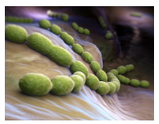
INTENDED USE

The Mouse Anti-Pneumococcal CWPS/22F (CW22) IgM ELISA Kit quantifies IgM antibodies against common wall polysaccharides (CWPS) and serotype 22F polysaccharides of *S. pneumoniae* in mouse serum/plasma of vaccinated, and/or infected hosts. The kit also provides for absorption of anti-CW22 antibodies for further assessment of anti-pneumococcal antibodies to capsular polysaccharides of various serotypes in other immunoassays.

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 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** (non-conjugated: **Pneumovax** by Merck). Pneumococcal conjugate vaccines (PCV) contain polysaccharides conjugated to diphtheria toxin **CRM197**, with three PCV vaccines currently available: **Prevnar-7** or PCV-7 (Wyeth) is a 7-valent vaccine; **Synflorix** (GSK) is a 10-valent vaccine (PCV-10); **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.

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 IgM ELISA requires an **adsorption step** to remove the non-protective, non-specific CWPS antibodies. Recently, the WHO recommended an extra absorption step with **22F CPS** to remove cross reactive antibodies and to have better measurement of CPS-specific antibodies. This kit provides ready-to-use CWPS/22F absorption mix and coated plates to verify the efficacy of the adsorption **before measuring the CPS-specific (7, 11, 13, 23 or individual CPS) IgG or IgM.**

PRINCIPLE OF THE TEST

The Mouse Anti-CW22 IgM ELISA kit is based on the binding of anti-CW22 IgM in samples to the CW22 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 CW22 antibody in samples, including absorbed samples), is determined relative to anti-CW22 specific Controls.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8oC 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 4°C for long term and RT for short term.
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-Mouse IgM-HRP Conjugate Concentrate (100x) Part: H-MsM.211, 0.15ml	Peroxidase conjugated anti-mouse IgM in buffer with detergents and antimicrobial. 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
Pneumococcal CWPS Coated Strip Plate	560-CWPS	8-well strips (12)	Coated with CWPS and 22F CWPS2 antigen, and post-coated with stabilizers.
Anti-Pneumococcal CW22 Controls			
Positive Control	560-CW-rPC	1.0ml	**High** level Anti-CW22, buffer, antimicrobial.
Sensitivity Control	560-CW-rSC	1.0ml	**Low** level Anti-CW22, buffer, antimicrobial.
CWPS/22F Absorbent	560-CW-Abs	0.55ml	CWPS + 22F CWPS2. Use 10ul/200ul serum (see Assay Design,p3)
Low NSB Sample Diluent (LSNB) 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	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.
- Disposable glass or plastic 5-15ml tubes
- Stock bottle to store diluted Wash Solution; 0.2 to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- ELISA reader at 450 nm and ELISA plate washer

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
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 (LSNB)**, which provides the lowest assay background, should be at least 5 times the initial dilution and performed the same day as the assay.

Assay Design
Review Interpretation of Results (p5-7) 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 lower than the **Sensitivity Control**. This is usually 1/50 or greater dilution for mouse serum/plasma with normal levels of IgG and IgM.
- Pre-adsorb samples and **Positive Control** with **CWPS/22F** antigen (see Example below):
  1. Dilute each sample 1:10 into **WSD** for stability. DO NOT dilute the Positive Control
  2. Add **CWPS/22F Absorbent** at 1:20, e.g. 10ul + 200ul sample (1:10). Mix and let stand for 1 hour or longer at ambient temperature (or overnite refrigerated). Stable for at least 2 months refrigerated.
  3. Dilute the absorbed sample (1:10) at least 5-fold in **LSNB**, e.g., 40ul (1:10) + 160ul LSNB = 200ul (1:50). DO NOT dilute the Positive Control.
  4. Assay the absorbed samples and Controls as per Procedure.
- 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 a set of **Controls**, which validate that the assay was performed to specifications: **Positive** should give a high signal (>1.5 OD); upon **Absorption**, OD should be less than the **Sensitivity,** a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5).

Example:

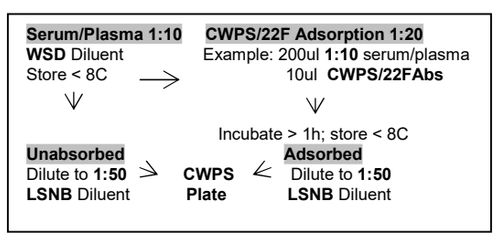


Plate Set-up
Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8 Calibrator wells and 2 wells for each sample 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-Mouse 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.

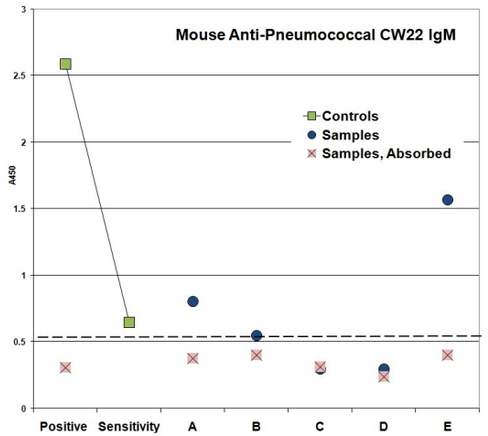
INTERPRETATION OF RESULTS

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-Pn CW22 IgM, from either natural encounter or vaccination, is controlled so that the **Sensitivity Control** 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:

Positive Control –anti- Pn CW22 antiserum, derived from Pn CPS vaccination, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.
Absorbed – absorbed with the **CWPS/22F Absorbent**, according to protocol; signal is below the threshold.

Sensitivity Control - 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.

Samples (1/50) A,B,C,D,E – 2 samples (**A, B**) from mouse sera pools appear at the threshold; absorbed are negative = below the threshold; 2 samples (**C,D**) from naïve mice are negative; sample **E** (1/1000) from PN CPS vaccinated mouse is positive = clearly above the threshold; absorbed sera is negative, indicating removal of anti-CW22 antibody.

The **Sensitivity Control** can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative:

- Divide each Sample net OD by the **Sensitivity Control** net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

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.

Example:

	Assay Net OD		Calculated Antibody Activity	
Sample	Control	Exptl	Control	Exptl
1	0.248	**P** 2.212	0.79	**7.04**
2	0.290	**S** 0.452	0.92	**1.44**
3	0.186	**I** 0.541	0.59	**1.72**
4	0.276	**U** 0.212	0.88	0.68
5	0.161	**U** 0.122	0.51	0.39
6	0.173	**M** 1.491	0.55	**4.75**
7	0.153	**M** 0.694	0.48	**2.21**
8	0.211	**N** 1.487	0.67	**4.74**
9	0.145	**N** 0.546	0.46	**1.74**
10	0.110	**U** 0.263	0.35	0.84
Mean	0.195			
SD	0.0595			
Mean +2 SD	**0.314**	= **Positive Index**		

Results

Experimental Samples are represented as follows:
P – Positive Control
S – Sensitivity Control
I – Internal Control; lab's threshold positive serum
U – Uninfected mouse sample
M – samples represent injection of Pn CPS antigen into mice; positive indicates presence of CW22 antigen in the inoculum.
N – Naturally infected mice samples.

INTERPRETATION OF RESULTS (cont)

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 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
The plate is coated with a mixture of the purified non-conjugated CWPS and 22F CWPS2; also used as the CWPS/22F Absorbent.

The anti-mouse IgM HRP conjugate specifically detects IgM, and does not react with IgG, IgA or IgE class antibodies.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Controls, 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

Instruction Manual No. M-560-435-C22

Mouse Anti-Pneumococcal Common Wall and 22F Polysaccharides (CW22) IgM ELISA Kit

Catalog # 560-435-C22, 96 Tests

For Absorption and Quantitation of Anti-CW22 Antibodies in Mouse Serum or Plasma

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

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ELISA Kit Components	Amount	Part #
Pneumococcal CWPS Coated Strip Plate	8-well strips (12)	560-CWPS
Anti-Pneumococcal Positive Control	1.0 ml	560-CW-rPC
Anti-Pneumococcal Sensitivity Control	1.0 ml	560-CW-rSC
CWPS/22F Absorbent	0.55 ml	560-CW-abs
Anti-Mouse IgM HRP Conjugate (100X)	0.15 ml	H-MsM.211
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-560-435-C22