INTENDED USE

The **Human Anti-Hepatitis B Core Antigen IgM** ELISA Kit detects and quantifies **HBcAg** (hepatitis B core antigen) IgM in human serum or plasma of vaccinated, immunized and/or infected hosts. Other biological samples may also be tested (e.g., saliva, mucous) with appropriate validation. 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/or standardizing vaccine batches and protocols.

For research use only (RUO), not for diagnostic use.

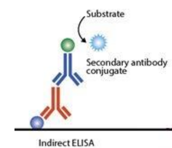
GENERAL INFORMATION

Hepatitis B virus (HBV) is an hepadnavirus—hepa from hepatotropic (attracted to the liver) and dna because it is a DNA virus. Although replication takes place in the liver, the virus spreads to the blood where viral proteins and antibodies against them are found in infected individuals.

The virus particle, (virion) consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity similar to retroviruses. The outer envelope contains embedded proteins which are involved in viral binding of, and entry into, susceptible cells. The virus is one of the smallest enveloped animal viruses There are four known genes encoded by the genome, called C, X, P, and S. The **core protein** is coded for by gene C **(HBcAg). HBeAg** is produced by proteolytic processing of the pre-core protein. The DNA polymerase is encoded by gene P. Gene S is the gene that codes for the **surface antigen (HBsAg).** The HBsAg gene is one long open reading frame but divided into three sections, pre-S1, pre-S2, and S. Because of the multiple start codons, polypeptides of three different sizes called large, middle, and small (pre-S1 + pre-S2 + S, pre-S2 + S, or S) are produced.

Hepatitis B core antibodies (anti-HBc Ab) appear shortly after the onset of symptoms of hepatitis B infection and soon after the appearance of hepatitis B surface antigen (HBsAg). Commercial vaccines consist of and produce antibodies to HBsAg, whereas the presence of antibodies to HBcAg indicates previous or ongoing infection with HBV in an undefined time frame.

PRINCIPLE OF THE TEST



The **Human Anti-HBcAg IgM** ELISA kit is based on the binding of antibody in samples to HBcAg immobilized on the microwells, and bound antibody is detected by anti-Ig's-specific antibody conjugated to HRP. After a washing step, TMB is added and color (blue) is developed which is directly proportional to the amount of antibody present in the sample. Stopping Solution is added to terminate the reaction, and A450nm is then measured using an ELISA reader. The activity of antibody in samples is determined relative to Calibrators.

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-HuM.2a11, 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
Hep B Core Ag Microwell Strip Plate	4586	8-well strips (12)	Coated with recombinant HBcAg , and post-coated with stabilizers.
Anti-HB Core Ag Calibrators			
1 U/ml 2.5 U/ml 5 U/ml 10 U/ml	4587B 4587C 4587D 4587E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-HBcAg in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial.
Anti-HB Core Ag Positive Control	4587PC	0.65ml	Anti-HBcAg diluted in buffer with protein, detergents and antimicrobial as stabilizers. [Value Range on Label]
Low NSB Sample Diluent	TBTm **Not** for HRP 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.

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

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 5 times the initial dilution and performed the same day 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 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 1:200 or greater dilution for human 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-HBcAg Positive Control**; value range is on the label.
- 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.
- 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.

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. 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 IgM 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.

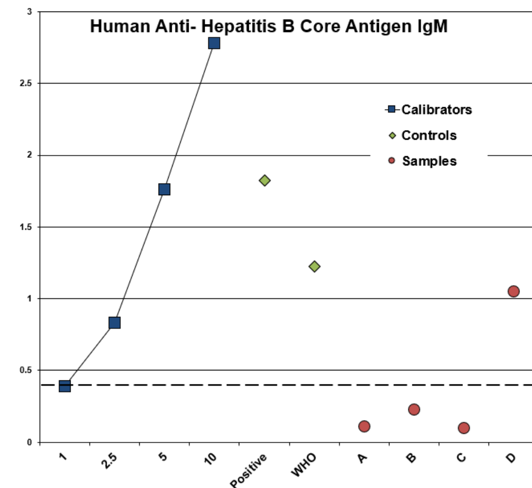
INTERPRETATION OF RESULTS

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-HBcAg IgM, 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 1:200 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of antiserum from anti-HBcAg 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 – an anti-HBcAg serum; value range is on the label. This Control can be used to gauge precision and to normalize between-assay variation.

WHO 1st International Standard B for **Hepatitis B core antigen** (NIBSC 95/522)) – antiserum reactive to HBcAg).
[3.5 U/ml anti-HBc IgM = **10** IU/ml WHO Std.]

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

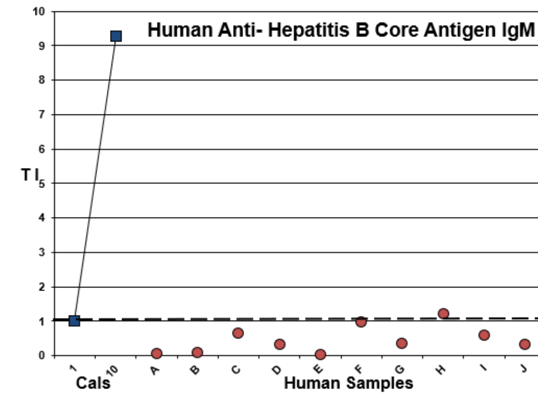
- Divide each Sample net OD by the 1 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

Example:

Human Serum/Plasma IgM

A panel of human serum/plasma of unknown history was tested for anti-HBcAg IgM (1:200 dilution in Low NSB Sample Diluent). **Threshold Index** was calculated using the **1** U/ml **Cal**.



Results

Anti- HBcAg IgM: eight samples were negative (below the **1.0** Threshold Index); 2 samples (F,H) were borderline.

Notes:

1. **Positives** may be due to prior encounter with the virus, similar antigen(s), or an aspect of innate immunity.

2. The **sensitivity** of the assay may be adjusted by changing the sample dilutions: a) increase dilution (e.g., 1:400) to lower the signals of borderline positives to negative; b) decrease dilution (e.g., 1:100) 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. If other biological specimens and/or HBcAg preparations are used for antibody determination, sample dilutions and positive/negative thresholds should be determined using similar 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

= IgM Antibody Activity Units

PRODUCT SPECIFICATIONS

Specificity

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

Assay Sensitivity

The HBcAg -coated plated, the anti-human IgM HRP concentration, and the Low NSB Sample Diluent are optimized to differentiate anti-HBcAg IgM from background (non-antibody) signal with human serum/plasma samples diluted 1:200.

Calibrator Values

The Calibrators are composed of antibody to HBcAg. Values are assigned as arbitrary anti-HBcAg activity units (see Limits of the Assay).

Instruction Manual No. M-4565

Human Anti-Hepatitis B Core Antigen (HBcAg) IgM

ELISA Kit Cat. No. 4565

For Quantitation of Anti-Hepatitis Core Antigen (HBcAg) IgM In Serum or Plasma

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

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ELISA Kit Components	Amount	Part
Hep B Core Ag Coated Microwell Strip Plate	8-well strips (12)	4586
Anti-HB Core Ag Positive Control	0.65 ml	4587PC
Anti-HB Core Ag Calibrator 1 U/ml	0.65 ml	4587B
Anti-HB Core Ag Calibrator 2.5 U/ml	0.65 ml	4587C
Anti-HB Core Ag Calibrator 5 U/ml	0.65 ml	4587D
Anti-HB Core Ag Calibrator 10 U/ml	0.65 ml	4587E
Anti-Human IgM HRP Conjugate (100X)	0.15 ml	H-HuM.2a11
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-4565