VaccZyme™ Anti-PCP IgG2 Enzyme Immunoassay Kit

For *in-vitro* research use

Product Code: MK013

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1 INTENDED USE

This assay is designed for the *in-vitro* measurement of specific IgG2 antibodies against Pneumococcal Capsular Polysaccharide (PCP) present in human serum.

Sufficient materials are supplied to allow a maximum of 41 samples to be tested in duplicate or 89 in single, with a calibration curve and two controls.

2 BACKGROUND

This kit is designed to measure IgG2 subclass antibody responses to pneumococcal vaccines incorporating 23 polysaccharides isolated from *Streptococcus Pneumoniae.*[1] These polysaccharides represent approximately 80% of the commonly encountered virulent serotypes. Immunisation with 23-valent vaccines is recommended for elderly patients to reduce death due to pneumonia and for children over 2 years that are at risk of life threatening pneumococcal infection.[2]
The response in 30% of subjects to vaccination with *S. pneumoniae* is attributable to C-polysaccharide (CPS) antibodies and not too specific anti-PCP IgG2. These CPS antibodies confer limited protection against pneumococcal infection, consequently CPS absorption has been incorporated in this assay.[3]
Pneumococcal polysaccharide antigens elicit the production of mainly IgG2 antibodies, via a thymus independent response.[4] Patients with repeated bacterial infections should be investigated for immunodeficiency and thus inability to respond to specific polysaccharide antigens (review[5/6]).
A patient's specific antibody response may be evaluated by the serological determination of their IgG2 anti-PCP antibody levels pre- and post-vaccination using this quantitative enzyme immunoassay.

3 PRINCIPLE OF THE ASSAY

Microwells are pre-coated with the PCP antigen (1-5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F –Danish nomenclature). Samples are diluted in a diluent containing CPS. The calibrators, controls and diluted patient samples are added to the wells and antibodies recognising the PCP antigen bind during the first incubation.

After washing the wells to remove all unbound proteins, purified peroxidase sheep labelled anti-human IgG2 (γ chain specific) conjugate is added. The conjugate binds to the captured human antibody and the excess unbound conjugate is removed by a further wash step. The bound conjugate is visualised with 3,3',5,5' tetramethylbenzidine (TMB) substrate which gives a blue reaction product, the intensity of which is proportional to the concentration of antibody in the sample. Phosphoric acid is added to each well to stop the reaction. This produces a yellow end point colour.

4 PRECAUTIONS

4.1 WARNING

All donors of human sera supplied in this kit have been serum tested and found negative for Hepatitis B surface antigen (HBsAg) and antibodies to human immunodeficiency virus (HIV 1 and HIV 2) and Hepatitis C virus. The assays used were either approved by the FDA (USA) or cleared for *in vitro* diagnostic use in the EU (Directive 98/79/EC, Annex II); however these tests cannot guarantee the absence of infective agents. Proper handling and disposal methods should be established as for all potentially infective material, including (but not limited to) users wearing suitable protective clothing at all times. Only personnel fully trained in such methods should be permitted to perform these procedures.
This product contains sodium azide and Proclin 300 and must be handled with caution; suitable gloves and other protective clothing should be worn at all times when handling this product. Do not ingest or allow contact with the skin (particularly broken skin or open wounds) or mucous membranes. If contact does occur wash with a large volume of water and seek urgent medical advice. Explosive metal azides may be formed on prolonged contact of sodium azide with lead and copper plumbing; on disposal of reagent, flush with a large volume of water to prevent azide build-up.
The buffers and serum supplied in this kit contain various enzyme inhibitors as listed below. These are hazardous and should be handled with care.

INHIBITOR	CONCENTRATION
Sodium Azide	0.099%
Proclin™ 300	0.02%-0.045%
Bromonitrodioxane	0.002%
Methylisothiazone	0.002%

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The stop solution contains 3M phosphoric acid, which is an irritant. Do not allow contact with skin or eyes.
Reagent spills should be cleaned up appropriately, observing local and environmental regulations.

4.2 CAUTION

This kit is for *in-vitro* research use only. Not for use in diagnostic procedures.
This product should only be used by appropriately trained personnel.
Strict adherence to the protocol is recommended. Any deviation may effect assay performance, and the results obtained. Pay attention to specific '**Notes**' and warnings throughout these Instructions for Use.
Reagents from different batch numbers of kits are **NOT** interchangeable. If large numbers of tests are performed care should be taken to ensure that all reagents are from the **SAME** batch. All strips used must be taken from the same foil pouch. Substitution of any component may lead to incorrect results.
To avoid reagent contamination, especially of the conjugate and calibrators, only use new or clean plastic / glassware. NEVER return unused reagents to the bottles.
Do **not** leave reagent bottles uncapped; any resulting evaporation or contamination will lead to inconsistent results.
TMB substrate must not be exposed to light or water.
Microbially contaminated, haemolysed or lipaemic serum and specimens containing particulate matter should not be used.
Inaccurate sample dilution cannot be checked, as kit controls are ready to use. The use of calibrated pipettes and appropriate internal QC samples is recommended.
When using automated assay systems, sample dilutors and other automated equipment, follow the manufacturer's instructions carefully. Particular attention should be given to the setup of the equipment and installation and connection to external services. All settings for automatic washers and readers must be followed carefully and equipment must be maintained and serviced according to the manufacturer's instructions.

4.3 STORAGE AND STABILITY

The kit should be stored at 2-8°C and should **not** be frozen. Inappropriate storage temperatures will affect the results.
Wash buffer diluted into a clean container can be stored at room temperature for a maximum of 4 weeks.
The expiry date of the kit is shown on the outer label.

5 SAMPLE COLLECTION AND STORAGE

Blood samples should be collected by venepuncture allowed to clot naturally and the serum separated.
The serum may be stored at 2-8°C for up to 48 hours prior to assay, or for prolonged storage kept undiluted at -20°C or below.
Repeated thawing and freezing should be avoided.
Serum samples should **not** be heat-inactivated, as this may give false positive results.

6 MATERIALS

6.1 MATERIALS SUPPLIED

6.1.1 Instruction Leaflet: Giving full assay details.
6.1.2 QC Certificate: Indicating the expected performance of the batch.
6.1.3 PCP Coated Wells: 12 breakapart 8 well strips coated with PCP antigen. Each plate is packaged in a re-sealable foil bag containing two desiccant pouches.
6.1.4 PCP IgG2 Sample Diluent: 2 bottles containing 50mL of CPS buffer for sample dilution. Coloured yellow, ready to use.
6.1.5 Wash Buffer: 1 bottle containing 50mL of a 20-fold concentrated buffer for washing the wells.
6.1.6 PCP IgG2 Calibrators: 5 bottles each containing 1.2mL of diluted human serum, with the following concentrations of anti-PCP antibody: 90, 30, 10, 3.3 and 1.11mg/L. Ready to use.
6.1.7 PCP IgG2 High Control: 1 bottle containing 1.2mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
6.1.8 PCP IgG2 Low Control: 1 bottle containing 1.2mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
6.1.9 PCP IgG2 Conjugate: 1 bottle containing 12mL of purified peroxidase labelled antibody. Coloured turquoise. Ready to use.
6.1.10 TMB Substrate: 1 bottle containing 14mL TMB substrate. Ready to use.
6.1.11 Stop Solution: 1 bottle containing 14mL of 3M phosphoric acid. Ready to use.

6.2 ADDITIONAL MATERIALS AND EQUIPMENT - not supplied

6.2.1 Automatic microplate plate washer: This is recommended, however, plate washing can be performed manually.
6.2.2 Plate reader: Capable of measuring optical densities at 450nm referenced on air.
6.2.3 Distilled or deionised water: This should be of the highest quality available.
6.2.4 Calibrated micropipettes: for dispensing 1000, 100 & 10µL.
6.2.5 Multichannel pipette: Recommended for dispensing 100µL volumes of conjugate, substrate and stop solution.
6.2.6 Glass/plastic tubes: For sample dilution.

6.3 PRE-ASSAY STEPS

1. Bring the kit to room temperature
The kit is designed for room temperature operation (20-24 °C).
Remove the kit from storage and stand at room temperature for approximately 60 minutes. Wells must **not** be removed from the foil bag until they have reached room temperature.
NOTE: The kits may be maintained at room temperature for up to 1 week.

2. Kit components
Gently mix each kit component before use.

3. Wash buffer dilution
Add 50mL of the wash buffer concentrate to 950mL of distilled water (1 in 20 dilution) into a clean container and mix. Smaller volumes can be diluted as appropriate.
NOTE: Diluted wash buffer can be stored at room temperature for up to 4 weeks, therefore only dilute the appropriate amount.

4. Sample dilution
Dilute 10µL of each sample with 1000µL of sample diluent and (1:100) and mix well.
NOTE: Diluted sample **must** be used within 8 hours.

5. Strip and frame handling
Place the required number of wells in the strip holder. Position from well A1, filling columns from left to right across the plate. When handling the plate, squeeze the long edges of the frame to prevent the wells falling out.
NOTE: Return unused wells to the foil bag immediately with the two desiccant pouches and re-seal tightly to minimise exposure to moisture.
Take care not to puncture or tear the foil bag, see below.
WARNING: Exposure of wells to moisture or contamination by dust or other particulate matter will result in antigen degradation, leading to poor assay precision and potentially false results.

7 ASSAY METHOD

Maintain the same dispensing sequence throughout the assay.

1. Sample addition
Dispense 100µL of each calibrator, control and diluted (1:100) sample into the appropriate wells of the plate provided. **NOTE:** Samples should be added as quickly as possible to the plate to minimise assay drift, and the timer started after the addition of the **last** sample.
Incubate at room temperature for 30 minutes.

2. Washing
The washing procedure is critical and requires special attention. An improperly washed plate will give inaccurate results, with poor precision and high backgrounds. After incubation remove the plate and wash wells 3 times with 250-350µL wash buffer per well. Wash the plate either by using an automatic plate washer or manually as indicated below. After the final automated wash, invert the plate and tap the wells dry on absorbent paper.

Plates can be washed manually as follows:
a. Flick out the contents of the plate into a sink.
b. Tap the wells dry on absorbent paper.
c. Fill each well with 250-350µL of wash buffer using a multichannel pipette.
d. Gently shake the plate on a flat surface.
e. Repeat a-d twice.
f. Repeat a and b.

3. Conjugate addition
Dispense 100µL of conjugate into each well, blot the top of the wells with a tissue to remove any splashes.
Note: **To avoid contamination, NEVER return excess conjugate to the reagent bottle.**
Incubate at room temperature for 30 minutes.

4. Washing
Repeat step 2.

5. Substrate (TMB) addition
Dispense 100µL of TMB substrate into each well, blot the top of the wells with a tissue to remove any splashes.
Note: To avoid contamination never return excess TMB to the reagent bottle.
Incubate at room temperature in the dark for 30 minutes.

6. Stopping
Dispense 100µL of stop solution into each well. This causes a change in colour from blue to yellow.

7. Optical density measurement
Read the optical density (OD) of each well at 450 nm on a microplate reader, within 30 minutes of stopping the reaction.

8 RESULTS AND QUALITY CONTROL

1. Quality control
In order for an assay to be valid, all the following criteria must be met:
- Calibrators and controls must be included in each run.
- The values obtained for the controls should be in the ranges specified on the QC Certificate.
- The curve shape should be similar to the calibration curve, shown on the QC Certificate.
  If the above criteria are not met, the assay is invalid and the test should be repeated.

2. Calculate mean optical densities (For assays run in duplicate only).
For each calibrator, control and sample calculate the mean OD of the duplicate readings. The percentage coefficient of variation (%C.V.) for each duplicate OD should be less than 15%.

3. Plot calibration curve
The calibration curve can be plotted either automatically or manually as follows by plotting the anti-PCP IgG2 antibody concentration on the log scale against the OD on the linear scale for each calibrator:-
- Automatic - Use appropriately validated software, and the curve fit that best fits the data.
- Manual - Using log/linear graph paper, draw a smooth curve through the points (**not** a straight line or point to point).

4. Treatment of anomalous points
If any one point does not lie on the curve, it can be removed. If the absence of this point means that the curve has a shape dissimilar to that of the sample calibration curve, or more than one point appears to be anomalous, then the assay should be repeated.

5. Calculation of the control values
Read the level of the anti-PCP IgG2 antibody in the controls directly from the calibration curve. The values should fall within the range given on the QC Certificate.

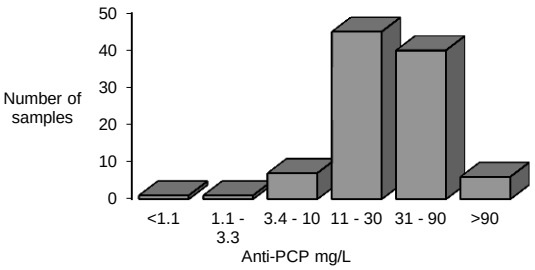
6. Calculation of antibody levels in diluted samples
Read the level of the anti-PCP IgG2 antibody in the diluted samples directly from the calibration curve.
NOTE: The calibrator values have been adjusted by a factor of 100 to account for a 1:100 sample dilution. No further correction is required.

7. Assay calibration
The assay is calibrated against an affinity purified preparation of human antiserum to PCP.

8. Limitations
Pre- and post-vaccination samples should be run simultaneously.
This kit is for *in-vitro* research use only. Not for use in diagnostic procedures.

9 TYPICAL VALUES

Anti-PCP IgG2 antibodies levels were measured in serum from 100 normal adult blood donors (of unknown vaccination and immune status). The results displayed below, are provided for illustration only, and should not be used to calculate a normal range.



10 PERFORMANCE CHARACTERISTICS

10.1 PRECISION
The intra- and inter-assay precision were measured using three samples within the range of the calibration curve. The standard deviations (SD) and % C.V. for each sample are given below:-

INTRA-ASSAY PRECISION		
n=16	**Concentration (mg/L)**	**% C.V.**
Sample 1	3.4	6.0
Sample 2	18.8	6.8
Sample 3	71.5	4.5

INTER-ASSAY PRECISION		
n=3	**Concentration (mg/L)**	**% C.V.**
Sample 1	2.7	5.8
Sample 2	17.9	4.3
Sample 3	58.9	6.3

10.2 ANALYTICAL SENSITIVITY
Sensitivity was determined as the mean concentration + 2 SD given by 20 determinations of the sample diluent. This equates to 0.45 mg/L.

10.3 MEASURING RANGE
The measuring range of the assay is 1.1 – 90 mg/L.

11 REFERENCES

1. Robbins J B *et al*. Considerations for formulating the second generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. *Journal of infectious diseases*, 1983: **148**: 1136-1159.

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4. Barret D J and Ayoub E M. IgG2 subclass restriction of antibody to pneumococcal polysaccharides. *Clin. Exp. Immunol*, 1986; **63**: 127-134.

5. Berger M. Immunoglobulin G subclass determination in diagnosis and management of antibody deficiency syndromes. *The Journal of Pediatrics*, 1987:**2**:325-328.

6. Jefferis R and Kumararatne D S. Selective IgG subclass deficiency: quantification and clinical relevance. *Clin. Exp. Immunol*, 1990; **81**: 357-367.