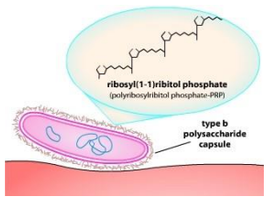
INTENDED USE

The Hemophilus influenzae type b Polyribosyl Phosphate (HIB-PRP) ELISA Kit is an immunoassay for quantifying free **HIB-PRP** in cell culture, vaccine formulations, other qualified samples **or PRP conjugated to carrier proteins (**tetanus toxoid and human albumin etc). For research use only (RUO).

GENERAL INFORMATION



Hemophilus influenzae type b (Hib) is a gram-negative coccoidal, capsular bacterium that causes, 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.

Hib Vaccines: Influenzae B **Comvax** (HepB/Hib; Merck), **PedvaxHib** (Hib-PRP-OMP)–Merck; **Trihibit** (DTAP/Hib), **ActHib** (Hib-PRP-Tetanus toxoid) - Sanofi Pasteur; **HibTiter** (Hib-Hboc) – Wyeth Lederle

PRINCIPLE OF THE TEST

The HIB-PRP ELISA kit is based on competitive binding of a fixed concentration of anti-PRP-HRP conjugate either to PRP coated on the plate or to PRP in the sample. Higher concentrations of PRP in the sample reduces the amount of anti-PRP-HRP that binds to the PRP on the plate. After a washing step, substrate is added and color (blue) is developed. Color intensity is indirectly proportional to the amount of PRP present in the sample. Adding stop solution terminates the reaction (yellow color). Absorbance is then measured on a microtiter well ELISA reader at 450 nm and the concentration of PRP in the sample is read from the standard curve.

STORAGE AND STABILITY

The microtiter well plate and all other reagents, if unopened, are stable at 2-8 ^{o} C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

KIT CONTENTS

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To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
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 Diluent** and store at 2-8ºC until the kit lot expires or is used up.
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 **Working Wash Solution** and store at ambient temperature until kit is used entirely.
Anti-HIB-PRP - HRP Conjugate Concentrate (100x) Part No. 980-PRP-h, 0.15ml	Peroxidase conjugated anti-PRP in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8ºC storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
HIB-PRP antigen Microwell Strip Plate	980-PRP	8-well strips (12)	Coated with HIB-PRP antigen, and post-coated with stabilizers.
HIB-PRP Standards			
10 ng/ml 30 ng/ml 100 ng/ml 300 ng/ml 1000 ng/ml	980-PRP-sB 980-PRP-sC 980-PRP-sD 980-PRP-sE 980-PRP-sF	0.25 ml 0.25 ml 0.25 ml 0.25 ml 0.25 ml	Five (5) vials, containing dilutions of the WHO Intl Std (NIBSC 02/208) for non-conjugated PRP
Positive Control [PRP] range on label	980-PRP-PC	0.25 ml	HIB-PRP of stated concentration range; diluted in stabilizers and antimicrobial.
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 Antibody HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 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

Standards, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains 1% 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
Culture medium, bioprocessing preparations, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference (See Limits of the Assay, page 6). For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature.

For all samples, clarify by centrifugation and/or filtration prior to dilution in Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Assay Validation
Validate the performance of the sample antigen and matrix in the assay system for recovery and parallelism (see Limits of the Assay, page 6), as follows:

Recovery – a measure of the interference of the sample matrix (diluent effect) in providing accurate quantitation of the sample HIB-PRP relative to the Standard curve.

Prepare and run a series of dilutions of the sample antigen (concentrations that will fall within the Standard range) in Working Sample Diluent to determine the dilutions that give consistent and accurate quantitation. For most buffer solutions a minimum 5-fold sample dilution is usually sufficient.

Parallelism – dilutions of the sample should read equivalent values from the top and bottom of the Standard curve to provide good assay precision.

Prepare a dilution series of the sample antigen that gives complete recovery and falls within the full range of the Standard curve. Sample readings from the upper and lower regions of the curve should differ by less than 25%.

Plate Set-up
Bring all reagents to room temperature (18-30^{o} C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 10 Standard 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. Sample Addition

- Add **20ul** of standards, samples and controls each to wells. **[20ul +]**

2. HRP Conjugate Addition **100ul]**

- Immediately: add **100ul** of Working Anti-PRP-HRP Conjugate to wells.
- Tap the plate gently to mix reagents and incubate for **60 minutes at room temperature (22-28º C).**

Note: Std, control & sample addition do not initiate the immunoassay reaction, so front-to-back timing is not a concern. Add the HRP Conjugate, which does initiate the reaction, before sample volumes can evaporate/dry.

- Wash wells 5 times and pat dry on fresh paper towels.

3. Substrate Incubation **[100ul – 15 min]**

- Add 100ul TMB Substrate to each well. Tap the plate gently for a few seconds. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes at room temperature in the dark, e.g., place in a drawer or closet.

Note: a) If your microplate reader does not register optical density (OD) above 2.0, incubate for less time; b) it is OK to stop the reaction early if the 1000 ng/ml Std has color, e.g., if the signal of the Blank or 10 ng/ml Std is becoming too high for the reader. (>2.5-3.00).

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.

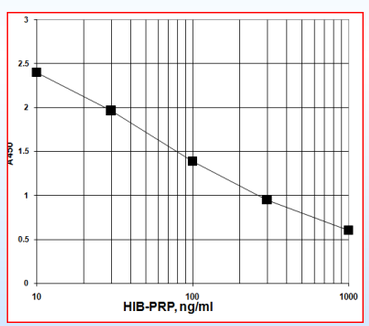
CALCULATION OF RESULTS

1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, HIB-PRP concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the standards (y-axis) against the concentration (ng/ml) of HIB-PRP (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
4. The HIB-PRP concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 1000 ng/ml standard should be further diluted and re-assayed.

Typical Results:

Wells	Calibrators & Samples	**A450 nm**
A1, A2	Diluent Blank	2.52
B1, B2	10 ng/ml **Calibrator**	2.34
C1, C2	30 ng/ml **Calibrator**	1.96
D1, D2	100 ng/ml **Calibrator**	1.49
E1, E2	300 ng/ml **Calibrator**	1.01
F1, F2	1000 ng/ml **Calibrator**	0.63
G1, G2	Positive Control [35 - 65]	1.75

Positive Control = **50.5** ng/ml



PERFORMANCE CHARACTERISTICS

Specificity
The antibodies used for the Anti-PRP HRP are from animals immunized with PRP conjugated to a protein different from the coated PRP protein conjugate. The assay is, therefore, specific for PRP, whether free or bound to another molecule.

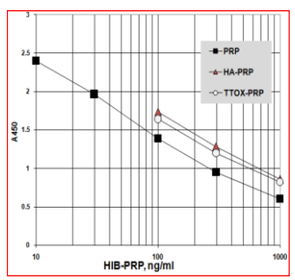
Precision
Samples containing low, medium and high concentrations of HIB-PRP were assayed multiple times in the same assay (n=10) to provide within-assay precision, and as duplicates in multiple assays (n=5) to obtain between-assay reproducibility. Coefficient of variations were calculated for the concentrations using a point-to-point curve-fitting program.

HIB-PRP concentrations were measured with 7.4 to 12.5% CV within-assay and 8.2 to 13.7% CV between-assay reproducibility.

Sample	**HIB-PRP** ng/ml	**Intra-assay** %CV	**Inter-assay** %CV
Low HIB-PRP	52.5	**12.5**	**15.4**
Medium HIB-PRP	264	**7.4**	**8.2**
High HIB-PRP	456	**9.2**	**13.7**

Parallelism & Recovery

Dilutions of the PRP-tetanus toxoid from the ActHIB vaccine (Sanofi-Pasteur) and PRP-human albumin (HA) conjugate (NIBSC) were compared with the PRP Standard curve. Mass values were provided by the respective sources.



Results

- **TTOX-PRP**: parallelism showed **99%** concordance between high and low values; recovery = **44%** of Std value.

Note: PRP bound to TTOX may present a structure to the anti-PRP HRP significantly different from that of the free, non-bound PRP standard; <100% recovery indicates a lower potency relative to the standards. Dilutions of a PRP-conjugate might be used as a standard curve if recovery and/or parallelism deviates significantly from the PRP standards.

- **HA-PRP**: parallelism showed **91%** concordance between high and low values; recovery = **52%** of Std value.

Note: The mass is of combined PRP + HA. The recovery represents a measure of the efficacy of PRP to carrier conjugation.

Hib-PRP conjugated to human albumin and Tetanus toxoid has been tested. ADI HIb-PRP kit is an antibody based assay; chemical conjugation of PRP to the proteins may alter reactivity with the antibody and give different concentration relative to the free HIb standards. Therefore, we suggest that preparations of HIb-carrier proteins (tetanus or diphtheria toxoid, OMP) be tested and concentration values assigned using an internal reference.

QUALITY CONTROL

Reagents Accurate and reproducible assay results rely on proper storage, handling and control of reagent and sample temperature. Store all reagents as indicated, and warm to room temperature only those to be used in the assay. Shelf-life of the critical reagents and samples will diminish with extended exposure to non-refrigeration, resulting in inaccurate assay results. All solutions should be clear. Cloudiness or particulates are indications of reagent contamination or instability and may interfere with proper performance of the assay. Do not use.

Sample Controls A Positive Serum Control is provided with the kit, assigned with an HIB-PRP concentration value range. Recovery in this range is an indicator of proper assay performance. Each lab should also assay internal control samples, which represent the lab's expected sample population and that are maintained stabilized. A Sample Diluent blank should also be run; OD should be >2.0 and higher than 10 ng/ml Standard OD.

Standard Curve The signal generated by the standards should be continuously decreasing in OD from the lowest Standard to the highest Standard, with a range greater than 1.2 OD. Non-uniform or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. Do not rely on results generated from an assay with these issues.

Technique Accurate and reproducible assay results rely on good lab technique regarding pipetting, plate washing and handling of samples and reagents.

Equipment Precision of results relies on uniform and effective washing techniques; an automatic washer may be used. ELISA reader and pipettes should be properly calibrated.

RELATED ITEMS

980-100-PHG Human Anti-H. Influenzae B (Hib) polyribosyl phosphate (PRP) IgG ELISA Kit, 96 tests

980-101-HNC Human anti-H. Influenzae B (Hib-PRP) IgG negative control serum

980-101-HPC Human anti-H. Influenzae B (Hib-PRP) IgG positive control serum

980-110-PHM Human Anti-H. Influenzae B (Hib) polyribosyl phosphate (PRP) IgM ELISA Kit, 96 tests

980-120-PMG Mouse Anti-H. Influenzae B (Hib) polyribosyl phosphate (PRP) IgG ELISA Kit, 96 tests

980-130-PRG Rabbit Anti-H. Influenzae B (Hib) polyribosyl phosphate (PRP) IgG ELISA Kit, 96 tests

980-140-PRM Rabbit Anti-H. Influenzae B (Hib) polyribosyl phosphate (PRP) IgM ELISA Kit, 96 tests

980-150-PKG Monkey Anti-H. Influenzae B (Hib) polyribosyl phosphate (PRP) IgG ELISA Kit, 96 tests

980-HIB-AG1 Human Anti-H. Influenzae B (Hib) polyribosyl phosphate (PRP) antigen (vaccine) ELISA Kit, 96 tests

980-VID-Hib-48 ID-VAC H. Influenzae B (Hib-PRP) vaccine Identification ELISA Kit (Confirm the presence of active ingredients in commercial vaccines), 48 tests

Instruction Manual No. M-980-HIB-AG1

Hemophilus influenzae type b Polyribosyl Phosphate (HIB-PRP)

ELISA Kit Cat. No. 980-HIB-AG1

For Quantitation of HIB-PRP in Solution or in Vaccines

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

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ELISA Kit Components	**Amount**	**Part**
HIB-PRP Coated Microwell Strip Plate	8-well strips (12)	HIB-PRP
HIB-PRP Positive Control	0.25 ml	980-PRP-PC
HIB-PRP Standard 10 ng/ml	0.25 ml	980-PRP-sB
HIB-PRP Standard 30 ng/ml	0.25 ml	980-PRP-sC
HIB-PRP Standard 100 ng/ml	0.25 ml	980-PRP-sD
HIB-PRP Standard 300 ng/ml	0.25 ml	980-PRP-sE
HIB-PRP Standard 1000 ng/ml	0.25 ml	980-PRP-sF
Anti-HIB-PRP HRP (100X)	0.15 ml	980-PRP-h
Sample Diluent Concentrate (20x)	10 ml	SD20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	980-HIB-AG1