ImmunoWELL™ CE

MYCOPLASMA PNEUMONIAE ANTIBODY (IgM) TEST

Product No. 3130

IVD **For *In Vitro* Diagnostic Use**

INTENDED USE

ImmunoWELL Mycoplasma Pneumoniae Antibody (IgM) Test is a qualitative enzyme immunoassay (EIA) for the detection of specific IgM antibodies *to M. pneumoniae* in serum and is an aid in the diagnosis of *M. pneumoniae* infection.

SUMMARY AND EXPLANATION

The order Mycoplasmatales includes approximately 70 species, most of which are not found in humans. The genus Mycoplasma contains two species commonly found in man, *M. pneumoniae* and *M. genitalium*. These two species share lipid antigen specificities, and are therefore antigenically related. Two other human pathogens, *M. hominis* and *Ureaplasma urealyticum* are not serologically related to these.

Mycoplasma pneumoniae is the only known mycoplasma species that is a primary pathogen in man. Clinical manifestations can range from asymptomatic respiratory infections to severe pneumonia[1]. *M. pneumoniae* accounts for 15 to 20% of total pneumonia[2,3]. Other symptoms associated with *M. pneumoniae* infection include abnormalities of the central nervous system (meningitis, encephalitis), cardiac involvement (myocarditis, pericarditis), hemolytic anemia, arthritis, G.I. inflammations, and mucocutaneous reactions[4]. *Mycoplasma pneumoniae* is identified as a common infectious cause of Stevens-Johnson Syndrome, a well-defined systemic disease that can develop into a life-threatening illness in children[5].

The *Mycoplasma pneumoniae* organism is sensitive to erythromycin and tetracyclines; however, it is resistant to drugs more routinely given in the treatment of acute pneumonia. Thus, a rapid and reliable diagnosis of *M. pneumoniae* infection is essential to proper patient management[6]. Culturing of *M. pneumoniae* is too difficult and slow for clinical diagnostic utility. Serology provides the primary diagnostic tool with current methods including complement fixation (CF), indirect immunofluorescence assays (IFA), immune adherence hemagglutination assay (IAHA) and enzyme immunosorbent assays (EIA).

ASSAY PRINCIPLE

The ImmunoWELL Test utilizes an EIA microtiter plate technique for the detection of antibodies. Antihuman IgG treated serum is added to antigen coated microtiter wells and allowed to react. After removal of unbound antibodies, horseradish peroxidase-conjugated antihuman IgM antibodies are allowed to react with bound antibodies. The bound peroxidase reacts with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS®), the chromogenic substrate, developing a color. Finally, the substrate reaction is stopped and the optical density is read with a spectrophotometric microwell reader.

REAGENTS

Reaction Wells coated with Mycoplasma pneumoniae, strain FH (ATCC #15531). The antigen is purified by chloroform and methanol extraction.
Specimen Diluent consisting of 0.01 M phosphate buffered saline (PBS, pH 6.2-7.6) and carrier protein containing <0.1% NaN_3
Calibrator consisting of human anti–M. pneumoniae prediluted 1:100 in Specimen Diluent
Positive Control consisting of human anti–M pneumoniae serum containing <0.1% NaN_3
Negative Control consisting of a nonreactive serum substitute containing <0.1% NaN_3
Absorbent consisting of antihuman IgG antibodies in 0.01 M PBS (pH 6.2-7.6) and carrier protein containing <0.1% NaN_3. The absorbent binds up to 15 mg/mL of human IgG and is used to prevent interference due to rheumatoid factor or competing IgG in patient specimens.
Wash Buffer Concentrate consisting of a 20X concentrate of 0.01 M PBS (pH 6.2-7.6) and 0.05% Tween
Conjugate consisting of peroxidase-conjugated goat antihuman IgM in PBS (pH 6.2-7.6) and carrier protein containing preservatives
Substrate Buffer consisting of 0.1 M sodium citrate (pH 4.4-4.6) and 0.01% hydrogen peroxide
Substrate Concentrate 2.19% 2-2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) in 0.1 M sodium citrate (pH 4.4-4.6)
Stop Solution 0.25 M Oxalic Acid

WARNINGS AND PRECAUTIONS

For *In Vitro* Diagnostic Use: ImmunoWELL reagents have been optimized for use as a system. Do not substitute other manufacturers' reagents or other ImmunoWELL Test reagents. Dilution or adulteration of these reagents may also affect the performance of the test. Do not use any kits beyond the stated expiration date. Close adherence to the test procedure will assure optimal performance. Do not shorten or lengthen stated incubation times since this may result in poor assay performance.
Some reagents contain sodium azide that may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.
Warning - Potential Biohazardous Material: Human sera used in the preparation of this product were tested using FDA approved procedures and found non-reactive for hepatitis B surface antigen and for antibodies to HIV-1, HIV-2and hepatitis C virus. Because no test method can offer complete assurance that infectious agents are absent, handle reagents and patient samples as if capable of transmitting disease.

RECONSTITUTION AND STORAGE

Kit is stored at 2-8°C until stated expiration date.
Reaction wells are removed from the foil pouch and unused wells are resealed in the pouch using the integral zip-lock. Reaction wells are stable for at last four weeks once opened. Actual stability depends on the storage environment. Run quality control, specifically the Low Limit value, assures all reagents including the microtiter plate yield acceptable performance.
Wash Buffer (pH 6.2-7.6) is prepared by adding the contents of the Wash Buffer Concentrate (20X) bottle into 1 liter of distilled/deionized water. After reconstitution, the 1X solution is stored at 2-8°C. Discard when visibly turbid.
IN SOME INSTANCES THE WASH BUFFER CONCENTRATE (20X) MAY DEVELOP CRYSTALS UPON STORAGE AT 2-8°C. IT IS IMPORTANT THAT THESE CRYSTALS ARE COMPLETELY REDISSOLVED BEFORE

DILUTION OF THE CONCENTRATE. THIS CAN BE ACCOMPLISHED BY WARMING THE CONCENTRATE TO 37°C IN A WATER BATH WITH OCCASIONAL MIXING.

Color Developer is prepared by adding one (1) drop of Substrate Concentrate to 1mL of Substrate Buffer. One mL of Color Developer is sufficient for one eight-well strip. **Use within one hour.**

SPECIMEN COLLECTION AND HANDLING

ImmunoWELL Test is performed on serum. The test requires 10 µL of serum. Lipemic, hemolyzed and icteric serum have not been shown to be acceptable specimens. Serum is collected according to standard practices and may be stored at 2–8°C for up to five days. Serum may be frozen below -20°C for extended periods.
A single specimen or serum pairs may be tested. If a serum pair is tested, two sera, one collected during the acute disease phase and the other collected at least one week later (convalescent), are used.

PROCEDURE

MATERIALS PROVIDED

Microtiter Wells in Carrier	Calibrator
Positive Control	Negative Control
Wash Buffer Concentrate (20X)	Conjugate
Substrate Buffer	Substrate Concentrate
Stop Solution	Absorbent
Specimen Diluent	

MATERIALS REQUIRED BUT NOT PROVIDED

Distilled or deionized water	Test tubes
Microwell washer	Pipets
Microwell spectrophotometer (405 nm), capable of measurement to 2.00 absorbance units	

PERFORMANCE CONSIDERATIONS

Reproducibility in the assay is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the assay procedure.
Positive and Negative Control Sera (undiluted) - These control sera are used to assure test performance.
Calibrator (prediluted 1:100) is used to standardize between run values.
Substrate Blank - All reagents, except serum, are added to the substrate blank well. This blank well is intended to baseline (zero) the microwell spectrophotometer.

ASSAY PROCEDURE

1. Allow all components including diluted Wash Buffer to warm to room temperature (22-27°C).
2. Determine the total number of specimens to be tested. Include one blank and duplicates of calibrator and controls in each run.
3. For each control and specimen, pipet 10 µL serum into a clean tube containing 1 mL Specimen Diluent and mix (1:100 dilution). **CAUTION: CALIBRATOR IS PREDILUTED. DO NOT DILUTE FURTHER.**

4. Pipet 100 µL of 1:100 diluted controls and specimens (step 3) into clean tubes each containing 20 µL Absorbent and mix. Pipet 200 µL prediluted calibrator into a clean tube containing 40 µL Absorbent and mix. Incubate at room temperature (22–27°C) for 30±2 minutes.
5. Determine the total number of wells to be run including blank, calibrators, controls and specimens. Well strips can be broken to the exact number needed to conserve reagent wells. Strips need to be completed with used wells to facilitate washing procedures.
6. Add 100 µL of Specimen Diluent into the first well as a substrate blank.
7. Pipet 100 µL of the prediluted calibrator, and diluted controls and specimens (step 3) into each assigned well.
8. Incubate at room temperature (22–27°C) for 60±2 minutes.
9. Aspirate the samples out of the wells.
10. Wash the wells three times by completely filling the wells with Wash Buffer (see Reconstitution and Storage) and aspirating the wells completely after washes.
11. Pipet 100 µL Conjugate into all wells.
12. Incubate the wells at room temperature (22–27°C) for 30±2 minutes.
13. Aspirate the conjugate out of the wells.
14. Wash the wells three times as described in step 10.
15. Prepare fresh Color Developer (see Reconstitution and Storage).
16. Pipet 100 µL of Color Developer into each well.
17. Incubate at room temperature (22–27°C) for 30±2 minutes.
18. Add 100 µL of Stop Solution to each well.
19. Inspect the outside bottom surface of the microwells for the presence of condensation, dried buffer salts or wash solution, which might interfere with the spectrophotometric reading. Carefully clean the well bottoms with a soft tissue.
20. Using the substrate blank to zero the spectrophotometer, read the optical density of each well at 405 nm within 30 minutes of completion of step 18.

IT IS RECOMMENDED THAT DUAL WAVELENGTH SPECTROPHOTOMETERS USE ONLY ONE WAVELENGTH, 405 NM.

QUALITY CONTROL

GenBio provides positive and negative controls with defined ranges. Interpretations should not be made unless the control results fall within these limits. In addition, the laboratory should act in accordance with laboratory accreditation requirements and/or individual laboratory monitoring programs.

INTERPRETATION

The assigned values (U/mL) may vary between lots. Assure the values listed below are used.

INITIAL INTERPRETATION (INDIVIDUAL SAMPLE)

If paired samples are received, they should be tested concurrently but initially interpreted as individual samples. If one or both are IgM Positive (>950 U/mL), further evaluation is not required. In order to eliminate the effects of washing variation, instrument variability, etc., specimen values are normalized according to the following calculation:

$$A_N = (A_S \times AV_C)/A_C$$

Where:

A_N	Normalized activity of the specimen (U/mL)
A_S	Absorbance of the specimen
A_C	Mean absorbance of the calibrator obtained in the assay
AV_C	Assigned Value (U/mL) of the calibrator given in the Supplement

RESULTS SHOULD NOT BE INTERPRETED IF CALIBRATOR ABSORBANCE IS BELOW THE LOW LIMIT. CONTACT GENBIO IF YOUR RESULTS ARE OUTSIDE OF THE EXPECTED RANGE. THE MAGNITUDE OF THE MEASURED RESULT, ABOVE THE CUTOFF, IS NOT INDICATIVE OF THE TOTAL AMOUNT OF ANTIBODY PRESENT.

Calibrator Assigned Value	**1885**	Units/mL
Calibrator Absorbance Low Limit	**0.24**	Absorbance units
Positive Control Expected Range	**2000 - 5800**	Units/mL
Negative Control Expected Range	**< 770**	Units/mL

Table 1: Interpretation Ranges

CLASSIFICATION	UNITS/ML	CLINICAL INTERPRETATION
Negative	<770	Clinically significant amount of *M. pneumoniae* antibody not detected.
Low Positive	770-950	*M. pneumoniae* specific IgM presumptively detected. It is recommended that another sample should be collected 1-2 weeks later to assure reactivity.
Positive	>950	Highly significant amount of *M. pneumoniae* specific IgM antibody detected.

The above ranges in Table 1 are determined by testing sera from blood donors (See Figure 1). Although most samples were negative, some subjects did report a low positive result. The *M. pneumoniae* antibody levels obtained from the assay are an aid to diagnosis only. In most cases, a positive antibody result will provide laboratory support of *M. pneumoniae* infection. However, specific IgM may persist for several months after initial infection or be absent during early infection or reinfection. Each physician must interpret these results in light of the patient's history, physical findings, and other diagnostic procedures.

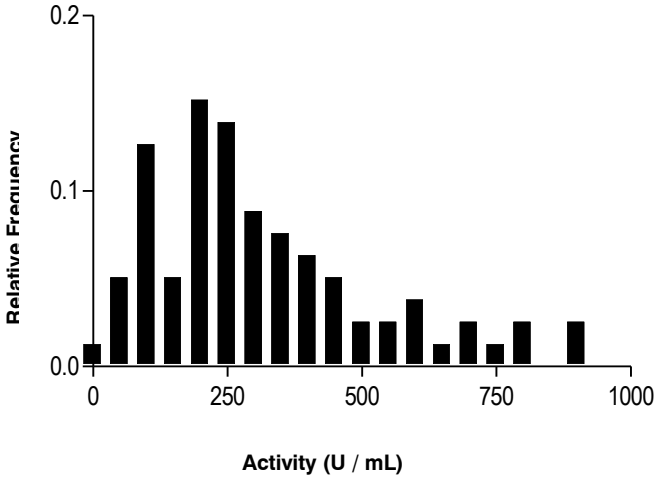
LIMITATIONS

Absorbent removes up to 15 mg/mL of human IgG (50% endpoint), but the presence of residual IgG in individuals with hyper-gammaglobulinemia may affect results adversely.

EXPECTED RESULTS

Seventy-nine normal samples from asymptomatic blood donors were tested and the reported activities (U/mL) are shown in Figure 1.

Figure 1: Normal Sera



It is reported that the percentage of pneumonias caused by *M. pneumoniae* is 10-33% in the general population, 35-74% in children (5-19 years old), 27-52% in college students, 8-54% in military recruits and 7-17% in civilian adults[7]. An increase of IgG relative to IgM antibodies occurs with time after onset of illness. Also, adults respond with higher IgG antibody ratios than do children[2]. IgM titers are significant in a high percentage of patients at admission.[1]

PERFORMANCE CHARACTERISTICS

Reference results (CF or IAHA method) are classified as: 1) greater than a four–fold titer increase, 2) single value above 1:1024 titer, 3) falling titer greater than four-fold, or 4) no significant change. Paired samples were not selected by the sites, but were frozen and are retrospectively evaluated.

THE MAJORITY OF SAMPLES ARE FROM REFERENCE LABORATORIES THAT MAY HAVE RECEIVED SELECTED SAMPLES. SINCE ASSAY PERFORMANCE DEPENDS ON THE PREVALENCE OF DISEASE IN A POPULATION, USERS MAY NOT SEE THE SAME PERFORMANCE IN THEIR LABORATORY.

SENSITIVITY

Assay relative sensitivity depends most on when during the course of infection the sample was collected. As would be expected, IgM antibody is less frequently detected (29%, range of 16-44%) at first and is more often positive in later disease (76%, range of 60-87%).

Table 2 compares reference method results to ImmunoWELL IgM results, not taking into account ImmunoWELL IgG test results. Combining the CF and IAHA rise, fall and greater than or equal to 1024 titer results as significant positives, the relative sensitivity is 79% (65-89%). The relative specificity is 89% (52-100%).

Table 2: Relative Performance (only ImmunoWELL IgM results), ImmunoWELL IgM Positive (>950 U/mL)

CF and IAHA Combined					
	Rise	Fall	1021 Titer	No Change	Total
Agree	27	8	6	8	49
Disagree	9	2	0	1	12
Total	36	10	6	9	61

Using the same pairs evaluated in Table 2, pairs showing an ImmunoWELL IgM positive result are excluded from IgG consideration. The relative performance of ImmunoWELL IgG to CF and IAHA results is shown in Table 3. There is 73% (39-94%) agreement with traditional serology testing for positive pairs and 100% (63-100%) agreement for negative pairs.

Table 3: Relative Performance (without IgM positives), ImmunoWELL IgG Ratio Results (without IgM Positive Pairs)

CF AND IAHA COMBINED					
	Rise	Fall	1024 Titer	No Change	Total
Agree	7	1	0	8	16
Disagree	2	1	0	0	3
Total	9	2	0	8	19

Overall relative performance following both ImmunoWELL IgM and IgG package insert guidelines is shown in Table 4. The sensitivity is 96% (87-100%) and specificity is 89% (52-100%).

Table 4: Relative Performance (IgM and IgG results), ImmunoWELL IgM and IgG Ratio Results

CF AND IAHA COMBINED					
	Rise	Fall	1024 Titer	No Change	Total
Agree	35	9	6	8	58
Disagree	1	1	0	1	3
Total	36	10	6	9	61

SPECIFICITY

Assay specificity, assessed by testing sera from asymptomatic blood donors (“normals”), is 95% (88-99%).

CROSS-REACTIVITY

The glycolipid antigen used in complement fixation assays may be cross-reactive with organ-specific antigens from brain, pancreas, and antigens from various organisms of group A *Neisseria meningitidis.* It is unknown whether such interactions occur with the purified form of glycolipid used in this assay. It is also unknown whether antibodies of organisms producing similar symptomatology (i.e., symptomatology consistent with *M. pneumoniae* infection) may cause cross-reactivity.

Two of fifteen autoimmune sera containing anti-ribonucleoprotein, an extractable nuclear antigen, were anti-*M. pneumoniae* reactive. In addition, samples from patients with symptomatically related diseases were tested and the results are reported in Table 5. It is unknown whether the reactivity is due to cross-reactivity or dual infection. It is also unknown whether there is cross-reactivity to chlamydia or streptococcus type organisms.

Table 5: Samples from Patients with Symptomatically Related Diseases

AGENT	NEGATIVE	LOW POSITIVE	POSITIVE
Respiratory Synctial Virus	15	1	1
Influenza	22	0	0
Legionella	5	0	0
Adenovirus	5	0	0
Parainfluenza	1	0	0

REPRODUCIBILITY

Twenty assay runs, testing samples in duplicate, using common reagents on twenty different days were made at GenBio. The assay's results are shown in Table 6. **EIA PRECISION MAY VARY BETWEEN LABORATORIES.**

Table 6: Assay Precision

U/ML	WITHIN RUN VARIATION (%CV)	BETWEEN DAY VARIATION (%CV)
1242	6%	12%
148	11%	5%
1291	3%	8%
382	5%	11%
120	5%	17%
279	4%	19%

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QUICK REFERENCE PROCEDURE

IMMUNOWELL MYCOPLASMA IGM

- Prepare Wash Buffer from Wash Concentrate
- Dilute each control and specimen 1:100 in Specimen Diluent
- Add 100 µL of 1:100 diluted controls and specimens to 20 µL Absorbent. Add 200 µL of prediluted calibrator to 40 µL Absorbent. Mix and incubate 30 min at room temperature.
- Add 100 µL of Specimen Diluent into the first well as a substrate blank.
- Pipet 100 µL of the treated calibrator, controls and specimens into coated microwells and incubate 60 min at room temperature.
- Aspirate microwells and wash microwells three times with Wash Buffer.
- Pipet 100 µL of Conjugate into microwells and incubate 30 min at room temperature.
- Aspirate microwells and wash microwells three times with Wash Buffer.
- Prepare fresh Color Developer
- Pipet 100 µL of Color Developer into microwells and incubate 30 min at room temperature
- Pipet 100 µL of Stop Solution into microwells and read results at 405 nm.

To place an order for ImmunoWELL products, contact your local distributor, or call GenBio directly for the distributor nearest you and for additional product information.
For assistance, please call toll-free 800-288-4368.
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