INTENDED USE

The Mouse Anti-Pneumococcal CPS23 IgG ELISA Kit quantifies IgG antibodies against capsular polysaccharides (CPS) of 23 serotypes of *S. pneumoniae* in Mouse serum or plasma of vaccinated and/or infected hosts. This test is suitable for:

- Determining **immune status** relative to controls;
- Assessing efficacy of **vaccines**, including dosage, adjuvantcy, route of immunization and timing;
- Qualifying and standardizing vaccine batches & protocols.

GENERAL INFORMATION

Streptococcus pneumoniae, or pneumococcus, a Gram-positive bacteria, is one of the most common causes, along with *N. meningitides*, of bacterial meningitis in adults and young adults, Pneumococcal strains have capsular polysaccharides (**CPS**) that acts as a virulence factor for the organism; more than 90 different serotypes are known. Serotype specific antibodies against the CPS provide protection against the corresponding strains. The pneumococcal vaccine most commonly used today consists of purified polysaccharides from **23 serotypes** (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F;non-conjugated: **Pneumovax** by Merck). Pneumococcal conjugate vaccine (PCV) contains polysaccharides conjugated to diphtheria toxin **CRM197**, with three PCV vaccines currently available: **Prevnar-7** or PCV-7 (Wyeth) is a 7-valent vaccine (4, 6B, 9V, 14, 18C, 19F, and 23F); **Synflorix** (GlaxoSmithKline) is a 10-valent vaccine (PCV-10: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F); **Prevnar 13** (Pfizer) is 13-valent (PCV-13: 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F); each are conjugated to carrier protein CRM197.

PRINCIPLE OF THE TEST

The Anti-CPS23 IgG ELISA kit is based on the binding of anti-CPS Ig's in samples to the purified CPS antigens coated on the microwells; bound antibodies are detected by specific antibody-HRP conjugated. After a washing step, chromogenic substrate (TMB) is added and color is developed, which is directly proportional to the amount of antibody present in the samples. Stopping Solution is added to terminate the reaction, and absorbance at 450nm (yellow color) is then measured using an ELISA reader. The activity of CPS antibody in samples is determined relative to anti-CPS specific IgG calibrators.

PRODUCT SPECIFICATIONS

Specificity

The plate is coated with a mixture of the purified non-conjugated 23 CPS used in Pneumovax (Merck); no antibodies to CRM197 will be detected. The anti-Mouse IgG HRP conjugate specifically detects IgG, and does not react with IgM, IgA or IgE class antibodies. ADI has other ELISA kits to detect antibodies to a given carbohydrate (e.g. 6B or others) and the carrier protein, CRM197.

All pneumococci, both virulent and avirulent strains possess a common polysaccharide, **CWPS** (Cell Wall PolySaccharide, teichoic acid). CPS antigens used in the vaccines or in the ELISA are contaminated with CWPS and other impurities. Therefore, the serotype specific anti-CPS pneumococcal IgG ELISA requires an **adsorption step** to remove the unprotective, non-specific CWPS antibodies. Recently WHO recommended an extra absorption step with **22F CPS** to remove cross reactive antibodies and to have better measurement of CPS-specific antibodies. **ADI provides a separate kit to perform and measure adsorption with CWPS/22F (#560-410-C22).**

Please consult the additional guidelines on testing samples for anti-CPS antibodies:
(http://4adi.com/objects/catalog/product/extras/ADI_Res_Bull_2013_Pneumococcal_Vaccine_tests.

Assay Sensitivity

The CPS23-coated plate, anti-Mouse IgG HRP concentration, and Low NSB Sample Diluent are optimized to differentiate anti-CPS23 IgG from control and immune when samples are tested at a dilution of 1:100 or higher.

KIT CONTENTS

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.

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 0.5ml + 9.5ml with distilled or deionized water as needed for HRP Conjugate and Sample Dilution. Label as **Working Sample Diluent** and store at 2-8^{o}C until the kit lot expires or is used up.
Anti-Mouse IgG-HRP Conjugate Concentrate (100x) Part No. : H-MsG-112b, 0.15ml	Peroxidase conjugated is in a 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 2-8^{o}C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Pneumococcal 23 Type CPS Microwell Strip Plate	560-161	8-well strips (12)	Coated with 13 serotypes of CPS antigen, and post-coated with stabilizers.
Anti-Pneumococcal Ig's 23 Calibrators			
10 U/ml 25 U/ml 50 U/ml 100 U/ml	560192B 560192C 560192D 560192E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-pneumococcal CPS IgG levels in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
Positive Control	560190-PC	0.65 ml	Mouse anti-CPS23 IgG positive control (unads.)
The Calibrators values are assigned as arbitrary anti-CPS23 antibody activity units (see Limits of the Assay).			
Low NSB Sample Diluent	TBTm	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 (100ul, 1-10ml) and multi-channel pipettor Disposable
- 5-15ml , bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength and ELISA washer.
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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 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-CPS23 activity, a combination of antibody concentration and avidity for the CPS23 antigens. Antibodies with substantially different total IgG concentrations may display similar anti-CPS23 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, 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 CPS23 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 50 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 Always wear gloves when handling serum-containing samples, including the standards and controls, and dispose of these samples and containers as biohazard waste.

Sample Adsorption and ELISA Specificity

This kit will measure anti-CPS Ig's in adsorbed or non-adsorbed samples. Mouse samples have high levels of non-specific anti-CWPS/22F antibodies that must be adsorbed to measure vaccine specific antibodies.

Antibody Stability

Initial dilution of serum into **Working Sample Diluent** (1XSD20T) is recommended to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for months, stored refrigerated or frozen. Further dilution into **Low NSB Sample Diluent** (TBTm), 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 (1/10): **10**ul serum + **90**ul 1XSD20 [or 0.1ml + 0.9ml]
Further (1/100): **10** ul initial (1/10) + **90**ul TBTm

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 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 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**, 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.

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 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, controls and samples and 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 Antibody-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 microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read 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

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., 50 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:

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

= **Positive Index**

CALCULATION OF RESULTS (continued)

Method B. Use of a Calibrator Curve

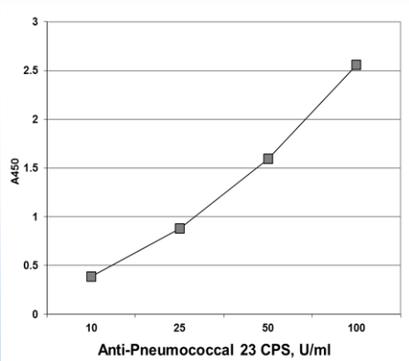
When the dilution curves of samples are parallel to the Calibrator curve (see Limits of the Assay), the anti-CPS23 activity units may be determined by interpolation from the Calibrator curve, as follows:

1. The results may be calculated using any immunoassay software package. If software is not available, anti-CPS23 activity concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the calibrators (y-axis) against the concentration (U/ml) of anti-CPS23 (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.
4. The anti-CPS23 activity concentrations in unknown samples and controls can be determined by interpolation from the calibrator curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 100 U/ml calibrator should be further diluted and re-assayed.

Typical Results:

Wells	Calibrators		A450 nm
A1,2	**Sample Diluent Blank**		0.13
B1,2	10 U/ml	**Calibrator**	0.39
C1,2	25 U/ml	**Calibrator**	0.88
D1,2	50 U/ml	**Calibrator**	1.59
E1,2	100 U/ml	**Calibrator**	2.56
F1,2	Sample	**1:100**	1.21

Sample Result: **34.5** U/ml x 100 dilution = **3.54 k**U/ml



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., 50 U/ml) can be used to normalize inter-assay values.

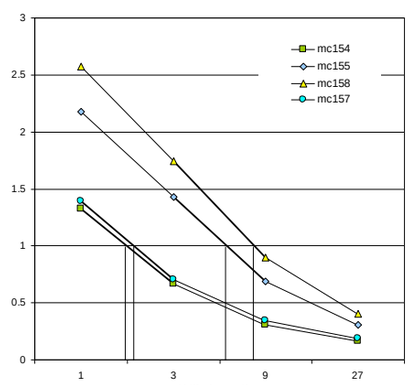
Calculations

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
   = **Total IgG Antibody Activity Units**

Example:

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



Titer Values
mc154 = 1.72 kU
mc155 = 5.70 kU
mc157 = 1.85 kU
mc158 = 7.90 kU

Instruction Manual No. M-560-180-23G

Mouse Anti-Pneumococcal CPS23 IgG

ELISA Kit #. 560-180-23G

For Quantitation of Anti-CPS IgG to 23 Serotypes in Mouse Serum or Plasma

ALPHA DIAGNOSTIC INTERNATIONAL

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Your Molecular & Cell Technology Partner

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ELISA Kit Components	**Amount**	**Part**
Pneumococcal 23 type CPS Coated Microwell Strip Plate	8-well strips (12)	560191
Anti-Pneumococcal 23 Calibrator 10 U/ml	0.65 ml	560191B
Anti-Pneumococcal 23 Calibrator 25 U/ml	0.65 ml	560191C
Anti-Pneumococcal 23 Calibrator 50 U/ml	0.65 ml	560191D
Anti-Pneumococcal 23 Calibrator 100 U/ml	0.65 ml	560191E
Anti-Pneumococcal 23 IgG **Positive Control**	0.65 ml	560190-PC
Anti-Mouse IgG HRP Conjugate (100X)	0.15 ml	
Sample Diluent (20X)	10 ml	SD20T
Low NSB Sample Diluent (LNSD)	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-560-180-13G

DRAFT MANUAL: PLEASE COSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.