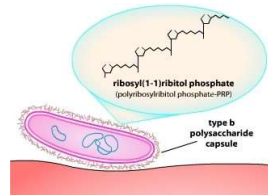


## INTENDED USE

The Rat Anti-Hib-PRP IgG ELISA Kit detects and quantifies Hib-PRP-specific IgG in Rat serum or plasma of vaccinated or immunized hosts. 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).

## GENERAL INFORMATION



*Hemophilus influenzae* type b (Hib) is a gram-negative coccoidal, capsular bacterium that causes, in Rats, especially in infants, bacteremia, pneumonia and acute bacterial meningitis. Vaccines prepared using the capsular polysaccharide (PRP:

polyribosyl ribitol phosphate) have been effective in providing protection and minimizing the disease in populations that are routinely immunized. The PRP conjugated to various proteins, such as tetanus and diphtheria toxoid and meningococcal outer membrane protein, have been highly effective in broadening the range of immunity against disease. The Hib vaccines are also available combined with other vaccines, with a pentavalent vaccine (Hib-diphtheria-pertussis-tetanus-hepatitis B) being widely used worldwide.

The ADI Anti-Hib-PRP ELISA will quantify antibodies specific to the PRP antigen, regardless of carrier protein used for immunization.

## PRINCIPLE OF THE TEST

The Rat Anti-Hib-PRP IgG ELISA kit is based on the binding of Rat anti-Hib-PRP IgG in samples to PRP immobilized on the microwells, and anti-Hib-PRP IgG antibody is detected by anti-Rat 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-Hib-PRP 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 Rat IgG antibody in samples is calculated relative to anti-Hib-PRP Calibrators.

## PRODUCT SPECIFICATIONS

### Specificity

Purified Hib-specific polysaccharide (PRP) conjugated to Rat albumin (HA) is used to coat the microwells; thus the assay is specific for antibodies directed to PRP. The anti-Rat IgG HRP conjugate reacts with Rat IgG antibodies that bind to PRP-HA on the plate. IgA, IgM and IgE class antibodies would not be measured above background signals.

### Assay Sensitivity

The PRP-HA coating level, HRP conjugate concentration and Low NSB Sample Diluent are optimized to differentiate anti-Hib-PRP IgG from background (non-antibody) signal with Rat serum samples **diluted 1:100**.

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## 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
<b>Wash Solution Concentrate (100x)</b> Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as <b>Wash Solution</b> and store at ambient temperature until kit is used entirely.
<b>Sample Diluent Concentrate (20x)</b> Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as <b>Working Sample/Conjugate Diluent</b> and store at 2-8°C until the kit lot expires or is used up.
<b>Anti-Rat IgG - HRP Conjugate Concentrate (100x)</b> Part H-HuG.211, 0.15ml	Peroxidase conjugated anti-Rat IgG in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of <b>Working Sample/Conjugate Diluent</b> 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
<b>HbO-HA Microwell Strip Plate</b>	980-131	8-well strips (12)	Coated with Hib-specific PRP conjugated to Rat albumin, and post-coated with stabilizers.
<b>Anti-Hib-PRP Calibrators</b>			
0.5 U/ml 1 U/ml 2.5 U/ml 5 U/ml	980-132A 980-132B 980-132C 980-132D	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-Hib-PRP levels in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
<b>Rat Anti-Hib-PRP IgG Positive Control</b>	980-102	0.65ml	Rat anti-Hib-PRP IgG; diluted in buffer with protein, detergents and antimicrobial as stabilizers. Net OD > 0.5
<b>Low NSB Sample Diluent</b> Reduces non-specific binding	TBTm  Not for HRP Conjugate dilution.	30 ml	Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for sample dilution. See <b>Assay Design</b> , page 3.
<b>TMB Substrate</b>	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
<b>Stop Solution</b>	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-Rat IgG HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2-1L.
- 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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## LIMITATIONS OF THE ASSAY

### Quantitation of Antibody in a Sample

The ELISA measures anti-Hib-PRP activity, a combination of antibody concentration and avidity for the Hib-PRP antigens. Antibodies with substantially different total Ig concentrations may display similar anti-Hib-PRP 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 Hib-PRP 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 2.5 U/ml Calibrator (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 Rat 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 Rat Anti-Hib-PRP IgG 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**.
- The Calibrators that are used for quantitation, e.g., for between-assay normalization, should be run in duplicate.

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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. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

### 1. 1<sup>st</sup> 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. 2<sup>nd</sup> Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-Rat IgG 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.

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## INTERPRETATION OF RESULTS

### Calculation of Results

Consider several data reduction methods to best represent the relationships among experimental and control groups, to determine **Positive Immune** and **Negative Non-immune**, and to **Quantitate** positive antibody levels.

**Method A. Antibody Activity** [ELISA Signal & Sample Dilution]  
Represent data as net OD units (A450 signal; blank subtracted) + dilution = **Total Activity Units**.

A Calibrator value in the mid-OD range (e.g., 1.0 U/ml) can be used to normalize inter-assay values.

### Method 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.

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 **quantifies** the positive Antibody Activity level.

### Example:

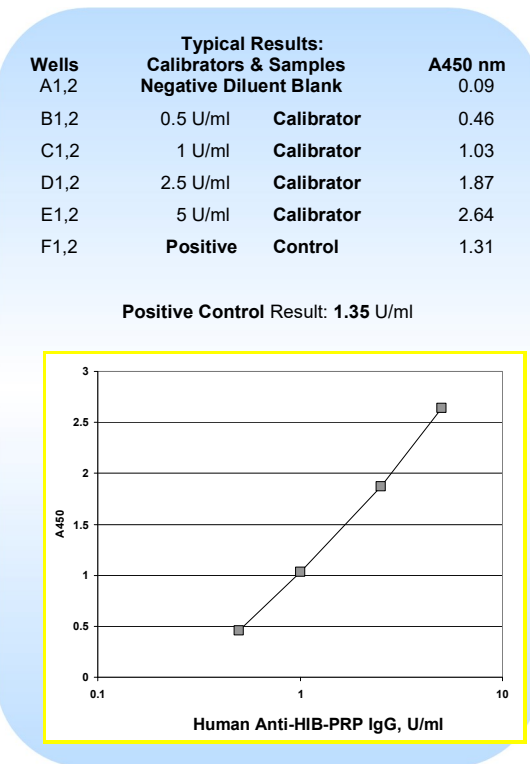
Sample	Assay Net OD		Calculation using Positive Index	
	Control	Exptl	Control	Exptl
1	0.243	2.358	0.49	<b>4.79</b>
2	0.351	0.597	0.71	<b>1.21</b>
3	0.286	1.421	0.58	<b>2.89</b>
4	0.357	1.268	0.73	<b>2.58</b>
5	0.512	0.857	<b>1.04</b>	<b>1.74</b>
6	0.342	1.296	0.70	<b>2.63</b>
7	0.298	0.608	0.61	<b>1.24</b>
8	0.285	0.369	0.58	0.75
9	0.157	0.864	0.32	<b>1.76</b>
10	0.187	0.543	0.38	<b>1.10</b>
Mean	0.302			
SD	0.095			
Mean +2 SD	<b>0.492</b>			<b>= Positive Index</b>

## CALCULATION OF RESULTS (continued)

### Method C. Use of a Calibrator Curve

When the dilution curves of samples are parallel to the Calibrator curve (see Limits of the Assay), the Anti-Hib-PRP activity units may be determined by interpolation from the Calibrator curve. The results may be calculated using any immunoassay software package. If software is not available, Anti-Hib-PRP activity concentrations may be determined as follows:

1. Calculate the mean OD of duplicate samples.
2. On graph paper plot the mean OD of the calibrators (y-axis) against the concentration (U/ml) of Anti-Hib-PRP (x-axis). Draw the best fit curve through these points to construct the calibrator curve. A point-to-point construction is most common and reliable.
3. The Anti-Hib-PRP activity concentrations in unknown samples and controls can be determined by interpolation from the calibrator curve.
4. Multiply the values obtained for the samples by the dilution factor of each sample.
5. Samples producing signals higher than the 5 U/ml calibrator should be further diluted and re-assayed.



### Calibrator Values

The calibrators are dilutions of Rat antibody reactive to Hib-PRP. Values are assigned as arbitrary anti-Hib-PRP activity units.

## CALCULATION OF RESULTS (continued)

### Method D. Titers from Sample Dilution Curves

The titer of 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:

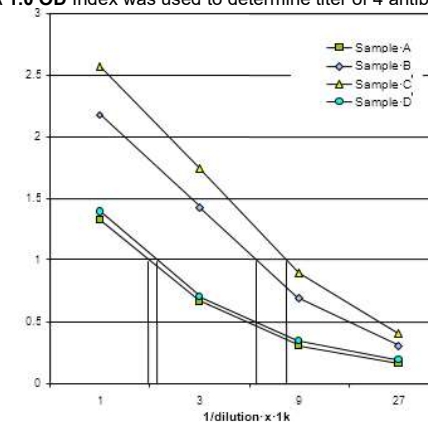
1. Use an OD value Index in the mid-range of the assay (2.0 – 0.5 OD); this provides the best sensitivity and reproducibility for comparing experimental groups and replicates. An arbitrary 1.0 OD is commonly used.
2. Prepare serial dilutions of each sample to provide a series that will produce signals 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. A Calibrator value in the mid-OD range (e.g., 1.0 U/ml) 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**

### Example:

II. A 1.0 OD Index was used to determine titer of 4 antibodies.



### Titer Values

Sample A = 1.72 kU      Sample B = 5.70 kU  
Sample C = 1.85 kU      Sample D = 7.90 kU

### 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](http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10)

## Rat Anti-Hib-PRP IgG (*Hemophilus influenzae* type b Polysaccharide, PRP) ELISA Kit

**Cat. 980-160-PRG, 96 tests**

**For Quantitation of Anti-Hib-PRP  
IgG in Serum or plasma or other  
biological fluids**

For research use only (RUO), not for diagnosis, cure or prevention of the disease.



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THE MANUAL SUPPLIED WITH THE KIT  
FOR ANY LOT SPECIFIC CHANGES.**