

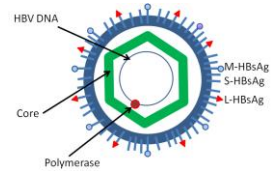
INTENDED USE

The Monkey Anti-HBsAg IgG ELISA Kit detects and quantifies **HBsAg** (hepatitis B surface antigen) IgG in monkey serum or plasma of 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, adjuvancy, route of immunization and timing;
- Qualifying and/or standardizing vaccine batches and protocols.

The kit has no live or killed virus or viral antigens. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

GENERAL INFORMATION



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. ADI has developed antibody ELISA kits to determine the efficacy of various existing vaccines and test new vaccines.

PRINCIPLE OF THE TEST

The Monkey Anti-HBsAg IgG ELISA kit is based on the binding of monkey anti-HBsAg in samples to HBsAg immobilized on the microwells, and anti-HBsAg IgG antibody is detected by anti-monkey IgG-specific antibody conjugated to HRP (horseradish peroxidase) enzyme. After a washing step, chromogenic substrate (TMB) is added and color is developed by the enzymatic reaction of HRP on the substrate, which is directly proportional to the amount of anti-HBsAg IgG present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microwell reader. The activity of monkey antibody in samples is determined relative to anti-HBsAg Calibrators.

PRODUCT SPECIFICATIONS

Specificity

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

Assay Sensitivity

The HBsAg -coated plated, the anti-monkey IgG HRP concentration, and the Low NSB Sample Diluent are optimized to differentiate anti-HBsAg IgG from background (non-antibody) signal with monkey serum samples diluted 1:100.

KIT CONTENTS

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.

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 Wash Solution and store at ambient temperature until kit is used entirely.
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°C until the kit lot expires or is used up.
Anti-Human IgM - HRP Conjugate Concentrate (100x) Part No. H-MsM.211, 0.15ml	Peroxidase conjugated anti-Human IgM in buffer with protein, 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°C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
HBsAg Microwell Strip Plate	4211	8-well strips (12)	Coated with recombinant HBsAg, and post-coated with stabilizers.
Anti-HBsAg Calibrators			
10 U/ml	4242B	0.65 ml	Four (4) vials, each containing anti-HBsAg in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
25 U/ml	4242C	0.65 ml	
50 U/ml	4242D	0.65 ml	
100 U/ml	4242E	0.65 ml	
Anti-HbsAg Positive Control	4242PC	0.65ml	Anti-HbsAg antibody; diluted in buffer with protein, detergents and antimicrobial as stabilizers. Net OD > 0.5
Low NSB Sample Diluent	TBTm Not for conjugate dilution	30 ml	Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for sample dilution
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. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Human IgM 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.

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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

LIMITATIONS OF THE ASSAY

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 non-parallel 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 Calculation of Results).

ASSAY DESIGN AND SET-UP

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 media, clarify the sample by centrifugation and/or filtration prior to dilution in Sample Diluent.

Antibody Stability

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 10 times the initial dilution and performed the same day as the assay.

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

Assay Design

Review Calculation of Results (p5-7) and Limits of the Assay (above) 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 <0.5 OD. This is usually 1/100 or greater dilution for monkey sera with normal levels of IgG and IgM.
- 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**).
- Run the Anti-HbsAg Positive Control; net OD > 0.5.
- 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 (above).
- 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**.
- Run samples in duplicate if used for quantitation; non-immunes that are significantly 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.

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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 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 (25-28oC). 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 **100 ul of blank, calibrators, samples and controls** each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for **60 minutes at room temp** (25-28oC).
- **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 Anti-Monkey IgG HRP** to each well.
- Incubate for **30 minutes at room temp**.
- **Wash wells 5 times** as in step 2.

3. 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 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 **100 ul 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.

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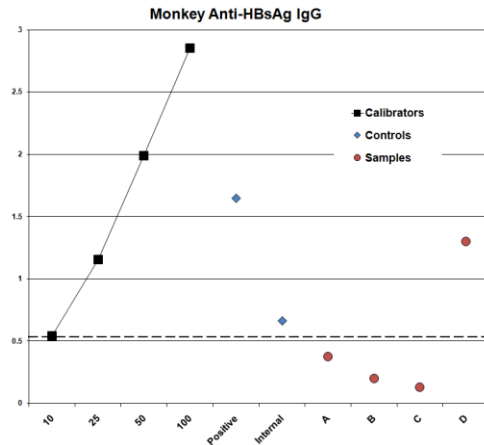
INTERPRETATION OF RESULTS

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 infection or vaccination, is controlled so that the **10 U/ml Calibrator** represents a threshold OD for most true positives in monkey serum diluted to 1:100 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.

Positive Control – an anti-HBsAg serum; net OD > **0.5**. This Control can be used to normalize between-assay variation.

Internal Control – a true positive from an immune monkey 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 (1:100) (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:

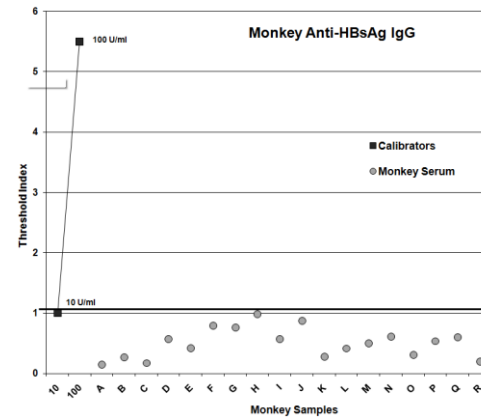
- ❖ Divide each Sample net OD by the 10 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

Example:

Monkey Serum IgG

A panel of sera from laboratory monkeys was tested for anti-HBsAg IgG (1:100 dilution). **Threshold Index** was calculated using the **10 U/ml Cal.**



Results

Anti- HBsAg IgG: all sera were negative or borderline (at or below the **1.0** Threshold Index).

Notes:

- Positives** may be due to prior encounter with the virus or anti-HBsAg immunization.
- 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).
- If other biological specimens and/or HBsAg preparations, are 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:

- Calculate the net OD mean + 2 SD of the Control/Non-immune samples = **Positive Index**.
- 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:

- 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.
- 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.
- 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.
- The Positive and Sensitivity Control values can be used to normalize inter-assay values.

Calculations

- 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).
- 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**

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Monkey Anti-HBsAg IgG

ELISA Kit Cat. No. 4250

For Quantitation of Anti-Hepatitis Surface Antigen (HbsAg) IgG In Serum or Plasma

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



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ELISA Kit Components	Amount	Part
HBsAg Coated Microwell Strip Plate	8-well strips (12)	4211
Anti-HbsAg Positive Control	0.65 ml	4242PC
Anti-HBsAg Calibrator 10 U/ml	0.65 ml	4242B
Anti-HBsAg Calibrator 25 U/ml	0.65 ml	4242C
Anti-HBsAg Calibrator 50 U/ml	0.65 ml	4242D
Anti-HBsAg Calibrator 100 U/ml	0.65 ml	4242E
Anti-Monkey IgG HRP Conjugate (100X)	0.15 ml	H-MkG.211
Sample Diluent (20X)	10 ml	SD20T
Low NSB Sample Diluent	30 ml	TBTm
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	M-4250