WHO Collaborating Center in Atlanta for alternative methods of treatment such as heat-inactivation at 64 °C for 30 minutes after RDE treatment, periodate and trypsin, or heat and periodate.

The majority of lab contaminants occur when influenza virus control reagents (positive control or research viruses, laboratory adapted strains and viruses distributed for laboratory certification) are distributed during the winter months and were processed at the same time as diagnostic specimens. The best safeguard is **not** to work with live laboratory strains and clinical materials submitted for isolation at the same time.

WARNING: ANY TIME A LABORATORY ADAPTED STRAIN IS PROPAGATED FOR DIAGNOSTIC OR RESEARCH PURPOSES, A RISK OF CONTAMINATING DIAGNOSTIC SPECIMENS OCCURS. (When it is necessary to grow live virus strains, precautions must be taken to reduce the likelihood of contaminating diagnostic specimens.)

B. References

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IV. Global Influenza Surveillance and Response System (GISRS)

Global influenza virological surveillance has been conducted through WHO's Global Influenza Surveillance and Response System (GISRS) for over half a century.

- WHO GISRS monitors the evolution of influenza viruses and provides recommendations in areas including laboratory diagnostics, vaccines, antiviral susceptibility and risk assessment.
- WHO GISRS also serves as a global alert mechanism for the emergence of influenza viruses with pandemic potential.

Established in 1952, the network currently comprises six WHO Collaborating Centers, four Essential Regulatory Laboratories and 142 institutions in 112 WHO Member States, which are recognized by WHO as National Influenza Centers, in addition to ad hoc groups established to address specific emerging issues.

The objectives of GISRS include:

- Isolation and identification of currently circulating influenza viruses followed by complete antigenic analysis for the purpose of yearly vaccine strain recommendations
- Early detection of influenza outbreaks and epidemics and the identification of associated epidemiological factors (ie, age, behavioral patterns, and contact with animals)
- Identification of high risk areas and population groups
- Early detection of novel influenza viruses with pandemic potential
- Monitoring of antiviral drug resistance

Worldwide influenza activity and antigenic characterization of circulating strains are made available as The Weekly Epidemiological Record (WER) on the WHO website www.who.int/wer/en. Surveillance and laboratory data from the United States can be accessed online at <http://www.cdc.gov/flu/weekly/fluactivitysurv.htm> and periodic updates regarding influenza are published in the MMWR Weekly Report at www.cdc.gov/mmwr/.

V. WHO Collaborating Centers

Inside GISRS, WHO Collaborating Centers (WHO CCs) and Essential Regulatory Laboratories are crucial elements of influenza surveillance and vaccine response.

The designated WHO Collaborating Centers are listed below. Participants of GISRS are encouraged to communicate with one of these centers on the level of influenza activity and provide influenza isolates for detailed antigenic analysis on a regular basis. The WHO Influenza Reagent Kit is provided so that influenza viruses may be accurately identified with standardized reagents.

WHO COLLABORATING CENTER FOR THE SURVEILLANCE, EPIDEMIOLOGY AND CONTROL OF INFLUENZA

Centers for Disease Control and Prevention
Influenza Division, G-16
1600 Clifton Road, NE
Atlanta, Georgia 30333 USA

Dr. Dr. Jacqueline Katz, Director
Dr. Xiyan Xu, Deputy Director (contact)

Telephone Number: 404-639-1657

WHO COLLABORATING CENTRE FOR REFERENCE AND RESEARCH ON INFLUENZA

Crick Worldwide Influenza Centre
The Francis Crick Institute, The Ridgeway, Mill Hill
LONDON NW7 1AA,
UNITED KINGDOM

Dr. John McCauley, Director
Dr. Rod Daniels, Deputy Director (contact)

Telephone Number: 44-208-816-2152

WHO COLLABORATING CENTER FOR REFERENCE AND RESEARCH ON INFLUENZA

Victorian Infectious Diseases Reference Laboratory
10 Wreckyn Street
North Melbourne 3051
Victoria, Australia

Dr. Ian Barr, Director (Acting)
Dr. Aeron Hurt, Deputy Director (contact)

Telephone Number: 61-3-9342-3944

WHO COLLABORATING CENTER FOR REFERENCE AND RESEARCH ON INFLUENZA

National Institute of Infectious Diseases
Department of Virology III
4-7-1 Gakuen, Musashi-Murayama-shi
Tokyo 208-0011, Japan

Dr. Takato Odagiri, Director
Dr. Shinji Watanabe, Deputy Director (contact)

Telephone Number: 81-42-561-0771

WHO COLLABORATING CENTER FOR REFERENCE AND RESEARCH ON INFLUENZA

Chinese National Influenza Center
National Institute for Viral Disease Control and Prevention
155# Changbai road, Changping district,
Beijing, P.R. China 102206 (New site)

Dr. Yuelong Shu, Director
Dr. Wang Dayan, Deputy Director (contact)

Telephone Number: 86-10-58900859

WHO COLLABORATING CENTER FOR STUDIES ON THE ECOLOGY OF INFLUENZA IN ANIMALS

Virology Division
Department of Infectious Disease
MS 330, Room E-9056
St. Jude Children's Research Hospital
262 Danny Thomas Place
Memphis, TN 38105-3678

Dr. Richard Webby, Director

Telephone Number: (901) 595-3014

VI. Specimen Submission

The reagents supplied in this kit are intended to provide the initial identification of field strains as influenza A(H3N2), influenza A(H1N1)pdm09, or influenza B. Due to the great heterogeneity of prevalent strains within each type and/or subtype, these reagents cannot be expected to antigenically characterize a strain. Therefore, representative field strains should be sent to an appropriate contract laboratory (U.S.) or WHO Collaborating Center for detailed antigenic analysis. Listed below are guidelines for selecting the strains to be forwarded. The WHO Center has the capability to analyze virus strains from all regions of the world and, as a result, can establish the characterization of strains prevalent worldwide.

A. Guidelines

1. **In countries where influenza is seasonal:**
(Sample submission guidelines have been sent to the Public Health labs in the U.S.)
 - a. **Any pre-season isolates and the first isolates (5-10) of the season.** These isolates can provide important information regarding circulation of strains as compared to the previous season, the match between vaccine and circulating strains, and information necessary for the vaccine formulation for the next year.
 - b. During the coming influenza season, submit one ***package containing up to thirty representative isolates along with their matching clinical materials every two months from your most recent cases, collected approximately within 4 weeks of shipment***
 - c. In order to support the biannual WHO vaccine composition recommendation, you are strongly encouraged to make one shipment in early **January** and one in middle **August**, at the latest, to ensure the best value from your viruses allowing inclusion of the analysis results in the very next consultation for WHO recommendation on vaccine composition.
2. **In countries where influenza occurs throughout the year: representative isolates should be sent at regular intervals but no more than ten collected in any two week period.**
3. **Isolates obtained during outbreaks.** Outbreaks may occur in immunized populations or in unimmunized populations where the attack rate is high. Submit no more than 3 viruses from one outbreak since viruses from an outbreak are usually very similar.
4. **Isolates that cannot be typed or subtyped by HAI testing with kit reagents.** Because it is important to rapidly detect new influenza variants, isolates which are negative when tested with kit antisera should be identified by IFA or real-time RT/PCR (if the technique is available in your lab). ***Any unsubtypeable influenza A isolates should be forwarded to a WHO Collaborating Center within 24 hours.***
5. **Isolates from persons receiving an antiviral agent or from their contacts that become ill.** The increased use of antiviral agents for treatment and prophylaxis of influenza in some areas of the world has created the potential for the emergence and spread of antiviral resistant viruses which must be monitored.
6. **Isolates from cases of suspected animal-to-human transmission of influenza viruses.** These are needed to monitor the characteristics of the viruses and to examine the potential for spread.

Laboratory and epidemiologic information about isolates should be submitted using the enclosed “The Influenza Specimen Submission” form and sent with the shipment of isolates. This form can be downloaded from the website: <http://www.influenzareagentresource.com/>

Recent database improvements allow us to log in your specimens with greater accuracy through the use of the electronic submission form.

PLEASE ATTEMPT HAI TESTING SO THAT THE HA SUBTYPE OF INFLUENZA A ISOLATES CAN BE DESIGNATED ON THIS FORM.

B. Shipping Information

1. Shipping Isolates. Isolates should be shipped to one of the five WHO Collaborating Centers listed on page 13-14. A completed “Influenza **Specimen Submission**” form for laboratory and epidemiological information should accompany the isolates. It is important to fill in the form as completely as possible, paying particular attention to **collection date**, **Passage History**, **Type/Subtype**, **level of activity**, and **laboratory information**.
2. International Shipments. When possible, it is especially important for the WHO Collaborating Center to be notified of the arrival time of the shipment by E-mail, fax, letter, or telephone. Import permits for shipments to the WHO Collaborating Center, Atlanta have been provided by e-mail in April 2015. Should additional permits be needed, please notify the contact person listed below.

C. Contact Persons for Receiving Isolates:

WHO Influenza Collaborating Center at Centers for Disease Control and Prevention in Atlanta, GA. USA
Dr. Xiyun Xu. Telephone: 404-639-1657 Fax: 404-639-0080

WHO Influenza Collaborating Center at National Institute for Medical Research, Mill Hill, London
Dr. Rod Daniels. Telephone: 44-208-816-2152 Fax: 44-208-906-4477

WHO Influenza Collaborating Center at Commonwealth Serum Laboratories, Victoria, Australia
Dr. Ian Barr. Telephone: 61-3-9342-3944 Fax: 61-3-9342-3939

WHO Influenza Collaborating Center at National Institute of Infectious Diseases, Tokyo, Japan
Dr. Takato Odagiri. Telephone: 81-42-561-0771 Fax: 81-42-561-0812

WHO Influenza Collaborating Center at National Institute for Viral Disease Control and Prevention
Dr. Wang Dayan. Telephone: 86-10-58900859

D. Request for Additional Reagents: www.influenzareagentresource.org

E. Request for Permits for Shipping to U.S.:

WHO Collaborating Center at Centers for Disease Control and Prevention in Atlanta, GA. USA –
Dr. Xiyun Xu (404-639-1657 or xxx1@cdc.gov) or **Ms. Teresa Wallis** (404-639-1687 or TWallis@cdc.gov).

F. Assistance in solving laboratory problems concerning the use of the reagents and questions on laboratory methodology:

WHO Collaborating Center at Centers for Disease Control and Prevention in Atlanta, GA. USA - **Xiyun Xu** (404-639-1657 or xxx1@cdc.gov)