Influenza (Av.)

H5-HA(Ag)

ELISA KIT FOR DETECTION OF HEMAGGLUTININ (HA) OF INFLUENZA A VIRUS, H5 STRAIN

Two-Step Incubation, Double Antibody Sandwich Principle

INSTRUCTIONS FOR USE, 2005

INTENDED USE

This kit is an enzyme-linked immunosorbent assay (ELISA) for qualitative detection of hemagglutinin (HA) of Influenza A Virus, H5 strain (also known as highly pathogenic avian influenza) in body excretes, nasopharyngeal aspirates, chicken embryo whole virus inoculation or viral lysates, etc. It is intended for clinical identification of the H5 strain of influenza A viruses.

SUMMARY

Influenza infection is an acute fever-like virus infection of the respiratory tract. The influenza virus and its toxin can lead to a serious inflammation of the bronchial mucosa and a damage of the tract.

The influenza viruses belong to the family of Orthomyxoviridae that have linear segmented (8) negative ssRNA genome with lipid envelope. Total genome length is 12000-15000 nucleotides (nt). The largest segment is 2300-2500 nt; the second largest is 2300-2500 nt; the third is 2200-2300 nt; the fourth is 1700-1800 nt; the fifth is 1500-1600 nt; the sixth is 1400-1500 nt; the seventh is 1000-1100 nt; the eighth is 800-900 nt. Genome sequence has terminal repeated sequences; repeated at both ends.

The virion envelope could be spherical, or filamentous with diameters of 50-120 nm, or 20 nm and 200-300(-3000) nm long. About 500 spikes are dispersed evenly over all the surface (i.e. hemagglutininesterase (HEF)), or dispersed equally over all the surface, but the various types are in clusters (i.e. hemagglutinin (HA) the major glycoprotein is interposed irregularly by clusters of neuraminidase (NA), with ratio of HA to NA about 4-5 to 1).

The Orthomyxoviridae family is divided into three types : A,B,C.

Type A influenza viruses are further divided into subtypes according to their Hemagglutinin (HA) and Neuraminidase (NA) proteins. Currently 15 (HA) subtypes and 9(NA) subtypes have been identified.

Type B influenza viruses produce less serious disease than does influenza type A and are not categorized as by H or N type as Influenza A is.

Type c influenza viruses were first isolated in 1949 and are not known to be responsible for epidemics

The infection in human mostly results from a droplet infection and appears as an epidemic which sometimes can be of pandemic proportions. After an incubation time of 1 - 3 days the symptoms appear suddenly- fast raise of temperature, often accompanied by shivering, the leading symptom of catarrhal inflammation appears, contributing to the clinical course of painful dry cough, tracheitis, laryngitis and frequently a rhinitis and conjunctivitis.
The appearance new Influenza epidemics and pandemics is facilitated by an antigen variability mainly in the HA and NA. In the past century, three major influenza epidemics have occurred:

1918-1919: (Spanish Flu, A (H1N1), 20-50 million deaths worldwide, nearly half were young, healthy adults.
1957-1958: Asian Flu (A (H2N2),1st identified in China Feb. 1957, 70,000 deaths in the United States.
1968-1969: Hong Kong Flu (A (H3N2), 1st detected in Hong Kong early 1968, virus still circulating today.

The 1997 outbreak of H5N1 avian influenza (or bird flu) in humans in Hong Kong caused alarm because it involved highly pathogenic strains of an influenza subtype A to which humans lack immunity. The H5N1 infected 18 humans, 6 of whom died (death rate of about 70 percent). Most of these cases occurred from contact with infected poultry or contaminated surfaces; however, it is thought that a few cases of human-to-human spread of H5N1 have occurred.

Avian influenza is an infectious disease of birds caused by type A strains of the influenza virus. The disease, first identified in Italy more than 100 years ago, now occurs worldwide. Infection triggers a wide spectrum of symptoms in birds, ranging from mild illness to a highly contagious and rapidly fatal disease resulting in severe epidemics. In the H5N1 bird flu in Hong Kong in 1997, patients had developed symptoms of fever, sore throat, cough and, in several of the fatal cases, severe respiratory distress secondary to viral pneumonia. Previously healthy adults and children, and some with chronic medical conditions, were affected.

More recently, outbreaks of avian influenza H5N1 occurred among poultry in eight countries in Asia (Cambodia, China, Indonesia, Japan, Laos, South Korea, Thailand, and Vietnam) during late 2003 and early 2004. At that time, more than 100 million birds in the affected countries either died from the disease or were killed in order to try to control the outbreak. By March 2004, the outbreak was reported to be under control. Beginning in late June 2004, however, new deadly outbreaks of influenza H5N1 among poultry were reported by several countries in Asia (Cambodia, China, Indonesia, Malaysia [first-time reports], Thailand, and Vietnam). It is believed that these outbreaks are ongoing.

Human infections of avian influenza H5N1 however, have been reported in Cambodia (1case/1death) Thailand (17cases/1 death) and Vietnam (51cases/ 33deaths) during both of these outbreak periods.

Hemagglutinin (HA) is a surface glycoprotein on Influenza A responsible for binding to N-AcetylNeuraminic Acid (NeuNAc) or commonly Sialic acid on host cell surface receptors. The Influenza viruses form the A virus group have principally similar morphological, chemical and biological features. The differentiation of the types is possible by the different antigenicity of their nucleo- and matrix proteins that have type-specific antigenicity. However, the essential immunodominant antigens and primary targets in diagnosis are the hemagglutinin (HA) and the neuraminidase (NA) antigens.

PRINCIPLE OF THE ASSAY

H5-HA(Ag) ELISA kit uses polystyrene microwell strips

pre-coated with antibodies (anti-HA"Ab") specific to the hemagglutinin (HA) of the H5 Influenza strain. Sample is added into the microwells. During the first incubation step, the specific anti-HA"Ab"-HA immunocomplex which forms in case of presence of H5 HA in the sample, is captured on the solid phase. After washing to remove sample proteins or unbound material, second H5 HA specific anti-HA"Ab" conjugated to horseradish peroxidase (anti-HA"Ab"HRP) is added. During the second incubations step, this anti-HA"Ab"HRP antibody will specifically bind to the anti-HA"Ab"-HA complex previously formed inside the wells.

Unbound anti-HA"Ab"HRP is removed after washing. Chromogen solutions containing tetramethylbenzidine (TMB) and urea peroxide are added into the wells.

In presence of the **(anti-HA"Ab" - HA - anti-HA"Ab"HRP)** "sandwich" immunocomplex, the colorless Chromogens are hydrolyzed by the bound HRP-conjugate to a blue colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color can be measured and is proportional to the amount of HA in the sample. Wells containing samples negative for H5 HA remain colorless.

Assay principle scheme:

Double antibody sandwich ELISA

Ab(p) + Ag(s) → [Ab(p)–Ag(s)] + (Ab)ENZ → [Ab(p)–Ag(s)–(Ab)ENZ]			→ blue→ yellow	(+)
Ab(p)	+ (Ab)ENZ→ [Ab(p)	]→	no color	(-)
Incubation1	Incubation2	Immobilized Complex	Coloring	Results
60 min.	30min.		30min.	

Ab(p)–pre-coated H5 specific anti-HA, or anti-HA"Ab"
Ag(s)– H5 hemagglutinin (HA) in sample;
(Ab)ENZ–HRP conjugated H5 anti-HA, or anti-HA"Ab"HRP

COMPONENTS

96 Tests

- **MICROWELL PLATE** 1plate

Blank microwell strips fixed on white strip holder. The plate is sealed in aluminum pouch with desiccant.
8×12 or 12×8-well strips per plate.
Each well contains antibodies (anti-HA"Ab") reactive to H5 HA. The microwell strips can be broken to be used separately. Place unused wells in the plastic sealable storage bag together with the desiccant and return to 2~8℃.

- **NEGATIVE CONTROL** 1vial

Blue liquid filled in a vial with green screw cap
1ml per vial.
Protein-stabilized buffer tested non-reactive for HA of H5 influenza-A. Preservatives: 0.1% ProClin 300.
Ready to use as supplied.
Once open, stable for one month at 2-8℃.

- **POSITIVE CONTROL** 1vial

Red color liquid filled in a vial with red screw cap.
1ml per vial.
Protein-stabilized buffer tested reactive for HA of H5 influenza-A. Preservatives: 0.1% ProClin 300.
Ready to use as supplied.
Once open, stable for one month at 2-8℃.

- **HRP-CONJUGATE REAGENT** 1vial

Red liquid filled in a white dropper vial with red screw cap.
12ml per vial.
Horseradish peroxidase-conjugated H5 anti-HA"Ab".
Ready to use as supplied. Once open, stable for one month at 2-8℃.

- **VIRUS LYSIS BUFFER** 2vials

Colorless liquid filled in a clear bottle with white screw cap.
50ml per vial

- **STOCK WASH BUFFER** 1bottle

Colorless liquid filled in a clear bottle with white screw cap.
50ml per bottle.
PH 7.4 20 × PBS (Containing Tween-20 as a detergent).
DILUTE BEFORE USE -The concentration must be diluted **1 to 20** with distilled/deionized water before use.
Once diluted, stable for one week at room temperature or for two weeks at 2-8℃.

- **CHROMOGEN SOLUTION A** 1vial

Colorless liquid filled in a white dropper vial with green screw cap.
6ml per vial.
Urea peroxide solution.
Ready to use as supplied.
Once open, stable for one month at 2-8℃.

- **CHROMOGEN SOLUTION B** 1vial

Colorless liquid filled in a black dropper vial with black screw cap.
6ml per vial.
TMB solution. Tetramethylbenzidine dissolved in citric acid.
Ready to use as supplied.
Once open, stable for one month at 2-8℃.

- **STOP SOLUTION** 1vial

Colorless liquid filled in a white dropper vial with yellow screw cap
6ml per vial.
Diluted sulfuric acid solution (2.0M H_2SO_4).

- **PLASTIC SEALABLE BAG** 1unit

For enclosing the strips not in use.

- **CARDBOARD PLATE COVER** 3sheets

To cover the plates during incubation and prevent evaporation or contamination of the wells.

- **PACKAGE INSERTS** 1copy

ADDITIONAL MATERIALS AND INSTRUMENTS REQUIRED BUT NOT PROVIDED

1. Freshly distilled or deionized water.
2. Disposable gloves and timer.
3. Appropriate waste containers for potentially contaminated materials.
4. Disposable V-shaped troughs.
5. Dispensing system and/or pipette (single or multichannel), disposable pipette tips
6. Absorbent tissue or clean towel.
7. Dry incubator or water bath, 37±0.5℃.
8. Microshaker for dissolving and mixing conjugate with samples.
9. Microwell plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm.
10. Microwell aspiration/wash system.

SPECIMEN PREPARATION

For liquid specimens –mix (1:1) each specimen with virus lysis buffer.

In case that solid specimens will be tested (e.g. fecal samples), such sample must be dissolved 1:10 with lysis buffer (for

example 0.1g of sample mixed with 1ml of lysis buffer).

For specimens collected into cotton swabs (e.g. throat swabs), elute the samples with 1ml of virus lysis buffer. Centrifugate and than use only the supernatant for testing.

SPECIAL INSTRUCTIONS FOR WASHING

1. A good washing procedure is essential to obtain correct and precise analytical data.
2. It is therefore recommended to use a good quality ELISA microplate washer, maintained at the best level of washing performances. In general, no less than 5 automatic washing cycles with dispensing of 350-400µl/well, are sufficient to avoid false positive reactions and high background (all wells turn yellow).
3. To avoid cross-contaminations of the plate with sample or HRP-conjugate, after incubation do not discard the content of the wells, but allow the plate washer to aspirate it automatically.
4. Anyway, we recommend calibrating the washing system on the kit itself in order to match the declared analytical performances. Assure that the microplate washer's liquid dispensing channels are not blocked or contaminated, and sufficient volume of Wash buffer is dispensed each time into the wells.
5. In case of manual washing, we suggest to perform at least 5cycles, dispensing 350-400µl/well and aspirating the liquid for 5times. If poor results (high background) are observed, increase the washing cycles or soaking time per well.
6. In any case, the liquid aspirated out the strips should be treated with a sodium hypochlorite solution(final concentration of 2.5%) for 24 hours, before liquids are disposed in an appropriate way.
7. The concentrated Washing solution should be diluted **1 to 20** before use. For one plate, mix 50 ml of the concentrate with 950ml of water for a final volume of 1000ml diluted Wash Buffer. If less than a whole plate is used, prepare the proportional volume of solution.

STORAGE AND STABILITY

The components of the kit will remain stable through the expiration date indicated on the label and package when stored between 2-8 ℃, **do not freeze**. To assure maximum performance of H5-HA(Ag) ELISA kit, during storage, protect the reagents from contamination with microorganism or chemicals.

PRECAUTIONS AND SAFETY

This kit is intended **FOR IN VITRO USE ONLY** IVD

FOR PROFESSIONAL USE ONLY

The ELISA assay is a time and temperature sensitive method. To avoid incorrect result, strictly follow the test procedure steps and do not modify them.

1. Do not exchange reagents from different lots, or use reagents from other commercially available kits. The components of the kit are precisely matched as to achieve optimal performance during testing.
2. Make sure that all reagents are within the validity indicated on the kit box and are of the same lot. Never use reagents beyond the expiry date stated on reagents labels or on the kit box.
3. **CAUTION - CRITICAL STEP:** Allow the reagents and samples to stabilize at room temperature (18-30℃) before use. Shake reagent gently before, and return to 2-8℃ immediately after use.
4. Use only sufficient volume of sample as indicated in the procedure steps. Failure to do so, may cause in low sensitivity of the assay.
5. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading.
6. When reading the results, ensure that the plate bottom is dry and there are no air-bubbles inside the wells.
7. Never allow the microplate wells to dry after the washing step. Immediately proceed to the next step. Avoid the formation of air-bubbles when adding the reagents.
8. Avoid assay steps long time interruptions. Assure same working conditions for all wells.
9. Calibrate the pipette frequently to assure the accuracy of samples/reagents dispensing. Always use different disposal pipette tips for each specimen and reagents as to avoid cross-contaminations. Never pipette solutions by mouth.
10. The use of automatic pipettes is recommended.
11. Assure that the incubation temperature is 37℃ inside the incubator.
12. When adding samples, avoid touching the well's bottom with the pipette tip.
13. When reading the results with a plate reader, it is recommended to determine the absorbance at 450nm or at 450nm with reference at 630nm.
14. All specimens from human origin should be considered as potentially infectious.
15. Materials from human origin may have been used in the kit. These materials have been tested with tests kits with accepted performance and found negative for antibodies to HIV ½, HCV, TP and HBsAg. However, there is no analytical method that can assure that infectious agents in the specimens or reagents are completely absent. Therefore, handle reagents and specimens with extreme caution as if capable of transmitting infectious diseases. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.
16. Bovine derived sera may have been used in this kit. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE free-geographical areas.
17. The pipette tips, vials, strips and sample containers should be collected and autoclaved for 1hour at 121℃ or treated with 10% sodium hypochlorite for 30minutes to decontaminate before any further steps for disposal.
18. The Stop solution (2M H_2SO_4) is a strong acid. Corrosive. Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes. ProClin 300 used as a preservative can cause sensation of the skin.
19. The enzymatic activity of the HRP-conjugate might be affected from dust, reactive chemical, and substances like sodium hypochlorite, acids, alkalins etc. Do not perform the assay in the presence of such substances.
20. Materials Safety Data Sheet (MSDS) available upon

request.

21. If using fully automated microplate processing system, during incubation, do not cover the plates with the plate cover. The tapping out of the remainders inside the plate after washing, can also be omitted.

ASSAY PROCEDURE

Step1 **Reagents preparation:** Allow the reagents to reach room temperature (18-30°C). Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37℃ until crystals dissolve. Dilute the stock Wash Buffer **1 to 20** with distilled or deionized water. Use only clean vessels to dilute the buffer.

Step2 **Numbering Wells:** Set the strips needed in strip-holder and number sufficient number of wells including three Negative control **(e.g. B1, C1, D1)**, two Positive control **(e.g. E1, F1)** and one Blank (**e.g. A1**, neither samples nor HRP-Conjugate should be added into the Blank well). Use only number of strips required for the test.

Step3 **Adding Sample**: Add **100µl** of Positive control, Negative control, and **samples pretreated with virus lysis buffer** (see the above specimen preparation instruction) into their respective wells. **Note: Use a separate disposal pipette tip for each specimen, Negative Control and Positive Control to avoid cross-contamination.**

Step4 **Incubating:** Cover the plate with the plate cover and incubate for **60 minutes at ROOM TEMPERATURE**. It is recommended to use micro shaker system and the shaking rate should be adjusted at low or medium speed.

Step5 **Washing:** At the end of the incubation, remove and discard the plate cover. Wash each well **5**times with diluted Wash buffer. Each time allow the microwells to soak for 30-60 seconds. After the final washing cycle, turn the strips plate down onto blotting paper or clean towel, and tap the plate to remove any remainders.

Step6 **Adding HRP-Conjugate:**Add **100µl HRP-Conjugate** to each well except the Blank and mix by tapping the plate gently.

Step7 **Incubating:** Cover the plate with the plate cover and incubate for **30 minutes at 37℃**.It is recommended to use water tank to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

Step8 **Washing:** At the end of the incubation, remove and discard the plate cover. Wash each well **5**times with diluted Wash buffer as in step 5.

Step9 **Coloring:** Dispense **50µl** of Chromogen A and **50µl** Chromogen B solution into each well including the **Blank**, and mix by tapping the plate gently. Incubate the plate at **37℃ for 30minutes avoiding light**. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in Positive control and Positive sample wells.

Step10 **Stopping Reaction:** Using a multichannel pipette or manually add **50µl** Stop Solution into each well and mix gently. Intensive yellow color develops in Positive control and Positive sample wells.

Step11 **Measuring the Absorbance:** Calibrate the plate reader with the Blank well and read the absorbance at **450nm**.If a dual filter instrument is used, set the reference wavelength at **630nm**. Calculate the Cut-off value and evaluate the results. (**Note:** read the absorbance within **10** minutes after stopping the reaction)

INTERPRETATION OF RESULTS AND QUALITY CONTROL

Each microplate should be considered separately when calculating and interpreting results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each sample optical density (OD) value to the Cut-off value (C.O.) of the plate. If the Cut-off reading is based on single filter plate reader, the results should be calculated by subtracting the Blank well OD value from the print report values of samples and controls. In case the reading is based on Dual filter plate reader, do not subtract the Blank well OD from the print report values of samples and controls.

1. Calculation of Cut-off value:

Cut-off value (C.O.) = *Nc +0.15

*Nc = the mean absorbance value for three negative controls.

Important: If the mean OD value of the negative control is lower than 0.03, take it as 0.03. If higher than 0.03 see the Quality control range.

Example:

1. Calculation of Nc:

Well No	B1	C1	D1
Negative controls OD value	0.02	0.012	0.016

Nc=0.016 (the Nc value is lower than 0.03 so take it as 0.03)

2. Calculation of Cut-off value: (C.O.) = 0.03 +0.15= 0.18

If one of the Negative control values does not meet the Quality control range specifications, it should be discarded and the mean value is calculated again using the remaining two values. If more than one control OD value does not meet the Quality control range specifications, the test is invalid and must be repeated.

2. Quality control range:

The test results are valid if the Quality Control criteria are verified. It is recommended that each laboratory must establish appropriate quality control system with quality control material similar to or identical with the patient sample being analyzed

1. The absorbance of the Blank well, which contains only Chromogens and Stop solution, is less than 0.080 at 450 nm.
2. The absorbance value OD of the Positive control must be equal to or greater than 0.500 at 450/630nm or at 450nm after blanking.
3. The absorbance value OD of the Negative control must be less than 0.100 at 450/630nm or at 450nm after blanking.

3. Interpretations of the results:

(S = the individual absorbance (OD) of each specimen)

Negative Results (S/C.O. <1): samples giving an absorbance less than the Cut-off value are considered negative, which indicates that H5 avian influenza hemagglutinin has not been detected with this kit.

Positive Results (S/C.O. ⩾ 1): samples giving an absorbance greater than or equal to the Cut-off value are considered initially reactive, which indicates that hemagglutinin of H5 avian influenza has probably been detected with this kit.

Any initially reactive samples must be retested in duplicates. Repeatedly reactive samples can be considered positive for H5 HA.

Borderline: Samples with absorbance to Cut-off ratio between 0.9 and 1.00 are considered borderline samples and retesting is recommended. Repeatedly positive samples can be considered positive for H5 HA.

LIMITATIONS

1. Non-repeatable positive result may occur due to the general biological and biochemical characteristics of ELISA method. The test is design to achieve performance characteristics of high sensitivity and specificity. HA may be undetectable during the early stages of the disease and in some immunosuppressed individuals.
2. Any positive results must be interpreted in conjunction with patient clinical information and other laboratory testing results.
3. Common sources for mistakes: kits beyond the expiry date, bad washing procedures, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
4. The prevalence of the marker will affect the assay's predictive values.

VALIDITY

Please do not use this kit beyond the expiry date indicated on the kit box and reagent labels.

INDICATIONS OF INSTABILITY OR DETERIORATION OF THE REAGENTS

1. Values of the Positive or Negative controls ,which are out of the indicated Quality control range, are indicator of possible deterioration of the reagents and/or operator or equipment errors. In such case, the results should be considered as invalid and the samples must be retested. In case of constant erroneous results classified as due to deterioration or instability of the reagents, immediately substitute the reagents with new ones, or contact Beijing technical support for further assistance.
2. If after mixing of the Chromogen A and B solutions into the wells, the, the color of the mixture turns blue within few minutes, do not continue carrying out the testing and replace the reagents with fresh ones.

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FOR RESEARCH USE ONLY

SUMMARY OF THE ASSAY PROCEDURE:	
Pre-treat the samples with virus lysis buffer	
Add Sample	**100µl**
Incubate ROOM TEMPERATURE	**60minutes**
Wash	**5times**
Add HRP-Conjugate	**100µl**
Incubate 37℃	**30minutes**
Wash	**5times**
Coloring	**50µl A + 50µl B**
Incubate 37℃	**30minutes**
Stop the reaction	**50µl stop solution**
Read the absorbance	**450nm or 450/630nm**

SUMMARY OF THE MAJOR COMPONENTS OF THE KIT:	
Microwell plate	**One/ 96 wells**
Negative/ Positive control	**One each/ 1ml**
HRP-Conjugate	**One/ 12ml**
Viral lysis solution	**One/100ml**
Wash Buffer	**One/ 50ml**
Chromogen A/B/ Stop Solution	**One each/ 6ml**
Note: the components of individual kits are not interchangeable	