# REAGENTS FOR THE TYPING OF HUMAN INFLUENZA ISOLATES 2017

This product was developed by the Victorian Infectious Diseases Reference Laboratory (VIDRL) in its capacity as a WHO Collaborating Centre for Reference and Research on Influenza, with material provided to VIDRL as part of the Global Influenza Surveillance and Response System.


**Prepared by the WHO Collaborating Centre for Reference and Research on Influenza**
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# Reagents for the Typing of Human Influenza Isolates for 2017

This product was developed by the WHO Collaborating Centre for Reference and Research on Influenza (VIDRL), with material provided to it as part of the WHO Global Influenza Surveillance and Response System (GISRS).

This kit contains non-infectious reagents for the laboratory-based typing and subtyping of human influenza viruses and is provided free of charge to your institute by the WHO Collaborating Centre for Reference and Research on Influenza at VIDRL, Melbourne, Australia.

Disclaimer:
The material in this kit is provided for laboratory use only. The kit is intended to be used for differentiating between different types and subtypes of human influenza virus. We do not guarantee the suitability of this kit for any other purpose and take no responsibility for results obtained through its use other than that described in the enclosed instructions. We also cannot guarantee against the loss of activity whilst in transit or subsequent storage.

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# I. The WHO Influenza Network

The WHO Global Influenza Surveillance Network was established in 1952.

The two major objectives of the WHO program at its outset were:

1. To study the origins of epidemic and pandemic influenza strains; and
2. To provide new virus strains quickly for the production of vaccines in the face of outbreaks.

Since then the WHO Global Influenza Surveillance Response System (GISRS) has grown to involve 143 National Influenza Centres in 113 countries and five WHO Collaborating Centres for human influenza located in London, Atlanta, Melbourne, Tokyo and Beijing.

The data and virus strains derived through this program are crucial for the regular updating of influenza vaccines and for monitoring the effectiveness of antiviral drugs, which together provide important measures against this ever-changing virus.

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**Internet Sites:**

The WHO Collaborating Centre, Melbourne, has a website at http://www.influenzacentre.org, which contains information, reports and useful links. Copies or any updates to the kit contents/performance will be listed here.

WHO reports on influenza activity worldwide can be obtained on the Internet:

- World Health Organisation website: http://www.who.int/topics/influenza/en/
- FluNet: http://www.who.int/influenza/gisrs_laboratory/flunet/en/ (FluNet is an interactive site which allows WHO National Influenza Centres and other surveillance groups to enter their data).

## II. Summary of recent influenza epidemiology

Type A and Type B influenza viruses are responsible for epidemic influenza in humans and are distinguishable based on their distinct internal nucleoprotein and matrix protein antigens.

The influenza A viruses can be further differentiated into 16 subtypes based on the major surface antigen, the haemagglutinin (H). However a second surface antigen, the neuraminidase (N), also exists in 9 different forms. All of these influenza A haemagglutinin and neuraminidase types can be found in aquatic birds (which appear to be the ancestral host), usually as harmless infections, while only certain subtypes have been found to establish transmissible infections in humans and other mammalian species. A number of subtypes are capable of infecting domestic poultry and two of these, H5 and H7, can cause serious disease with high mortality.

Viruses circulating in the human population display frequent antigenic changes, referred to as 'antigenic drift', through mutation in their surface antigens. Infrequently, a new subtype of influenza A enters the human population and if there is efficient human-to-human transmission a pandemic may result; this is referred to as 'antigenic shift'.

Surveillance for human influenza is undertaken in order that vaccines can be regularly updated to contain the most relevant virus strains and to provide early warning of emerging pandemic strains.

In March – April 2009 a new influenza A virus emerged from swine that was fully transmissible in man. This led to a world-wide pandemic with this virus now referred to as pandemic H1N1 (or A(H1N1)pdm09) and these viruses replaced the previously circulating seasonal influenza A(H1N1) viruses.

A(H3N2) viruses continue to circulate and have recently been the predominant influenza A subtype in many countries in the last few years compared to A(H1N1)pdm09. Influenza B viruses have continued to co-circulate with influenza A viruses.

There are two lineages of influenza B, B/Victoria/2/87-like and B/Yamagata/16/88-like viruses and both lineages have been detected in most parts of the world. B/Victoria-like viruses have predominated in recent years in some countries while B/Yamagata viruses have predominated in other countries. Further details on the current epidemiology of influenza are available on the WHO website at:
http://www.who.int/influenza/surveillance_monitoring/updates/latest_update_GIP_surveillance/en/

Since late 2003 highly-pathogenic influenza A(H5N1) infections in poultry have been widespread in Asia and a small number of human infections have been observed. For latest data on human infection numbers see http://www.who.int/influenza/human_animal_interface/en/

To date there has been evidence of only limited human to human transmission. WHO has issued a series of guideline documents regarding diagnosis of influenza A(H5), handling samples from infected persons etc., which can be accessed at:
http://www.who.int/csr/resources/publications/surveillance/WHO_CDS_EPR_ARO_2006_1/en/ and
http://www.who.int/influenza/gisrs_laboratory/molecular_diagnosis/en/

Similarly since March 2013 A(H7N9) has been detected in poultry and in a number of humans in China especially in 2016-17. Infection with H7N9 in humans can cause severe disease and death. To date there has been no evidence of sustained human to human transmission, with most cases having prior exposure to poultry or birds. See the WHO website for more information at:
http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/index.html

**It is important that National Influenza Centres and other surveillance groups immediately forward any human influenza A viruses that prove difficult to subtype, or that have unusual properties, to one of the WHO Collaborating Centres for Influenza.**

**Circulating Strains:**

**Type A(H3N2):** During the past year, strains antigenically related to A/Hong Kong/4801/2014 have circulated widely. These viruses are clearly genetically distinguishable and also show some antigenic differences from the previous vaccine virus A/Switzerland/9715293/2013, although these can be difficult to distinguish using the HI assay and may require other assays such as plaque reduction or virus neutralisation assays. Some A(H3) viruses have also been difficult to grow and may give low or no haemagglutination titres when assaying cell grown viruses using chicken or turkey red blood cells (RBC) or even human “O” RBC or guinea pig RBC. Addition of other agents such as oseltamivir carboxylate into the HI assay may also reduce HA titres.

This makes it very difficult to use this kit to type some current H3N2 viruses as the HI assay relies on the ability of virus to agglutinate RBC. If the virus does not agglutinate human O/Guinea Pig RBC then alternative methods (e.g. real time PCR) should be used to subtype the virus. The A(H3) antiserum in the 2017 kit will react with all of these recently circulating A(H3) viruses (and viruses should be type-able provided they agglutinate the RBC being used in the assay).

**Type A(H1) (Seasonal) viruses:** Seasonal A(H1N1) viruses have not been detected anywhere in the world since 2011, hence these reagents are **NOT** included in the 2017 kit.

**Type A(H1N1)pdm09 or Swine-like viruses:** The A(H1N1)pdm09 viruses currently circulating in Australia and many other countries are now most closely related to A/Michigan/45/2015. The A(H1N1)pdm09 antiserum in the 2017 kit **will not** react with the earlier seasonal A(H1N1) viruses.

**Type B viruses:** Over recent years, two antigenically and genetically distinct lineages, B/Victoria/2/87 (represented by B/Brisbane/60/2008) and B/Yamagata/16/88 (represented by B/Phuket/3073/2013), have co-circulated. Both lineages from the B/Victoria lineage and the B/Yamagata lineage have been recently detected in many countries. It is likely that both lineages will persist with proportions varying from year to year and from country to country, therefore reagents to identify both lineages are included in the 2017 kit. The antisera to both of these lineages have been tested against recent viruses from each of the lineages and have been found to perform satisfactorily. These reagents therefore will allow one to determine the lineage of the circulating B viruses using influenza virus isolates.

Note that some recent B/Victoria lineage viruses (MDCK-grown) that have been detected in the USA, such as B/New York/52/2016, which have 2 deletions in the HA gene (K162 and N163) are likely to give very low titres using the reagents supplied in this kit, (see table on page 14).

## III. The haemagglutination-inhibition (HAI) test for isolate identification

### A. Background

Influenza virus contains on its surface two glycoprotein antigens, haemagglutinin (HA) and neuraminidase (NA). Haemagglutinin binds specifically to sialic acid-containing receptors on the surface of susceptible cells and facilitates the infection process. Similar receptors are present on the red blood cell (RBC) plasma membrane. When RBCs are mixed with influenza virus in the appropriate ratio, the virus bridges the RBCs, resulting in agglutination of the cells (haemagglutination) and a change in their settling behaviour. Antibodies specific to the viral haemagglutinin interfere with this reaction and this is the basis of the haemagglutination-inhibition (HAI) test which allows the identification of virus isolates and the differentiation of the variant strains which frequently appear.

The HAI test was originally developed in the 1940s and has now been adapted to microtitre plates. It is the test most frequently used for the antigenic analysis of influenza isolates since it is simple to perform and requires only a small amount of unconcentrated antigen. However, it is not without practical problems.

Sera from many animal species contain non-specific inhibitors which, unless removed, can lead to false-positive results and confuse the correct identification of new virus isolates. Such non-specific inhibitors may be inactivated by a variety of methods; treatment with *V. cholerae* neuraminidase (receptor destroying enzyme [RDE]) is the most common.

**Note that the sera provided in this kit have been treated with RDE to remove non-specific inhibitors.**

In the test a standardised amount of virus HA antigen is mixed with serial dilutions of reference antisera which have been treated to remove non-specific inhibitors. Following incubation a red blood cell suspension is added and the test is interpreted by the patterns formed when the RBCs have settled.

## B. Haemagglutination-inhibition assay reagents

Influenza surveillance and vaccine formulation are based principally on antigenic analysis of the major viral surface antigen, HA, using the haemagglutinin-inhibition test. The reagents in this kit are designed to allow laboratories to identify influenza A virus isolates as belonging to the A(H1N1)pdm09 or A(H3) subtype or to one of the two circulating lineages of influenza B. Because antigenic variation is ongoing within the circulating viruses, the reagents are prepared to be broadly reactive and will not necessarily detect subtle changes in antigenicity. Therefore representative viruses that react with the reagents are of interest for more detailed antigenic and genetic analysis by the WHO Collaborating Centres. Viruses that have been identified as influenza A or B (by IFA or real time PCR for example) and react poorly or not at all with the reference antisera provided may represent important antigenic variants or novel sub-types. National and other reference centres are encouraged to submit such isolates to a WHO Collaborating Centre without delay and to retain a frozen stored portion of the original clinical sample which will permit isolation of a potential vaccine strain under conditions acceptable to regulatory authorities.

**Influenza HA Antigens:** The influenza A(H1N1)pdm09, A(H3N2) and B antigens provided in the kit consist of egg-grown viruses which have been concentrated, partially purified and inactivated by treatment with beta-propiolactone. They are suspended in phosphate buffered saline and contain 0.1% sodium azide as preservative. The antigens should be stored according to the recommendations below.

1x10mL each of the reference antigens are included in the 2017 kit:

**HA Titre of Supplied Antigens with RBC Type**

| **Antigens** | **Turkey** | **Fowl** | **Guinea Pig** | **Human "O"** |
|---|---|---|---|---|
| A/Hong Kong/4801/2014 A(H3N2)-like | 800 | 400 | 800 | 1200 |
| A/Michigan/45/2015 A(H1N1)pdm09-like | 800 | 400 | 500 | 250 |
| B/Phuket/3073/2013-like Yamagata-lineage | 500 | 500 | 125 | 250 |
| B/Brisbane/60/2008-like Victoria-lineage | 500 | 500 | 125 | 125 |

*The antigens are relatively stable at 2-8°C and contain 0.1% sodium azide as preservative.* ***They should not be frozen****. Some loss of titre may be experienced on storage; however, this can generally be reversed by mild sonication.*

## RDE Treated Influenza Antisera

**Immune Sera:** Immune sera have been prepared by inoculating rabbits with concentrated purified influenza virus and then RDE treated to remove inhibitors.

1x10mL of the four reference influenza antisera are included in the 2017 kit:

| **<u>Antisera</u>** | **Homologous HAI Titre**** |
|---|---|
| Influenza A/Hong Kong/4801/2014 A(H3N2)-like | 512 |
| Influenza A/Michigan/45/202015 A(H1N1)pdm09 –like | 128 |
| Influenza B/Phuket/3073/2013-like | 256 |
| Influenza B/Brisbane/60/2008-like | 128 |

*The RDE treated antisera contain 0.1% sodium azide as preservative and are stable for many months at 2-8°C.* ***They should not be repeatedly frozen and thawed*** *as this may generate non-specific inhibitors. If you wish to store the serum frozen it is recommended that it be dispensed into smaller aliquots.*

******Titre is specified as the further dilution of the treated serum as provided at which an HAI endpoint is usually found using 4 HA of homologous antigen.

## HAI Reactions of the 2017 Reagents

| | | Reference Antisera | | | |
|---|---|---|---|---|---|
| Reference Antigens | | 1 A/HK/ 4801 | 2 A/Mich/ 45 | 3 B/Phuk/ 3073 | 4 B/Bris/60 |
| **1** | **A/Hong Kong/4801/2014 -like** | 512 | <1 | <1 | <1 |
| **2** | **A/Michigan/45/2015-like** | <1 | 128 | <1 | <1 |
| **3** | **B/Phuket/3073/2013-like** | <1 | <1 | 256 | 2 |
| **4** | **B/Brisbane/60/2008-like** | <1 | <1 | 32 | 128 |
| | | | | | |
| **Field Isolates (cell grown)** | | | | | |
| A/Brisbane/273/2016* | | 64 | <1 | <1 | <1 |
| A/South Australia/40/2016* | | 16 | <1 | <1 | <1 |
| A/Tasmania/32/2016 | | <1 | 256 | <1 | <1 |
| A/New Caledonia/33/2016 | | <1 | 64 | <1 | <1 |
| B/Sri Lanka/31/2016 | | <1 | <1 | 128 | <1 |
| B/Fiji/13/2016 | | <1 | <1 | 32 | <1 |
| B/Brisbane/27/2016 | | <1 | <1 | <1 | 32 |
| B/Townsville/8/2016 | | <1 | <1 | <1 | 32 |
| B/New York/52/2016 | | <1 | <1 | <1 | 2 |

<1 = no inhibition observed in the presence of undiluted treated serum
* = isolates assayed using Guinea Pig RBC, all others using Turkey RBC

## Virus Isolates

Influenza viruses may be isolated in embryonated chicken eggs or in cell culture. The most commonly used continuous cell line for influenza isolation are MDCK and MDCK-SIAT-1 cells; some laboratories use primary cultures of monkey kidney cells. Viruses grown in the egg allantoic cavity readily agglutinate RBC from a wide variety of species and fowl cells are usually used because nucleated avian RBC settle more quickly than mammalian RBC and the settling patterns provide a much clearer distinction between agglutinated and unagglutinated cells. Viruses grown in cell culture often agglutinate fowl cells poorly but agglutinate guinea pig, human group “O” or turkey RBC to an acceptable titre. Because they are nucleated, turkey cells have the same advantages as fowl cells and are used in preference if available and virus can agglutinate the turkey RBC.

Note that the WHO Collaborating Centre, Melbourne, continue to see in recent cell culture influenza A(H3N2) isolates with low or undetectable HA titres with fowl and turkey RBCs, which have improved titres with guinea pig or human group “O” RBCs. Hence guinea pig or human group “O” RBCs should be used wherever possible for A(H3N2) cell grown viruses. See page 8 for more information.

## Other Materials/Reagents Required (not supplied)

RBC suspension
Phosphate buffered saline pH 7.2 (e.g. Dulbecco)
Micro-titration equipment

## C. Methods for haemagglutination-inhibition test

The following procedure is based on the use of the HAI test in 96-well microtitre plates. For avian RBC either V-shaped or U-shaped plates are acceptable (V-shaped preferred); for mammalian RBC U-shaped plates should be used.

### Summary

1. Standardisation of RBC
2. Treatment of Sera for Inactivation of Non-Specific Inhibitors
3. HA titration of Reference Antigens and Field Isolates
4. Preparation of Standard Antigen for HI Test
5. Haemagglutination-Inhibition Test for Identification of Isolates
6. Interpretation of Haemagglutination Patterns

### 1. Standardisation of RBC

Fowl and turkey cells are prepared at a final 1% concentration for use in the test as described below where 25μl of cell suspension is added per well. Some procedures use 50μl of 0.5% cell suspension. Where mammalian RBCs are used slightly higher cell concentrations may be used to make reading easier.

(a) Blood for RBC preparation is generally collected into Alsever's solution or into lithium heparin.

(b) Transfer the blood to 10mL centrifuge tubes and centrifuge at 600xg for 10 minutes.

(c) Aspirate the supernatant and buffy coat from the surface of the packed cells. Add PBS and resuspend the packed cells by gentle inversion. Centrifuge at 600xg for five minutes.

(d) Repeat this procedure at least twice more or until the buffy coat is no longer visible.
(e) Resuspend packed cells in PBS to an appropriate concentration based on the packed cell volume.
(f) Check and adjust cell concentration using a suitable method such as haematocrit tubes.

## 2. Treatment of Sera for Inactivation of Non-Specific Inhibitors

The antisera contained within this kit have been RDE treated. No further treatment is required.

## 3. HA titration of Reference Antigens and Field Isolates

(a) Dispense 25µl of PBS pH 7.2 into wells 2 through 12 of each lettered row on a microtitre plate.
(b) Dispense 50µl of each reference and each test antigen into the first well of the lettered row which will be diluted. Ensure the well has been labelled to identify the antigen under test. Include a RBC control well containing 25µl PBS on each plate.
(c) Make serial two-fold dilutions of each antigen by transferring 25µl from well to well with mixing across the plate.
(c) Add 25µl of RBC suspension into each well including the controls. Gently tap to mix. Allow plates to stand on a vibration-free surface at room temperature (approx 22°C) until the control RBC have settled (usually around 30 min for avian cells, 60 min for mammalian cells). Read and record agglutination patterns.
(e) Interpretation of HA Patterns (see Section 6 for diagram of haemagglutination patterns):

The end point of the titrations is the highest dilution of the virus which causes complete agglutination and this represents one haemagglutination unit. The titre of the virus is expressed as the reciprocal of the endpoint dilution keeping in mind the first well is neat and each subsequent well is a two-fold dilution. For example, if the last dilution showing complete agglutination is 1:160, then the HA titre is the reciprocal of the dilution which is 160.

### 4. Preparation of Standard Antigen for HI Test

Control reference antigens and field isolate antigens must be standardised to 4 HA units/25µl for use in the HAI test. Adjust the concentration of the antigen until 4 HA units/25µl is obtained checking the antigen level after every adjustment. Store the diluted antigen at $4^{o}$C and use on the day of preparation.

### 5. Haemagglutination-Inhibition Test for Identification of Isolates

(a) Add 50µl of each treated antiserum to the appropriate first well of the lettered row i.e. Reference Serum A into well A1, B into B1 and so on.

(b) Add 25µl of PBS pH 7.2 into wells 2 through 12 of each lettered row on a microtitre plate. Leave the last row as a RBC control.

(d) Serially dilute the sera across the plate from wells 1 through to 11, transferring 25µl from well to well.

(d) Dispense 25µl of antigen containing 4 HA units to all wells of the plate designated for that antigen. Do not add antigen to the control well. Add 25µl of PBS to RBC control wells. Mix contents by gentle tapping. Leave plates to incubate at room temperature for 45 minutes.

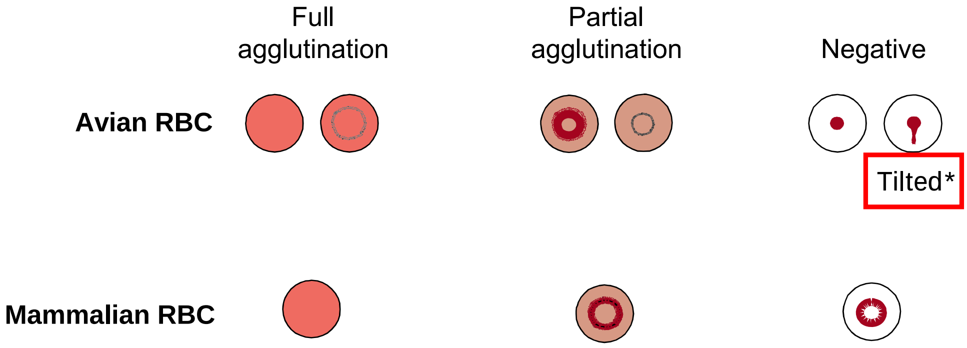
(e) Add 25µl of suspended 1% RBC to all wells. Mix by gentle tapping.

(f) Cover plates and allow the RBC to settle at room temperature until cells in the RBC control form a compact negative pattern (usually around 30 minutes). Read and record agglutination patterns.

(g) Interpretation of results:
The titre of a serum is the highest dilution of that serum which completely inhibits the agglutination of erythrocytes by the virus i.e. gives a negative haemagglutination pattern. (See diagram of haemagglutination patterns below).

## 6. Interpretation of Haemagglutination Patterns

**Note that determination of totally unagglutinated (a true negative pattern) for avian cells is assisted by gently tilting the plates to an angle of approximately 60$^{o}$ upon which unagglutinated cells should stream into a characteristic tear-drop pattern (see diagram*).**



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