INTENDED USE

The **Mouse Anti-Pneumococcal 03 CPS (Pn03) IgG ELISA Kit** quantifies IgG antibodies against the capsular polysaccharide of serotype 03 of *S. pneumoniae* in mouse serum or plasma of vaccinated, or infected hosts. This immunoassay 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

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

GENERAL INFORMATION

Streptococcus pneumoniae, or pneumococcus, a Gram-positive bacterium, 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 act 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 is conjugated to carrier protein: Tetanus or Diphtheria toxoid or Hemophilus D protein.

PRINCIPLE OF THE TEST

The Mouse Anti-Pneumococcal serotype CPS IgG ELISA kit is based on the binding of mouse anti-CPS in samples to the purified CPS antigen coated on the microwells; bound antibodies are detected by anti-mouse IgG-specific antibody conjugated to HRP enzyme. After a washing step, chromogenic substrate (TMB) is added and color (blue) 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 mouse antibody in samples is determined relative to anti-CPS specific IgG calibrators.

PRODUCT SPECIFICATIONS

Specificity
The plate is coated with purified non-conjugated type 03 CPS as contained in the vaccines described above; this preparation is contaminated with **CWPS** (Cell Wall PolySaccharide, techoic acid), which is common to all pneumococci, both virulent and avirulent strains. While sera from most normal humans contain antibodies to CWPS, laboratory mice would normally acquire reactivity upon vaccination. An **adsorption step** is required to remove these antibodies to allow discrimination from antibodies to the CPS. **ADI provides a separate kit to perform and measure adsorption with CWPS/22F (#560-430-C22).** The anti-Mouse IgG HRP conjugate specifically detects IgG, and does not react with IgM, IgA, or IgE class antibodies.

Assay Sensitivity
The CPS 03-coated plate, anti-Mouse IgG HRP concentration, and Low NSB Sample Diluent are optimized to differentiate anti-03 CPS IgG from control and immune animals when samples are tested at a dilution of 1:50 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 **Working 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.2a11, 0.15ml	Peroxidase conjugated anti-Mouse IgG 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 2-8^{o}C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Pneumococ-cal Type 03 CPS Coated Strip Plate	560-03	8-well strips (12)	Coated with Pn03 CPS antigen and post-coated with stabilizers.
Anti-Pneumococcal 03 Calibrators			
1 U/ml 2.5 U/ml 5 U/ml 10 U/ml	560-03-rSB 560-03-rSC 560-03-rSD 560-03-rSE	0.65ml 0.65ml 0.65ml 0.65ml	Four (4) vials, each containing anti-Pn03 CPS; in buffer with antimicrobial.
Anti- 03 CPS Positive Control	560-03-PC	0.65ml	Anti-Pn03 CPS; diluted in buffer with protein, detergents and antimicrobial. [value range on vial]
Low NSB Sample Diluent Reduces non-specific binding	TBTm **Not** for HRP Conjugate dilution	30 ml	Buffer with protein, detergents, and antimicrobial. Use as is for sample dilution. See **Assay Design**, page 3.
TMB Substrate	80091	12 ml	HRP substrate (TMB) ready to use
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-Mouse IgG 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.

ASSAY DESIGN AND SET-UP

Sample Collection and Handling
Serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Antibody Stability & Dilution
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): **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 Interpretation of Results (p. 5 & 6) before proceeding:

- Pre-adsorb samples with **CWPS/22F** antigen, if necessary (see Specificity, page 1)
- 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 lower than the **1 U/ml Calibrator**. This is usually 1/50 or greater dilution for mouse serum 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. Blank OD should be <0.3.
- Run the Anti-Pneumococcal 03 **Positive Control**; value range on label.
- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **10 U/ml** should give a high signal (>1.5 OD); **1 U/ml** should give a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5).

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 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 1-5 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, 1x diluent (blank) 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-Mouse IgG HRP to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 1.

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.

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.

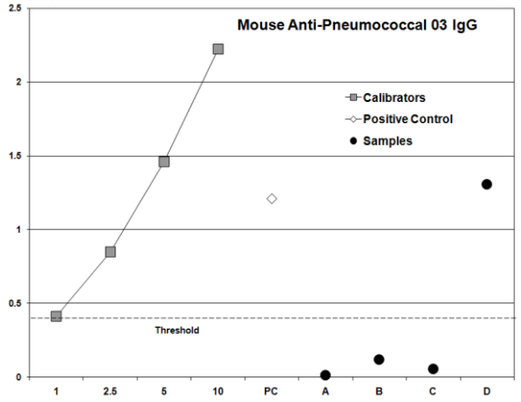
INTERPRETATION OF RESULTS

Method 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-Pn 03 CPS IgG, from either natural infection or vaccination, is controlled so that the **1 U/ml Calibrator** represents a threshold OD for most true positives in mouse serum diluted to 1:50 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of an anti-Pn 03 CPS antiserum, derived from Pn CPS vaccination, 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 – antiserum with reactivity to Pn 03 CPS; net OD > **0.5**. This Control may be used to normalize between-assay variation.

Samples A,B,C,D – 3 samples (1/50) (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 is **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

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 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 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 Calibrator 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

= IgG Antibody Activity Units

PLATE SPECIFICITY PERFORMANCE

Pneumococcal CPS Type-Specific Antisera

Rabbit antisera (Statens Serum Inst.), each specific for several Pn CPS types and absorbed of anti-Cell Wall Polysaccharide (**CWPS**) activity, were assayed at 1:10k dilution on plates with various mixes of CPS types:
03 [CPS type 03 only]
7 pool [4,6B,9V,14,18C,19F,23F (PCV-7)];
13 pool [same as 7 pool + 1,3,5,6A,7F,19A (PCV-13)];
23 pool [same as 13 pool (-6A) + 2,8,9N,10A,11A,12F, 15B,17F, 20,22F,33F (PneumoVax)]

A [1, 2, 4,18]	-	O	O	O	-
P [1,7,14,19]	-	O	O	O	-
Q [6,18, 23]	-	O	O	O	-
R [3, 4, 9,12]	O	O	O	O	-
S [5, 8,10,15,17]	-	-	O	O	-
T [2,11, 20, 22, 33]	-	-	-	O	-
Pneumococcal CPS Type:	03	7 pool	13 pool	23 pool	cwps

Reaction: Positive (O), Weak (±), Negative (-)

Results
CPS Type **03** – reacted only with R, which has specificity for this CPS type; no reaction with other specificities.

Pneumococcal Vaccine-specific Antisera

The 03 and pool plates were reacted with cwps-absorbed antisera from rabbits immunized with commercial Spn vaccines, and the WHO International Standard (NIBSC 007) from humans vaccinated with Pneumovax (PCX-23). CPS types in each vaccine are shown above; PCV-10 (Synflorix) contains the same types as PCV-7 + 1,5,51.

PCV-7	-	O	O	O	-
PCV-10	-	O	O	O	-
PCV-13	O	O	O	O	-
PCX-23	±	O	O	O	-
WHO 007	O	O	O	O	-
Pneumococcal CPS Type:	03	7 pool	13 pool	23 pool	cwps

Results
CPS Type **03** – positive reactivity with anti-PCV-13 (1:1000) and anti-PCX-23 and the WHO Std (each contains 03) at a 1:100 dilution.

LIMITS OF THE ASSAY

- The sensitivity of the assay may be increased to perhaps convert a borderline sample to a positive by using a lower dilution of the sample, e.g., 1/25. The values of negatives may increase, so an alternative threshold should be established using known negatives to develop a **Positive Index** (page 6), or by using known **Internal Controls** as discriminator for a **Threshold Control** (instead of the kit Calibrators).
- Anti-Spn antibody levels of an infected or immunized animal may be below detection threshold related to the time course of the infection, e.g., too early for positive titer development.

Instruction Manual No. M-560-200-03G

Mouse Anti-Pneumococcal Type 03 CPS IgG

(Pn03/03/17-X)

Cat. #560-200-03G

For Quantitating Anti-Capsular Polysaccharide (CPS) IgG to Serotype 03 in Mouse Serum/Plasma

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

ALPHA DIAGNOSTIC INTERNATIONAL

4638 N Loop 1604 W • San Antonio • Texas 78249 • USA

Phone (210) 561-9515 • Fax (210) 561-9544

Toll Free (800) 786-5777

Email: service@4adi.com

ELISA Kit Components	Amount	Part #
Pneumococcal Type 03 CPS Coated Strip Plate	8-well strips (12)	560-03
Anti-Pneumococcal 03 Positive Control	0.65 ml	560-03-rPC
Anti-Pneumococcal 03 Calibrator 1 U/ml	0.65 ml	560-03-rSB
Anti-Pneumococcal 03 Calibrator 2.5 U/ml	0.65 ml	560-03-rSC
Anti-Pneumococcal 03 Calibrator 5 U/ml	0.65 ml	560-03-rSD
Anti-Pneumococcal 03 Calibrator 10 U/ml	0.65 ml	560-03-rSE
Anti-Mouse IgG HRP Conjugate (100X)	0.15 ml	H-MsG.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-560-200-03G