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User's Manual

Human H5N1 IgG ELISA Kit

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RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

Human H5N1 IgG ELISA kit measures IgG antibody is serum or plasma samples. The kit employs purified recombinant H5N1 protein to specifically detect antibodies to influenza subtype H5N1. The kit is particularly suited to assess the H5N1 antibody levels in vaccinated and non-vaccinated samples or to determine the efficacy of new vaccines or formations. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

General Description

Influenza A virus subtype H5N1, also known as A(H5N1) or simply H5N1, is a subtype of the influenza A virus which can cause illness in humans and many other animal species. A bird-adapted strain of H5N1, called HPAI A(H5N1) for highly pathogenic avian influenza virus of type A of subtype H5N1, is the highly pathogenic causative agent of H5N1 flu, commonly known as avian influenza ("bird flu"). It is enzootic in many bird populations, especially in Southeast Asia. One strain of HPAI A(H5N1) is spreading globally after first appearing in Asia. It is epizootic (an epidemic in nonhumans) and panzootic (affecting animals of many species, especially over a wide area), killing tens of millions of birds and spurring the culling of hundreds of millions of others to stem its spread.

The influenza infection is an acute feverish virus infection, which principally leads to an illness of the respiratory tract and appears as an epidemic or pandemic. The infection mostly results from a droplet infection. The virus spreads from the mucous membrane of the upper respiratory to the whole bronchial tract. There the virus and its toxin can lead to a serious inflammation of the bronchial mucosa and a damage of the vessels. After incubation time of 1 to 3 days the symptoms appear suddenly: Followed by a fast increase of temperature, often accompanied by shivering, the catarrhal leading symptom appears, which contribute to the clinical course beside painful dry cough, tracheitis, laryngitis and frequently a rhinitis and conjunctivitis. The Influenza viruses form a virus group with principally similar morphological, chemical and biological features. The types A, B and C were defined, from which many other variants are known. The distinction of the types will be possible by the different antigenicity of their nucleoproteins, which are coated by a matrix protein with type-specific antigenicity, too. However, both internal antigens are of less importance for the immunity. The essential antigens are the hemagglutinin and the neuraminidase. Both are surface antigens and subject to a permanent change of their antigenicity, which is called drift or shift. The appearance of permanent new Influenza epidemics and pandemics are particularly facilitated by an antigen variability, because the new drift or shift variants infect a population which is only partly immune or in an extreme case completely susceptible to the disease. The determination of the Influenza type (A, B, and C) gives both the clinician and epidemiologist important indications for further actions.

Research has shown that a highly contagious strain of H5N1, one that might allow airborne transmission between mammals, can be reached in only a few mutations, raising concerns about a pandemic and bioterrorism. Several H5N1 vaccines have been developed and approved, and stockpiled by a number of countries, including the United States and other countries for use in an emergency.

Principles of Testing

Human H5N1 IgG ELISA Kit is based on the principle of the enzyme immunoassay (EIA). Influenza antigens

are bound on the surface of the microtiter strips. Diluted patient serum or ready-to-use standards are pipetted into the wells of the microtiter plate. A binding between the IgG antibodies of the serum and the immobilized Influenza A takes place. After an incubation step, the plate is rinsed with wash solution, in order to remove unbound material. Bound antibodies are detected with anti-human-IgG HRP conjugate. Aftera further washing step, the substrate (TMB) solution is added. The color development is terminated by the addition of a stop solution, which changes the color from blue to yellow. The color is measured using an ELISA reader at 450 nm. The concentration of the IgG antibodies is directly proportional to the intensity of the color. Results are obtained by comparing the A450 of the samples with the supplied calibrators and positive calibrators.

Reagents And Materials Provided

1. Influenza A H5N1 protein antigen coated strip plate, (8 x 12 strip or 96 wells) 1 plate
2. Influenza A IgG Calibrator A (1 U/ml) 0.65 ml 1 vial
3. Influenza A IgG Calibrator B (2.5 U/ml) 0.65 ml 1 vial
4. Influenza A IgG Calibrator C (5 U/ml) 0.65 ml 1 vial
5. Influenza A IgG Calibrator D (10 U/ml) 0.65 ml 1 vial
6. Influenza A IgG Positive Control 0.65 ml 1 vial
7. Anti-Human IgG-HRP Conjugate (100×) 0.15 ml 1 bottle
8. Sample Diluent (20×) 10 ml 1 bottle
9. Wash Buffer (100×) 10 ml 1 bottle
10. TMB Substrate Solution 12 ml 1 bottle
11. Stop Solution 12 ml 1 bottle
12. Low NSB Sample Diluent 30 ml 1 bottle

Materials Required But Not Supplied

1. Pipettors and pipettes that deliver 100 µl and 1-10 ml.
2. Disposable glass or plastic 5-15 ml tubes for diluting samples and Anti-Human IgG HRP Concentrate.
3. Stock bottle to store diluted Wash Solution; 0.2 to 1 L.
4. ELISA plate reader at 450 nm wavelength and ELISA plate washer.

Storage

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

Specimen Collection And Preparation

Sample Collection

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.

Antibody 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 µl serum+ 40 µl WSD (or 0.1ml + 0.4 ml)

Further (1/50): 10 µl Initial (1/5) + 90 µl LNSD (1/50).

Plate Preparation

1. Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
2. Add 200-300 µl 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.

Reagent Preparation

1. Wash Solution: Dilute the entire volume 10 ml + 990 ml with distilled or deionized water into a clean stock bottle. Label as Working Wash Solution and store at 4°C for long term and ambient temp. for short term.
2. Sample Diluent: Dilute the entire volume, 10 ml + 190 ml with distilled or deionized water into a clean stock bottle. Label as Working Sample/Conjugate Diluent and store at 2-8°C until the kit lot expires or is used up.
3. Anti-Human IgG-HRP Conjugate: 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 100× to 2-8°C storage.

Assay Procedure

Design

1. 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.
2. Run the Anti-Flu H5N1 IgG Positive Control; the value range is on the label.
3. 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.
4. 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.

Steps

1. Add 100 µl of calibrators, samples and controls each to predetermined wells.

2. Tap the plate gently to mix reagents and incubate for 60 minutes.
3. 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.
4. Add 100 µl of diluted Anti-Human IgG HRP to each well.
5. Incubate for 30 minutes.
6. Wash wells 5 times.
7. Add 100 µl TMB Substrate to each well. The liquid in the wells will begin to turn blue.
8. 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).

9. Add 100 µl of Stop Solution to each well.
10. Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.
11. Use any commercially available microplate reader capable of reading at 450 nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
12. Read absorbance of the entire plate at 450 nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630 nm to normalize well background.

Quality Control

The test results are only valid if the test has been performed following the instructions. All standards and kit controls must be found within the acceptable ranges as stated on the vials. The positive control value range is on the label. If criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls. In case of any deviation the following technical issues should be proven (reagents, protocol, equipments, etc).

Calculation

Method 1

Human serum is diluted to 1:100 or greater.

The 1 U/ml Calibrator can be used to calculate a Threshold Index that numerically discriminates Positive/Negative: 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.

Method 2

1. Positives may be due to prior 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.
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.

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/Nonimmune 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.

Method 3

A sample value would be Positive if significantly above the value of the pre-immune serum sample or a suitably determined nonimmune 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.

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

Sensitivity

<1 U/ml

Linearity

79-114%

Recovery

100-114%

Precautions

1. All reagents in the kit package are for research use only.
2. The reagents must be kept between 2 - 8°C. The reagents cannot be guaranteed if the shelf-life dates have expired or if they have not been kept under the conditions described in this insert.
3. The concentrated wash solution may be stored at room temperature. Once diluted, this solution remains stable for six weeks if kept between 2 - 8°C.
4. Unused strips must be stored immediately in the aluminium envelope, taking care to keep the desiccant dry and the envelope's seal airtight. If these precautions are taken, the strips' activity can be conserved up to the kit's shelf-life date.
5. Do not use reagents from other kits.
6. The quality of the water used to prepare the various solutions is of the utmost importance. Do not use water that may contain oxidants (e.g., sodium hypochlorite) or heavy metal salts, as these substances can react with the chromogen.
7. Discard all solutions contaminated with bacteria or fungi.
8. All materials and disposable equipment that come in contact with the samples must be considered potentially infectious and be disposed of in compliance with the legislation in force in the country.
9. To guarantee the reliability of the results, one must follow the protocol to the letter. Special care must be taken in observing the incubation times and temperatures, as well as measuring the volumes and dilutions accurately.