



## **THE 2017-2018 WHO INFLUENZA REAGENT KIT FOR IDENTIFICATION OF INFLUENZA ISOLATES**

Produced and distributed by:

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**In Collaboration with  
International Reagent Resource (IRR) and American Type Culture Collection (ATCC)  
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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/Michigan/45/2015 NYMC X-275), BPL inactivated  
Lot 1718H1AG (Cat. No. FR-1565)
- 1 @ 1mL - Influenza A(H1N1)pdm09 Reference Goat Antiserum  
Lot 1718H1AS (Cat. No. FR-1561)
- 1 @ 10mL - Influenza A(H3) Control Antigen (A/Hong Kong/4801/2014), BPL inactivated  
Lot 1718H3AG (Cat. No. FR-1566)
- 1 @ 1mL - Influenza A(H3) Reference Goat Antiserum  
Lot 1718H3AS (Cat. No. FR-1562)

#### **Influenza B reagent list for HAI:**

- 1 @ 10mL - Influenza B Control Antigen, Yamagata lineage (B/Phuket/3073/2013), BPL inactivated  
Lot 1718BYAG (Cat. No. FR-1569)
- 1 @ 1mL - Influenza B Reference Goat Antiserum, B/Yamagata lineage  
Lot 1718BYAS (Cat. No. FR-1564)
- 1 @ 10mL - Influenza B Control Antigen, Victoria lineage (B/Brisbane/60/2008), BPL inactivated  
Lot 1718BVAG (Cat. No. FR-1567)
- 1 @ 1mL - Influenza B Reference Ferret/Goat Antiserum, B/Victoria lineage  
Lot 1718BVAS (Cat. No. FR-1582)

#### **Other reagents for HAI:**

- 1 @ 1mL - Influenza Normal Control Goat Serum, Lot 64242011 (Cat. No. FR-1377)
- 1 @ 25mL - Receptor Destroying Enzyme (DENKA), Lot 527071 (Cat. No. FR-50)

***Store antigens at 2°C to 8°C and store reconstituted antisera in aliquots at -30°C±10°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 A(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/Michigan/45/2015 NYMC X-275), BPL inactivated  
Lot 1718H1AG (Cat. No. FR-1565)
- 1 @ 10mL - Influenza A(H3) Control Antigen (A/Hong Kong/4801/2014), BPL inactivated  
Lot 1718H3AG (Cat. No. FR-1566)
- 1 @ 5mL - Influenza B Control Antigen, Yamagata lineage (B/Phuket/3073/2013), [Ether Extracted](#)  
Lot 1718BYEX (Cat. No. FR-1570)
- 1 @ 5mL - Influenza B Control Antigen, Victoria lineage (B/Brisbane/60/2008), [Ether Extracted](#)  
Lot 1718BVEX (Cat. No. FR-1568)

***Store antigens at 2°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.

A previous study 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). HA is 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). The HAI test is performed by mixing the standardized viral antigen with serial dilutions of serum and then adding the 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/](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 2°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 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 2°C to 8°C and reconstituted sera at -30°C±10°C.**

**Receptor Destroying Enzyme (RDE).** The RDE supplied is a commercial preparation and should be used according to the suppliers' protocol. 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. **Store the lyophilized RDE at 2°C to 8°C and reconstituted RDE at -30°C±10°C.**

**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 are 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 100ml:  
2.74g dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) and 0.79g 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:  
40ml of 25X phosphate buffer and 8.5 g of sodium chloride (NaCl)
  - c. After thorough mixing, check pH =  $7.2 \pm 0.1$ . 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 2°C to 8°C for no longer than 3 weeks.
3. Physiological saline, 0.85%.
  - a. Prepare a 20X stock solution by dissolving 170g of NaCl in deionized water q.s. to 1000mL.

- b. Sterilize by autoclaving at 121°C.
- c. To prepare physiological saline, 0.85% NaCl, add 50mL 20X stock solution to 950mL deionized water.
- d. Sterilize by autoclaving at 121°C.
- e. Store opened physiological saline at 2°C to 8°C for no longer than 3 weeks.

4. Microtitration equipment including plates (u or v-bottom) 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 (8cm) side and 1-12 on the 5 inch (12cm) side, so that well #1 is designated as A1 ([Figure 1](#)). The HAI test procedure involves the treatment of sera for removal of nonspecific inhibitors, adsorption of the sera for the removal of nonspecific agglutinins, HA titration of control reference antigens and field strains, preparation of standardized antigen, and the addition of standardized antigen to diluted sera for characterization 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 used at WHO Collaborating Centers is included as an example.

1. RBCs are usually supplied in Alsever's Solution. To wash cells, filter a volume (45mL) through gauze, and centrifuge 1,200RPM (about 300 x g) for 5min. (~500 x g for guinea pig red blood cells).
2. Aspirate Alsever's and add 50mL PBS (pH 7.2 to 7.4) for wash. Swirl gently to mix and centrifuge at 1,200RPM (about 300 x g) for 5min 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 1,200RPM (~300 x g) for 10min. (~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 (a 0.5% concentration is equivalent to  $4 \times 10^7$  cells/mL; a 0.75% concentration is equivalent to  $6 \times 10^7$  cells/mL).

**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 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 see Troubleshooting p. 11. Use the following procedure with the RDE supplied in this kit.

##### RDE treatment procedure

1. Reconstitute the RDE with 25mL physiological saline.
2. Add 3 vol RDE to 1 vol serum (0.3mL RDE + 0.1mL serum). Incubate 12-18 hours at 37°C.
3. Heat 56°C for 30min. Add 6 volumes physiological saline. (Additional 0.6mL saline to 0.4mL. 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). 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 2°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. Repeat adsorption 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 50µL of PBS (pH 7.2) 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 100µL 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 50µL of PBS. These wells serve as indicators of a complete settling pattern without antigen.
4. Make serial two-fold dilutions of each antigen, transferring 50µL from well to well and discarding the final 50µL after row 10. The 2-fold dilutions will be 1:1 through 1:512.
5. Add 50µL 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 30min if using avian cells or 60min for guinea pig RBCs to allow complete settling of cells.
7. Record the results and interpret.

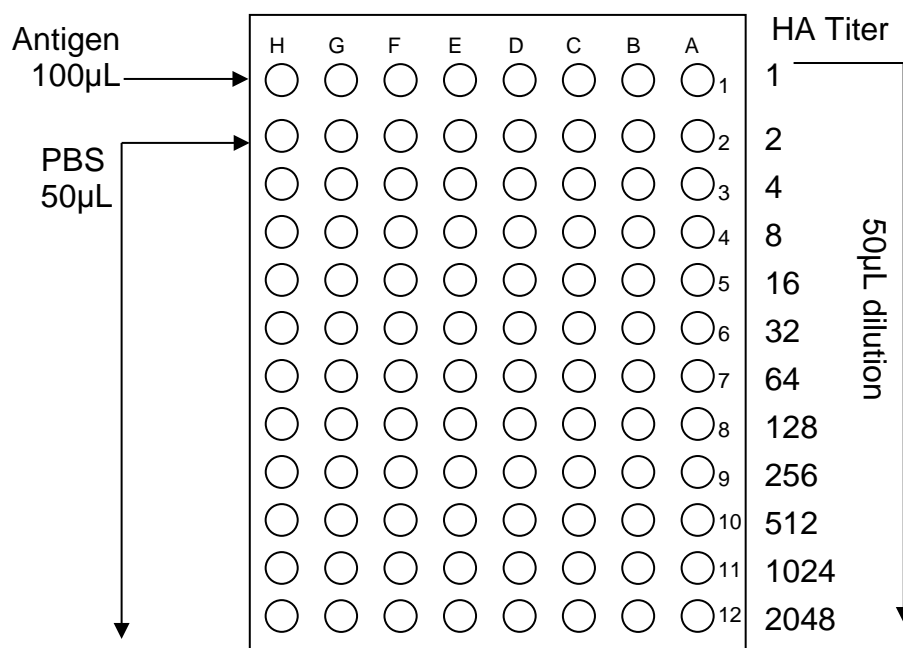
Hemagglutination occurs when the RBCs are in suspension after the RBC control has settled completely. This is usually recorded using a "+" symbol. An "o" symbol is used to record the absence of hemagglutination when a compact button on the bottom of the wells is formed; this button will run if the plate 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 (in U-bottomed plates). 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, 1mL of antigen will test 5 sera, each of which is diluted in 8 wells, with 25µL of antigen added to each well (5 sera X 8 wells X 25µL = 1 mL of standardized antigen). Prepare an additional 1.0mL 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 25µL of antigen is used in the test, a virus dilution that contains 4 HA units/25µL or 8 HA units/50µL is needed. Calculate the antigen dilution by dividing the HA titer, which is based on 50µL, 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 0.1mL antigen to 1.5mL 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/50µL. 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/ 25µL (8 units/50µL) is obtained. **Store the diluted antigen at 2°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/Michigan/45/2015 NYMC X-275)	A - Field Isolate #1
B - Influenza A(H3) Control Antigen (A/Hong Kong/4801/2014)	B - Field Isolate #2
C - Influenza B Control Antigen, YAM (B/Phuket/3073/2013) <sup>1</sup>	C - Field Isolate #3
D - Influenza B Control Antigen, VIC (B/Brisbane/60/2008) <sup>1</sup>	D - Field Isolate #4
E - Influenza B Ether Extracted Control Antigen (B/Phuket/3073/2013) <sup>2</sup>	E - Field Isolate #5
F - Influenza B Ether Extracted Control Antigen (B/Brisbane/60/2008) <sup>2</sup>	F - Field Isolate #6
G - Blank	G - Blank
H - Cell Control	H - Cell Control

<sup>1</sup>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 Victoria/02/1987 lineage.

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

**II. Add 50µL 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



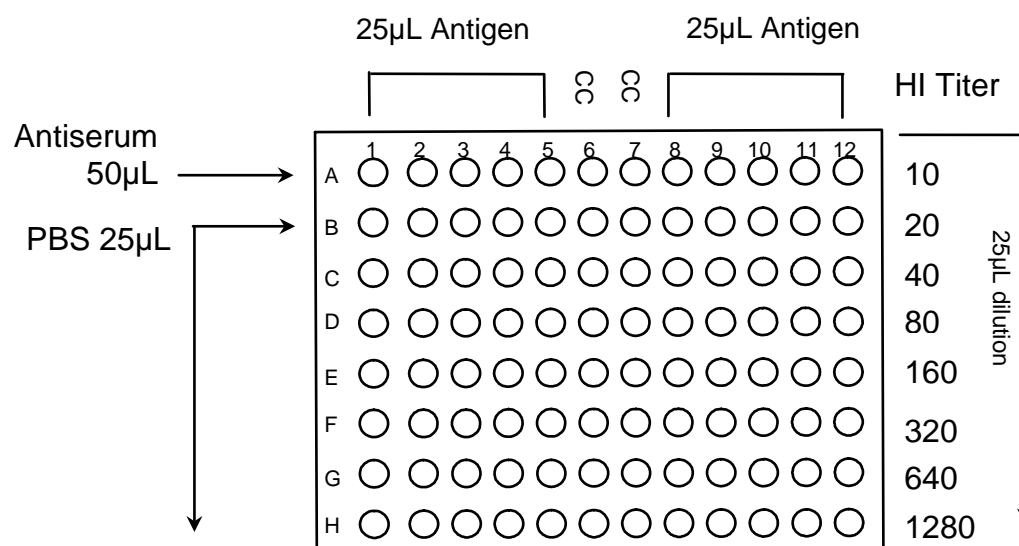
against each antiserum as shown in Table 1 of Section III. Interpretation of HAI results using the 2017-2018 Kit Reagents (page 10) for the type/subtype identification is based on the highest HAI titer obtained, which must be at least 4-8 fold or greater than any cross-reaction pattern observed.

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 $\mu$ 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 $\mu$ 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 $\mu$ 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 $\mu$ L from the first well of numbered columns 1-12 to successive wells. Discard the final 25 $\mu$ L after row H.
4. Add 25 $\mu$ 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 $\mu$ L because the HA unit calculations were based on a volume of 50 $\mu$ L.
5. Add 25 $\mu$ 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 10sec or by agitating the plates manually.
7. Cover the plates and incubate at room temperature (22° to 25°C) for 15min.
8. Add 50 $\mu$ L of standardized RBCs to all wells. Mix as before.
9. Cover the plates and incubate at room temperature (22° to 25°C.) for 30min if using avian cells or 60min for guinea pig RBCs to allow complete settling of cells.
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 Goat Antiserum
- 2- Influenza A(H3) Reference Goat Antiserum
- 3 - Influenza B Reference Goat Antiserum, B/Yamagata lineage
- 4 - Influenza B Reference Ferret/Goat Antiserum, B/Victoria lineage
- 5 - Negative Control Antiserum
- 6 - Cell Control (50µL PBS)
- 7 - Cell Control (50µL PBS)
- 8 - Influenza A(H1N1)pdm09 Reference Goat Antiserum
- 9 - Influenza A(H3) Reference Goat Antiserum
- 10 - Influenza B Reference Goat Antiserum, B/Yamagata lineage
- 11 - Influenza B Reference Ferret/Goat Antiserum, B/Victoria lineage
- 12 - Negative Control Antiserum

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

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

**III. Incubate.**

**IV. Add 50µL standardized RBCs.**

**V. Incubate, record results, and interpret.**

### III. Interpretation of HAI results using the 2017-2018 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 2017-2018 WHO Influenza Reagents**

2017-2018 WHO KIT REFERENCE ANTIGENS	H3N2	H1N1pdm09	B-YAM	B-VIC	NEGATIVE <sup>1</sup>	INTERPRETATION <sup>2</sup>
FR-1566 INFLUENZA A(H3) CONTROL AG	<b>5120</b>	<10	40	10	<10	A(H3N2)
FR-1565 INFLUENZA A(H1N1)pdm09 CONTROL AG	80	<b>1280</b>	40	<10	<10	A(H1N1)pdm09
FR-1569 INFLUENZA B CONTROL AG, YAMAGATA LINEAGE	<10	10	<b>320</b>	20	<10	B/YAMAGATA LINEAGE
FR-1567 INFLUENZA B CONTROL AG, VICTORIA LINEAGE	80	<10	40	<b>320</b>	20	B/VICTORIA LINEAGE
<b>TEST ANTIGENS</b>						
ISOLATE #1	1280	<10	<10	<10	<10	A(H3N2)
ISOLATE #2	640	<10	<10	<10	<10	A(H3N2)
ISOLATE #3	1280	<10	<10	<10	<10	A(H3N2)
ISOLATE #4	640	<10	<10	<10	<10	A(H3N2)
ISOLATE #5	2560	<10	<10	<10	<10	A(H3N2)
ISOLATE #6	<10	2560	<10	<10	<10	A(H1N1)pdm09
ISOLATE #7	<10	1280	<10	<10	<10	A(H1N1)pdm09
ISOLATE #8	<10	1280	<10	<10	<10	A(H1N1)pdm09
ISOLATE #9	<10	1280	<10	<10	<10	A(H1N1)pdm09
ISOLATE #10	<10	5120	<10	<10	<10	A(H1N1)pdm09
ISOLATE #11	<10	<10	320	20	<10	B/YAMAGATA LINEAGE
ISOLATE #12	<10	<10	320	20	<10	B/YAMAGATA LINEAGE
ISOLATE #13	<10	<10	320	20	10	B/YAMAGATA LINEAGE
ISOLATE #14	<10	<10	160	10	<10	B/YAMAGATA LINEAGE
ISOLATE #15	<10	80	320	20	20	B/YAMAGATA LINEAGE
ISOLATE #16	<10	<10	<10	320	<10	B/VICTORIA LINEAGE
ISOLATE #17	<10	<10	<10	320	10	B/VICTORIA LINEAGE
ISOLATE #18	<10	<10	10	320	20	B/VICTORIA LINEAGE
ISOLATE #19	<10	<10	<10	320	10	B/VICTORIA LINEAGE
ISOLATE #20	<10	<10	<10	320	<10	B/VICTORIA LINEAGE

<sup>1</sup> The negative is uninfected goat serum. All sera are treated with the WHO Reagent Kit RDE.

<sup>2</sup> 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 Section 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/25µL
  - 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. 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 143 institutions in 113 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](http://www.who.int/wer/en). Surveillance and laboratory data from the United States can be accessed online at <http://www.cdc.gov/flu/weekly/fluactivitiesurv.htm> and periodic updates regarding influenza are published in the MMWR Weekly Report at [www.cdc.gov/mmwr/](http://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 30329 USA

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. Kanta Subbarao, Director  
Dr. Ian Barr, 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. Wang Dayan, Director (Acting)

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:**  
*(The U.S. Public Health labs should follow the CDC Sample submission guidelines which have been sent via APHL)*
  - a. **Any pre-season specimens.** These specimens 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 shipments each containing thirty to forty specimens representing all circulating influenza A (H1N1pdm09 and H3N2) and influenza B (B/Victoria and B/Yamagata lineages) viruses. Please also consider representatives by types/subtypes, age groups, geographic regions, both ambulatory and hospitalized cases, cases receiving antiviral therapy (e.g. oseltamivir), and deaths/outbreaks. Submit the most recent specimens collected (within last 1-2 months).
  - c. In order to support the biannual WHO vaccine composition recommendation, laboratories are strongly encouraged to make one shipment which could be received by CDC in early **January** and another one for receipt by CDC in middle **August**, at the latest, to ensure the best value from collected 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:** [www.internationalreagentresource.org](http://www.internationalreagentresource.org)

Recent database improvements allow specimen logging with greater accuracy through the use of the electronic submission form.

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



## **B. Shipping Information:**

1. Shipping Clinical materials or virus isolates should be shipped to one of the five WHO Collaborating Centers listed on pages 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 in advance of the expected arrival times of shipments by e-mail, fax, letter, or telephone.

Import permits for shipments to the WHO Collaborating Center, Atlanta were provided by e-mail in April 2017. 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. Xiyan 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:**

International Reagent Resource [www.internationalreagentresource.org](http://www.internationalreagentresource.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. Xiyan Xu** (404-639-1657 or xxx1@cdc.gov) or **Ms. Wendy Sessions** (404-639-3211 or gra6@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 - **Xiyan Xu** (404-639-1657 or xxx1@cdc.gov), **Thomas Rowe** (404-639-4007 or txr2@cdc.gov) or **Wendy Sessions** (404-639-3211 or gra6@cdc.gov).