Version 18-11/25

User's Manual

Mouse Anti-Hepatitis B Surface Antigen (anti-HBsAg) IgG ELISA kit

REF **DEIASL263**

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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

The Mouse Anti-HBsAg IgG ELISA Kit detects and quantifies HBsAg specific IgG in mouse serum or plasma of vaccinated, immunized and/or infected animals. This immunoassay is suitable for:

1. Determining immune status relative to non-immune controls;
2. Assessing efficacy of vaccines, including dosage, adjuvantcy, route of immunization and timing;
3. Qualifying and/or standardizing vaccine batches and protocols.

The kit contains no virus (live or killed) or viral proteins to avoid infection. This kit is for research use only (RUO) and not for diagnosis cure or prevention of the disease.

General Description

Hepatitis B is an infectious disease caused by hepatitis B virus (HBV). Hepatitis, the acute illness, inflames the liver, causing jaundice, vomiting and (rarely) death. Chronic hepatitis B, however, can cause cirrhosis and liver cancer—a fatal disease. Although viral replication occurs in the liver, HBV spreads to the blood where virus-specific antigens and antibodies may be found in the infected host. Blood tests for these antigens and antibodies are used to diagnose the infection. Acute and chronic hepatitis B can be prevented by vaccination.HBV is divided into four major serotypes (adr, adw, ayr, ayw) based on antigenic epitopes presented on its envelope proteins, and into eight genotypes (A-H) according to overall nucleotide sequence variation of the genome. Genotypes differ by at least 8% of their sequences, differences which affect severity of disease and response to treatment and possibly vaccination. The hepatitis B surface antigen (HBsAg) is the first detectable viral antigen to appear during infection, and is most frequently used to screen for the presence of infection. HBsAg is also the basis for several recent vaccines, which use synthetic recombinant HBsAg and contain no blood products. Therefore, they cannot cause HBV infection, a problem with the original vaccine prepared from plasma from patients with long-term HBV infection. Following vaccination, HBsAg may be detected in serum for several days. These vaccines have provided protection for 85-90% of individuals. HBV Vaccine common brands available are: Engerix-B (GSK), Elovac B (Human Biologicals Institute, A division of Indian Immunologicals Limited), Genevac B (Serum Institute), Shanvac B etc. These vaccines are given intramuscularly.

Principles of Testing

The Mouse Anti-HBsAg IgG ELISA kit is based on the binding of antibody in samples to HBsAg virus antigen immobilized on the microwells, and antibody is detected by anti-mouse IgG-specific antibody-HRP conjugate. After a washing step, substrate (TMB) is added and color (blue) is developed, which is directly proportional to the amount of antbody present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm (yellow color) is then measured using an ELISA reader. The amount of mouse Anti-HBsAg IgG in samples is determined relative to mouse anti HBsAg calibrators.

Reagents And Materials Provided

1. Wash Solution Concentrate(100×), 10ml. Dilute the entire volume 10ml + 990ml with distilled or

deionized water into a clean stock bottle. Label as Wash Solution and store at ambient temperature until kit is used entirely.

2. Sample Diluent Concentrate (20×), 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°C until the kit lot expires or is used up.

3. Anti-Mouse IgG-HRP Conjugate Concentrate(100×), 0.15ml. Peroxidase conjugated anti-mouse IgG in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10µl 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.

4. HBsAg Microwell Strip Plate, 8-well strips(12). Coated with recombinant HBsAg and post-coated with stabilizers.

5. Anti-HBsAg Calibrators, 0.65 mL. Four (4) vials, each containing anti-HBsAg in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers. 10 U/ml, 25 U/ml, 50 U/ml, 100 U/ml

6. Anti-HbsAg Positive Control, 0.65 mL. Anti-HbsAg antibody, diluted in buffer with protein detergents and antimicrobial as stabilizers. [Value range on label].

7. Low NSB Sample Diluent, 30 mL. Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for sample dilution.

8. TMB Substrate, 12 mL. Chromogenic substrate for HRP containing TMB and peroxide.

9. Stop Solution, 12 mL. Dilute sulfuric acid.

Materials Required But Not Supplied

Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.

Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Mouse IgG HRP Concentrate.

Graduated cylinder to dilute Wash Concentrate; 200ml to 1L.

Stock bottle to store diluted Wash Solution; 200ml to 1L

Distilled or deionized water to dilute reagent concentrates.

Microwell plate reader at 450 nm wavelength.

Storage

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

Specimen Collection And Preparation

Sample Collection and Handling

For serum, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For other samples, including tissue culture medla, clarify the sample by centrifugation and/or filtration prior to dilution in Sample Diluent.

Sample Dilutlon & Antibody Stability

Prepare an initial sample dilution (1:10 or 20 ul sample into 180 ul) of Working Sample Diluent in order to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for months, stored refrigerated or frozen. Additional dilution (1:10 of the initial stock for a final dilution of 1:100) into Low NSB Sample Diluent provides low assay background and good discrimination of specific signal. It is possible to change the testing dilution to 1:50-1:500 depending upon the actual sample background. All sample dilutions in Low NSB should be at least 5 times the initial dilution and performed the same day as the assay. Do not store test dilutions.

Assay Design

Review Calculations and Limits of the Assay before proceeding:

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 <0.5 OD. This is usually 1:50 or greater dilution for mouse sera with normal levels of lgG and lgM.
2. 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 (See Method A).
3. Run the Anti-HbsAg Positive Control; value range is on the vial label.
4. Run a set of Calibrators. Calibrators validate that the assay was performed to specifications; results can be used to normalize between-assay variation for enhanced precision. Reading values off a Calibrator curve, Method B, has limitations. See Limits of the Assay.
5. Run a range of sample dilutions for expected higher positives that allows calculation of antibody Titer (when specific titer is at least 4-fold higher than non-immune). See Method C.
6. Run samples in duplicate if used for quantitation; non-immunes that are signifcantly lower than immunes may be run in singlicate. The Calibrators that are used for quantitation, e.g.. for between-assay normalization, should be run in duplicate. When determining titer from a dilution curve, singlicates can be run if more than two dilution points are used for titer calculations.

Plate Preparation

Plate Set-up

Bring all reagents to room temperature (18-30°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 and controi to be assayed.

Remove the appropriate number of microwell strips ftrom 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 durmp the liquid and pat dry on a paper towel before sample addition.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE (25-28°C). After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st incubatlon [100ul-60 min;4washes]

Add 100 ul of blank, callbrators, samples and controls each to pre-determined wells.

Tap the plate gently to mix reagents and incubate for 60 minutes at room temp (25-28°C).

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 100 ul of diluted Antl-MouselgG HRP to each well.

Incubate for 30 minutes at room temp 0.

Wash wells 5 times as in step 2.

3. 3rd Substrate Incubation [100ul-15 min]

Add 100 ul 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 densily (OD) above 2.0, incubate for less time, or read OD at 405-410nm (results are valid).

4. 4th Stop Step [Stop: 100ul]

Add 100 ul of Stop Solutlon to each well.

Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

5. 5th Absorbance Reading

Use any commercially available microplate reader capable of reading at 45Onm wavelength. Use a program suitable for obtaining OD readings, and data calculations If available.

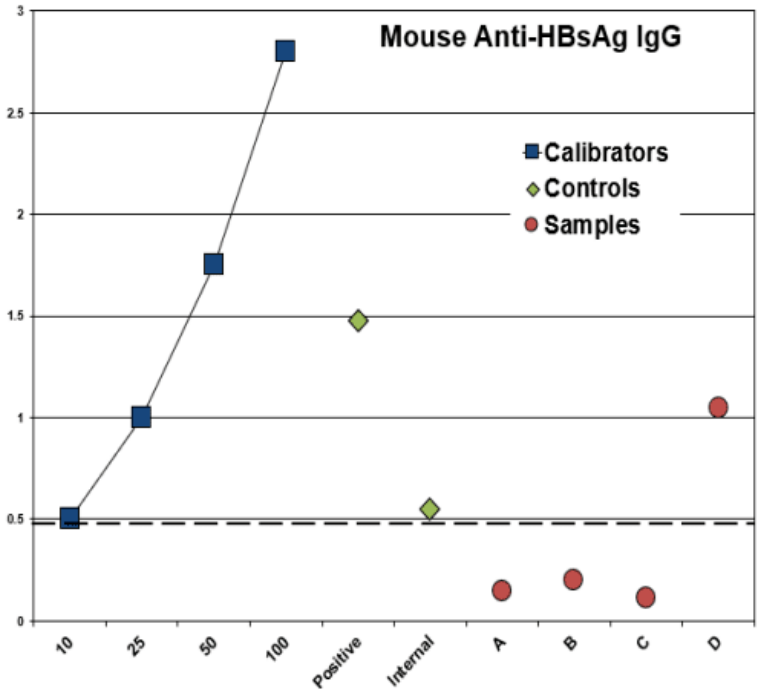
Read absorbance of the entire plate at 45Onm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to nomalize well background.

Calculation

A. Antibody Activity Threshold Index

Compare Samples to 10 U/mL Calibrator or Internal Control= Positive/Negative Cut-off.

Example:



Results:

The sensitivity of the assay to detect anti-HBsAg IgG, from either natural exposure or vaccination, is controlled so that the 10 U/ml Calibrator represents a threshold OD for most true positives in mouse serum diluted to 1:50 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators - dilution curve of antiserum from anti-HBsAg immunization, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.

10 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.

Positve Control - an anti-HBsAg serum; value range is on the vial label. This Control can be used to assess reproducibility and to normalize between-assay variation.

Internal Control - a true positive from an immune mouse 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 10 U/ml Calibrator for Positive/Negative discrimination purposes.

Samples A,B,C,D - 3 samples (1:50) (A, B, C) are negative: below the threshold; 1 sample (D) is positive: clearly above the threshold.

The 10 U/ml Calibrator can be used to calculate a Threshold Index that numerically discriminates Positive/Negative:

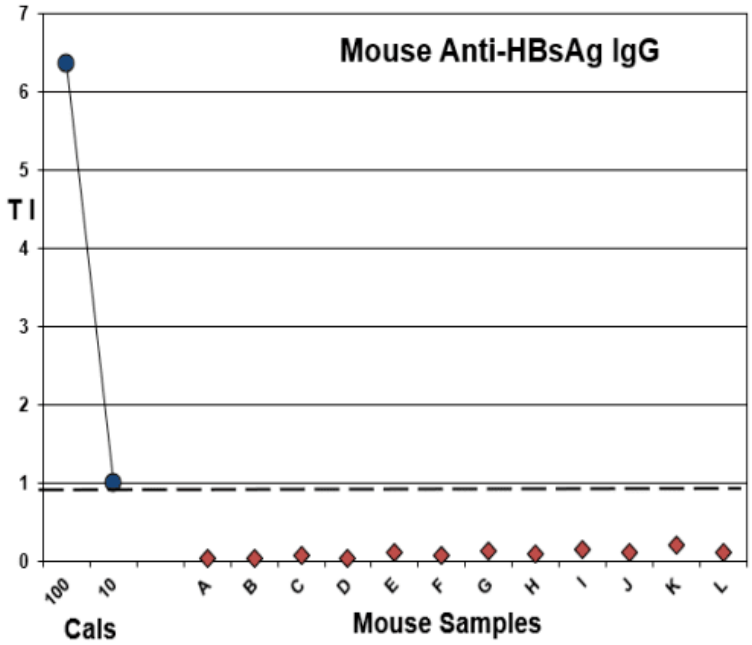
> Divide each Sample net OD by the 10 U/ml Calibrator net OD. Values above 1.0 are a measure of Positive Antibody Activity; below 1.0 are Negative for antibody.

Example:

Mouse Serum IgG:

A panel of pooled and individual sera from laboratory mice was tested for anti-HBsAg IgG (1:100 dilution).

Threshold index was calculated using the 10 U/mL Cal.



Results:

Anti- HBsAg IgG: all twelve sera were negative (below the 1.0 Threshold Index).

Notes:

1. Positives may be due to prior encounter with the virus or non-HBsAg proteins with common epitopes.
2. The sensitvity of the assay may be adjusted by changing the sample dilutions: a) increase dilution (e.g, 1:200) 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 altemative threshold should be considered using known negatives to develop a Positive Index (see below) or use an Internal Control.
3. When the Positive Index is above 5.0, using a dilution curve to calculate titer is a more accurate quantitation method (see Method C).

B. Positlve Index

Experimental sample values may be expressed relative to the values of Control or Non-immune samples, by calculation of a Positve 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.O are a measure of Posltive Antibody Activity; below 1.0 are Negative for antibody.

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 positve 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

Sensitivity

Assay Sensitivity

The HBsAg -coated plated, the anti- mouse IgG HRP concentration, and the Low NSB Sample Diluent are optimized to differentiate anti-HBsAg IgG from background (non-antibody) signal with mouse serum samples diluted 1:50

Specificity

Purifed recombinant HBsAg is used to coat the microwells; stabilizing postcoat contains BSA; thus, no other antibody specificity is detectable in the assay. The anti-mouse IgG HRP conjugate specifically detects IgG, and will not react with IgM, IgA or IgE class antibodies.

Precautions

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, wv). 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.

Limitations

Quantitation of Antibody in a Sample

The ELISA measures anti-HBsAg activity, a combination of antibody concentration and avidity for the HBsAg antigens. Antibodies with substantially different total Ig concentrations may display similar anti-HBsAg activities, due to differences in avidity. The quantitation or activity of the samples is, therefore, appropriately expressed in activity Units (titer), rather than mass units of IgG (e.g., ug/ml).

Calibrator Curve Quantitation

To quantitate antibody activity from a calibrator curve (such as provided with the kit), the dilution curve of the samples must be parallel to the calibrator curve, to avoid different values being obtained from different regions of the curve. Antibodies that are not matched in HBsAg avidity will often have nonparallel dilution curves. In these cases, antibody activity is best expressed as a titer relative to a reference positive such as the 25 U/ml Calibrator, or another Calibrator in the kit (see Calculations).

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