INTENDED USE

Human Anti-influenza Virus (H5N1) Hemagglutinin IgG ELISA Kit is an immunoassay suitable for quantifying IgG antibody activity specific for H5N1 hemagglutinin (HA) in serum, plasma or other qualified biological samples from vaccinated, immunized and/or infected hosts.

This immunoassay is suitable for:

- Determining **immune status** relative to non-immune controls;
- Assessing efficacy of **vaccines**, including dosage, adjuvantcy, route of immunization and timing;
- Qualifying and standardizing vaccine batches & protocols.

The kit contains recombinant protein and no virus or proteins from live or killed virus. The assay is for research use only (RUO) and is not intended nor validated for diagnosing disease.

GENERAL INFORMATION

Influenza A (Flu A) viruses are -ssRNA viruses of the family *Orthonyoviridae*; subtypes cause disease in a variety of hosts, including humans, birds, swine, equine and canine. Influenza A virus subtype **H5N1**, also known as A(H5N1) or simply H5N1, is a subtype of the influenza A virus. A bird-adapted strain of H5N1, called **HPAI A(H5N1)**, is the highly pathogenic causative agent of H5N1 flu, commonly known as avian influenza ("**bird flu**"). It is enzootic (maintained in the population) in many bird populations, especially in Southeast Asia. A bird-adapted strain of H5N1, HPAI A(H5N1), is spreading globally after first appearing in Asia. It is epizootic (an epidemic in nonhumans) and panzootic, killing tens of millions of birds and spurring the culling of hundreds of millions of others to stem its spread. Many references to "bird flu" and H5N1 in the popular media refer to this strain.

Eleven outbreaks of H5N1 were reported worldwide in June 2008 in five countries (China, Egypt, Indonesia, Pakistan and Vietnam) compared to 65 outbreaks in June 2006 and 55 in June 2007. In July 2013 the WHO announced a total of 630 confirmed human cases which resulted in the deaths of 375 people since 2003.

Several **H5N1 vaccines** have been developed and approved, and stockpiled by a number of countries, including the United States (in its National Stockpile), Britain, France, Canada, and Australia, for use in an emergency but the continual mutation of H5N1 may renders them of limited use. There is no highly effective treatment for H5N1 flu, but oseltamivir (Roche, Tamiflu), is somewhat effective in inhibiting the influenza virus replication. Three H5N1 vaccines for humans have been licensed as of June 2008 (Sanofi Pasteur's, GSK Prepandrix and CSL Limited's vaccine Panvax.

PRINCIPLE OF THE TEST

The Anti-H5N1 IgA/IgG/IgM ELISA kits are based on the binding of antibodies in samples to the purified H5N1 HA antigen immobilized on the microwells. Bound antibody is detected by anti-IgG/IgA or IgM-HRP conjugate (species specific). After a washing step, chromogenic substrate (TMB) is added and color (blue) developed, which is directly proportional to the amount of antibody present in the sample. Stop Solution is added to terminate the reaction, and Absorbance is then measured using an ELISA reader at 450nm. The presence of antibody (IgA/IgG/IgM) in samples is determined relative to anti-Flu A HA Calibrators and Controls.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8^{O}C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at 4^{o}C for long term and ambient temp. for short term.
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample/Conjugate Diluent** and store at 2-8^{o}C until the kit lot expires or is used up.
Anti-Human IgG-HRP Conjugate Concentrate (100x) Part: H-HuG.2a11, 0.15ml	Peroxidase conjugated anti-Human IgG in buffer with detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample/Conjugate Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8^{o} C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Flu A H5N1 HA Coated Strip Plate	920301	8-well strips (12)	Coated with H5N1 HA protein, and post-coated with stabilizers.
Anti-Flu A H5N1 Calibrators			
1 U/ml 2.5 U/ml 5 U/ml 10 U/ml	920302B 920302C 920302D 920302E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-Flu A HA; in buffer with antimicrobial.
Anti-Flu A H5N1 Positive Control	920302-PC	0.65 ml	Anti-H5N1 HA diluted in buffer with protein, detergents and antimicrobial. [Value range on label]
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Human IgG HRP Concentrate.
- Stock bottle to store diluted Wash Solution; 0.2 to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength and ELISA plate washer

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Caution: Human serum and other bodily fluids may contain infectious material. Always wear gloves when handling human samples, including the standards and controls (which have been tested non-reactive for HbsAg and Anti-HIV), and dispose of these samples and containers as biohazard waste.

Antibody Stability & Dilution

Initial dilution of serum into **Working Sample Diluent** (WSD) is recommended to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for years, stored refrigerated or frozen. Further dilution into **Low NSB Sample Diluent** (LNSD), which provides the lowest assay background, should be at least 5 times the initial dilution and performed the same week as the assay.

Example: Initial (1/5): **10**ul serum + **40**ul WSD [or 0.1ml + 0.4ml]
Further (1/50): **10**ul initial (1/5) + **90**ul LNSD (1/50)

Assay Design

Review Interpretation of Results (p5-7) before proceeding:

- Select the proper sample dilutions accounting for expected potency of positives and minimizing non-specific binding (NSB) and other matrix effects; for example, net signal for non-immune samples should be lower than the **1 U/ml Calibrator**. This is usually 1:100 or greater dilution for Human serum with normal levels of IgG, IgM and IgA.
- Run the Anti-Flu A H5N1 Positive Control; the value range is on the label.
- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy, and is used for net OD calculations, if required. Blank OD should be <0.3.
- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **10 U/ml** should give a high signal (>1.5 OD); **1 U/ml** should give a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5).

Plate Set-up

Bring all reagents to room temperature (18-30^{o} C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8 Calibrator wells and 2 wells for each sample control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation [100ul – 60 min; 4 washes]

- Add 100ul of calibrators, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-Human IgG HRP to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

3. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

4. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

5. Absorbance Reading

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website: http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

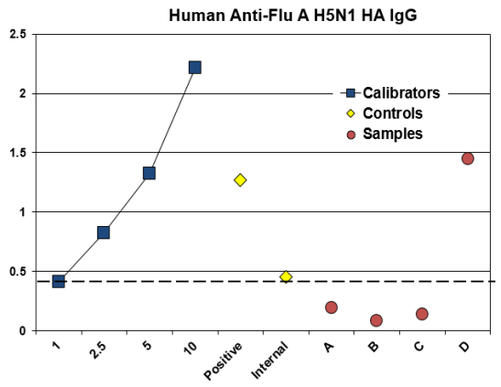
INTERPRETATION OF RESULTS

Method A. Antibody Activity Threshold Index

Compare Samples to **1 U/ml Calibrator** or **Internal Control**

= Positive/Negative Cut-off.

Example:



Results

The **sensitivity** of the assay to detect anti-H5N1 IgG, from either natural infection or vaccination, is controlled so that the **1 U/ml Calibrator** represents a threshold OD for most true positives in human serum diluted to 1:100 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of an anti- H5N1 antibody, derived from HA immunization, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.
1 U/ml: a 'Cut-off' line has been drawn to indicate a threshold distinguishing between **Positive/Negative**. This is not a clear-cut threshold, rather a low OD area that could represent either low positives or high background negatives.

Positive Control – serum showing reactivity to H5N1 HA; the value range is on the label. This Control may be used to gauge precision and to normalize between-assay variation.

Internal Control – a true positive from an immune Human that represents the investigator's experience in distinguishing low positive from negative samples (not in kit). This should be run in each assay to supplement the 1 U/ml Calibrator for Positive/Negative discrimination purposes.

Samples A,B,C,D – 3 samples (A, B, C) are negative: below the threshold; 1 sample (D) is positive: clearly above the threshold.

The **1 U/ml** Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative (see p6):

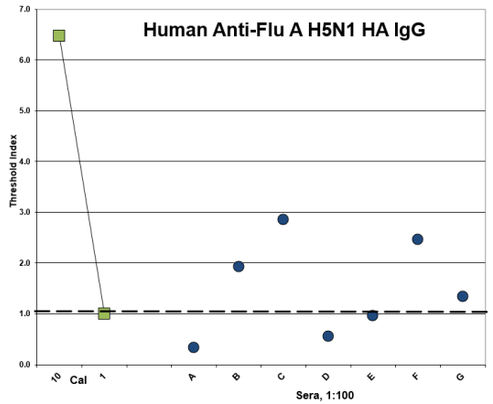
- Divide each Sample net OD by the 1 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

Example:

Human Serum IgG

A panel of human sera of unknown history was tested for anti-H5N1 HA IgG (1:100 dilution in Low NSB Sample Diluent). **Threshold Index** was calculated using the **1** U/ml **Cal**.



Results

Anti- H5N1 HA IgG: two sera (**A,D**) were negative at 1:100 (lower than 1.0 index); one was borderline (**E**); four were positive (**B,C,F,G**: above the 1.0 index) .

Notes:

1. **Positives** may be due to prior encounter with the virus or from influenza immunization.
2. The **sensitivity** of the assay may be adjusted by changing the sample dilutions: a) increase dilution (e.g., 1/500) to lower the signals of borderline positives to negative; b) decrease dilution (e.g., 1/50) to convert borderline samples to positive. With the latter, the values of negatives may increase, so an alternative threshold should be considered using known negatives to develop a **Positive Index (**see below) or use an **Internal Control** (Page 5).
3. Other biological specimens, such as eggs, may be used for antibody determination. Sample dilutions and positive/negative thresholds should be determined using specimens from non-immune or pre-immune populations.

B. Positive Index

Experimental sample values may be expressed relative to the values of Control or Non-immune samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the net OD mean + 2 SD of the Control/Non-immune samples = **Positive Index**.
2. Divide each sample net OD by the Positive Index. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

A sample value would be **Positive** if significantly above the value of the pre-immune serum sample or a suitably determined non-immune panel or pool of samples, tested at the same sample dilution.

This calculation also **quantifies** the positive Antibody Activity level, assigning a higher value to samples with higher Antibody Activity, and vice versa.

Method C. Titers from Sample Dilution Curves

The titer of elevated antibody activity calculated from a dilution curve of each sample is recommended as the most accurate quantitative method. Best precision can be obtained using the following guidelines:

1. Use an OD value Index in the mid-range of the assay (2.0 – 0.5 OD); this provides the best sensitivity and reproducibility for comparing experimental groups and replicates. An arbitrary 1.0 OD is commonly used.
2. Prepare serial dilutions of each sample to provide a series that will produce signals higher and lower than the selected index. With accurate diluting, duplicates may not be required if at least 4 dilutions are run per sample.
3. A 5-fold dilution scheme is useful to efficiently cover a wide range which produces ODs both above and below 1.0 OD. The dilution scheme can be tightened to 3-fold or 2-fold for more precise comparative data.
4. The Positive and Sensitivity Control values can be used to normalize inter-assay values.

Calculations

1. On a log scale of inverse of Sample Dilution as the x-axis, plot the OD values of the two dilutions of each positive sample having ODs above and below the OD value of the Index (arbitrary or selected Calibrator).
2. From a point-to-point line drawn between the two sample ODs, read the dilution value (x-axis) corresponding to the OD of the selected Index

= IgG Antibody Activity Units

PRODUCT SPECIFICATIONS

Specificity

Recombinant, purified, full length H5N1 hemagglutinin [Influenza A virus (A/chicken/India/NIV33491/06(H5N1))] is used as antigen. It is highly conserved (99%) in Asian, Russian, African, Middle East, Europe, and Korean H5N1 strains.

The Anti-Human IgG HRP conjugate reacts specifically with human IgG class antibodies that bind to H5N1 HA antigen. IgA, IgM and IgE antibody would not be measured above background signals.

Assay Sensitivity

The H5N1 HA antigen coating level, HRP conjugate concentration, and Low NSB Sample Diluent are optimized to differentiate anti-H5N1 HA IgG from background (non-antibody) signal with human serum samples diluted 1:100.

Instruction Manual No. M-920-080-H5G

Human Anti-Influenza A Virus (H5N1) HA IgG ELISA Kit

ELISA KIT # 920-080-H5G

For Quantitation of Anti-H5N1 Hemagglutinin IgG in Human Serum or Plasma

For research use only, not for diagnostic or therapeutic use.

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ELISA Kit Components	Amount	Part
Flu A H5N1 HA Coated Strip Plate	8-well strips (12)	920301
Anti- Flu A H5N1 Positive Control	0.65 ml	920302PC
Anti- Flu A H5N1 Calibrator 1 U/ml	0.65 ml	920302B
Anti- Flu A H5N1 Calibrator 2.5 U/ml	0.65 ml	920302C
Anti- Flu A H5N1 Calibrator 5 U/ml	0.65 ml	920302D
Anti- Flu A H5N1 Calibrator 10 U/ml	0.65 ml	920302E
Anti-Human IgG HRP Conjugate (100X)	0.15 ml	H-HuG.2a11
Sample Diluent (20x)	10 ml	SD20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	920-080-H5G