Mycoplasma pneumoniae IgG Enzyme Immunoassay

For Individual Laboratory to Complete:

Laboratory Name		
Adopted		
Reviewed		
Reviewed		
Revised		
Supercedes		

Method: **Diamedix Corp., Immunosimplicity®**
Manual or in conjunction with one of the Diamedix Automated EIA Systems such as the MAGO Plus, the DSX, or the DS2. For *In Vitro* Diagnostic Use.

Clinical Significance

Mycoplasmas are members of the Mollicutes class of bacteria and are the smallest self-replicating organisms. These bacteria lack a cell wall making them resistant to many antibiotics. The primary human pathogen is *Mycoplasma pneumoniae*, which is known to cause a wide range of clinical symptoms,ranging from mild respiratory infections “walking pneumonia"), to tracheobronchitis and severe atypical pneumonia (1). Unlike other respiratory infections, *M. pneumoniae* infections tend not to be seasonal. In large populations, disease is endemic year-round with periodic increases in incidence, whereas in smaller populations, outbreaks appear as epidemics (2,3). The elderly and children are at elevated risk of infection.

Diagnostic tests for *M. pneumoniae* infections include cold agglutinins, complement fixation,and culturing. Detecting the cold agglutinins is fast, simple to perform, and relatively inexpensive, but these are found in only 30 to 50% of *M. pneumoniae* infections (1,5). Complement fixation (CF) assays, though widely performed, detect predominantly “early” IgM antibodies and only to a minor extent IgG antibodies. The diagnostic value of the CF test may be limited to the initial *M. pneumoniae* infection. Another disadvantage of the CF assay is the antigen used is a crude extract, which is not specific for *M. pneumoniae*. Similar antigens (glycolipids) are found in human tissues and streptococci extracts, which may lead to false positive results(4). *M. pneumoniae* culturing is difficult due to the slow growth rate. The use of enzyme immunoassays offers several advantages over the other assay methods. Increased specificity is obtained by using purified detergent-treated *M. pneumoniae* extracts as antigens, which minimize the glycolipid cross-reactivity (4). Isotype-specific enzyme conjugates provide antibody information and the assay can be optimized for high sensitivity.

The Diamedix immunosimplicity® *Mycoplasma pneumoniae* IgG Test Kit is an EIA procedure intended for the qualitative detection of IgG antibodies to *M. pneumoniae* antigen. The test can be performed either manually or in conjunction with one of the Diamedix Automated EIA Systems.

Principle of the Procedure

Purified *Mycoplasma pneumoniae* antigen is bound to microwells. Diluted patient sera, Cut-Off Calibrator, and controls are placed in the microwells and incubated. Anti-*M. pneumoniae* IgG antibodies, if present, will bind to the antigen forming antigen-antibody complexes. Residual sample is eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG) is added and will bind to these complexes. Unbound conjugate is removed by aspirating and washing. Substrate is then added and incubated. In the presence of bound enzyme, the substrate is converted to an end product. The absorbance of this end product can be read spectrophotometrically at 450 nm (reference 600-630 nm) and is directly proportional to the concentration of IgG antibodies to *M. pneumoniae* present in the sample.

Specimen Collection

Whole blood should be collected by accepted medical techniques. Separated serum should remain at 22°C for no longer than 8 hours. If assays are not completed within 8 hours,serum should be refrigerated (2 - 8°C). If assays are not completed within 48 hours, or the separated sample is to be stored beyond 48 hours, samples should be frozen at -20°C. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen sera to room temperature slowly and mix gently,avoiding foam formation. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Grossly contaminated, hemolyzed, lipemic, or icteric specimens should not be used. The NCCLS provides recommendations for collecting and storing blood specimens (6).

CAUTION: Serum samples must not be heat-inactivated prior to use.

Reagents

Antigen Wells — Twelve, 8-well microwell breakapart strips, color-coded dark green, coated with purified *M. pneumoniae* antigen (strain FN).

Cut-off Calibrator — One vial with blue cap containing 0.5 ml of human serum or defibrinated plasma, weakly reactive for *M. pneumoniae* IgG antibodies, 0.1% sodium azide. The Cut-Off Calibrator is used to determine the cut-off of the assay.

Low Positive — One vial with white cap containing 0.25 ml of reactive

Control human serum or defibrinated plasma, 0.1% sodium azide. Assigned range printed on label. The Positive Control is used to control the low positive range of the assay.

Negative Control One vial with black cap containing 0.25 ml of non-reactive human serum or defibrinated plasma, 0.1% sodium azide. Assigned range printed on label. The Negative Control is used to control the negative range of the assay.

Sample A Diluent One bottle with blue cap containing 60 ml Phosphate buffer with protein stabilizers. Contains 0.2% sodium azide, Proclin™ 300, 90 ppm active ingredient. Color-coded blue.

Wash T Concentrate Two bottles with clear caps containing 50 ml of Tris buffer with detergent and Proclin™ 300, 15 ppm active ingredient. Each bottle is sufficient to make 1 liter of wash solution.

Conjugate One bottle with red cap containing 25 ml goat anti-human immunoglobulin G labeled with horseradish peroxidase. Also includes protein stabilizers and Proclin™ 300, 30 ppm active ingredient. Color-coded pink.

Substrate HRP One amber bottle with brown cap containing 25 ml buffered TMB solution (3,3',5,5' tetramethylbenzidine).

Stop N Solution One bottle with white cap containing 30 ml of 1N Sulfuric Acid. CAUTION: Acids are corrosive. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water.

Store these reagents at 2 to 8° C.

Other Materials Required

Manual Users:

1. Wash bottle or automated microplate washer
2. Pipettors capable of dispensing appropriate volumes
3. Timer
4. One liter graduated cylinder
5. One liter wash solution reservoir
6. Deionized or distilled water
7. Absorbent toweling
8. Tubes or microwell plate for sample dilution
9. Reader capable of reading absorbance at 450nm, reference at 600 or 630 nm.

Diamedix Automated EIA System Users:

1. One liter graduated container
2. Deionized or distilled water
3. Dilution containers as appropriate to system
4. Sample and Reagent tips required by system
5. Reagent containers required by system

Warnings:

1. Handle samples, Calibrator, controls and the materials that contact them as potential biohazards. Each donor unit in the Calibrator and controls has been found negative for Hepatitis B surface antigen, Hepatitis C and HIV-1 &2 antibodies by FDA-approved third generation tests. However, because no method can offer complete assurance that HIV-1&2, Hepatitis B virus, Hepatitis C, or other infectious agents are absent, these materials should be handled at the Biosafety Level 2 as recommended for any potentially infectious serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories", 1993.
2. Never pipette by mouth.
3. Avoid contact with open skin and mucous membranes.
4. Certain of the test reagents contain Proclin™ 300 as a preservative. When disposing of reagents containing Proclin™ 300, flush drains with copious amounts of water to dilute the active components below active levels.
5. Serum components contain sodium azide as preservative. Azides are reported to react with lead and copper in plumbing to form compounds that may become explosive. When disposing of solutions containing sodium azide, flush with copious amounts of water to minimize the build up of metal azide compounds.
6. Sodium azide inhibits horseradish peroxidase enzyme activity. Care must be taken to ensure that azide is not carried over from other reagents into conjugate and substrate steps.
7. Avoid contamination of the TMB Substrate Solution with conjugate or other oxidants, which will cause the solution to change color prematurely.
8. The substrate contains 3,3'5,5' tetramethylbenzidine (TMB) which has shown possible mutagenic effects in laboratory experiments.

Calibration

This test uses an in-house reference standard (or Calibrator). The Calibrator has been derived from weakly positive sera and is titrated to an absorbance value equivalent to the cut-off of the assay. Samples whose absorbances exceed this value are considered positive for *M. pneumoniae* IgG antibodies and samples whose absorbances are less than this value are considered negative for *M. pneumoniae* IgG antibodies. To account for the inherent variations in enzyme immunoassays an equivocal range of ±10% has been included at the assay cut-off.

Quality Control

a) The Positive and Negative Controls must be included in each test run.

b) The absorbance of the Blank must be < 0.25.

c) The absorbance of the Cut-Off Calibrator must be ≥ 0.10.

d) The Positive and Negative Controls must be within their assigned ranges.

If any of these criteria is not met, the run is invalid and must be repeated.

Note: Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations. For guidance on appropriate quality control practices please refer to NCCLS C24-A, Internal Quality Control Testing: Principles and Definitions.

Procedure

Allow all test components and patient samples to warm to room temperature before use. Invert reagent bottles gently several times before use. Return promptly to the refrigerator after use.

Prepare Wash Solution by adding 50 ml of Wash Concentrate to one liter with deionized or distilled H_20.

MANUAL USERS:

1. Prepare 1:101 dilutions of the Cut-Off Calibrator (in triplicate), controls, and patient samples in Sample Diluent (e.g., by addition of 5 µl sample to 500 µl Sample Diluent.)

2. Mix sample dilutions gently by withdrawing and expelling in a pipette tip 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 µl of diluted calibrator, controls, and diluted patient samples, to the antigen wells. Avoid formation of bubbles when transferring diluted samples.

NOTE: Include one well, which contains 100 µl of Sample Diluent only as the reagent blank. This will ultimately be used to “zero” the photometer before reading the test results.

3. Allow the wells to incubate at room temperature (18 - 30°C) for 30 ± 5 minutes.

4. Aspirate or discard the contents of the wells. Remove any excess moisture in the wells by tapping on paper toweling if necessary. Wash the wells by rinsing 3 times with at least 300 µl per well of Wash Solution. Remove excess moisture from the wells after washing. When using an automated washer, follow the manufacturer’s instructions.

5. Place 100 µl of Conjugate into each well, avoiding bubble formation.

6. Allow the wells to incubate uncovered at room temperature (18 - 30°C) for 30 ± 5 minutes.

7. Wash the wells as described in Step 4 above.

8. Place 100 µl of Substrate into each well, avoiding bubble formation.

9. Allow the wells to incubate uncovered at room temperature (18 - 30°C) for 30 ± 5 minutes.

10. Place 100 µl of Stop Solution into each well, avoiding bubble formation.

11. Read the absorbance of each well at 450 nm and zero against the reagent blank. A suitable reference wavelength (e.g., 600-630 nm) reading should be used. Read the plate within 30 minutes of adding Stop Solution.

Diamedix Automated EIA System USERS:
If using one of Diamedix's Automated EIA Systems, please refer to the corresponding Operating Manual for the test setup, procedure, and accessories/consumables needed.

Calculation of Results

Calculate the mean absorbance of the Cut-Off Calibrator. Note: When calculating the mean absorbance value for the Cut-Off Calibrator exclude any absorbance value that deviates by more than 15% from the mean of the three absorbance values. Use the mean of the remaining two replicates in calculations. Exclusion of more than one of the three absorbance values invalidates the run.
Determine the Index Value for each patient specimen or control using the following formula:

$$\frac{\textbf{Absorbance of Sample}}{\textbf{Mean Absorbance of Cut-off Calibrator}} = \textbf{Index Value}$$

The Diamedix Automated EIA Systems will calculate results using the above formula and print them automatically.

Example: Absorbance values obtained for Cut-Off Calibrator: 0.276, 0.288, 0.258 (after subtraction of blank)
Mean Absorbance of Cut-Off Calibrator = 0.274
Sample Absorbance = 1.150
Index Value = 1.150/0.274 = 4.2

Reference Ranges

Index < 0.90	No detectable IgG antibodies to *M. pneumoniae.*
Index 0.90 to 1.09	Equivocal for IgG antibodies to *M. pneumoniae.* Samples can be retested by another method or a

new sample can be tested.

Index ≥1.10 IgG antibodies to *M. pneumoniae* detected.

Reporting Results

When the Index Value is reported for a single specimen the following statement should be included "The following results were obtained with the *Mycoplasma pneumoniae* IgG Test Kit. The magnitude of the measured result, above the cut-off, is not indicative of the total amount of antibody present. The magnitude of the reported IgG level cannot be correlated to an endpoint titer".

Procedure Notes

1. Do not interchange reagents from different reagent lots except for Sample **A** Diluent, Wash **T** Concentrate, Substrate **HRP** and Stop **N** Solution.
2. Do not use reagents beyond their expiration date.
3. Store unused reagents at 2 to 8°C.
4. Incubations above or below the recommended temperatures or times may give erroneous results.
5. The EIA method is a very sensitive technique. Maintain consistent pipetting technique, incubation times, and temperature conditions throughout the test procedure. Cross contamination between reagents can invalidate the test.
6. Antigen coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.
7. (*Manual Procedure Only)* The washing procedure is very important and requires special attention. (Please refer to the Procedure section)
   NOTE: *Improperly washed* wells *may* give *erroneous results.*
8. The reported concentration of anti- *M. pneumoniae* IgG in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

Limitations

1. The results obtained with the Is-*Mycoplasma pneumoniae* IgG Test Kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves.
2. Assay performance characteristics have not been established for visual result determination. Kit procedures or practices outside those in this package insert may yield questionable results.
3. The test should be performed on serum. The use of whole blood or plasma has not been established.
4. If the testing of a particular specimen occurs early during the primary infection, no detectable IgG may be evident. If a Mycoplasma infection is suspected, a second sample should be taken at least 14 days later. Negative results do not rule out the diagnosis of *M. pneumoniae*-associated disease. The specimen may have been drawn before the appearance of detectable antibodies. Negative results in suspected early disease should be repeated in 4-6 weeks.

5. The use of hemolytic, lipemic, bacterially contaminated or heat inactivated specimens should be avoided.
6. Performance of this assay has not been established on spectrophotometry utilizing a single wavelength.
7. A single positive result only indicates previous immunologic exposure. The level of antibody response or class of antibody may both be required to determine active infection or disease stage.
8. The performance of this device has not been established on neonates and immunocompromised patients.
9. False positive results may occur with sera from patients with Ureaplasma, *Mycoplasma hominis*, *Mycoplasma genitalium*, pancreatitis, bacterial meningitis and other acute inflammatory disease. Cross-reactivity of this assay with antibodies to the above disease states has not been determined. Epidemiology of case, symptoms and other laboratory tests can help in differentiating these conditions from *Mycoplasma pneumoniae* infection.
10. *Mycoplasma pneumoniae* infection can have a long incubation period, thus elevated antibody titers in the acute specimen are common, and reinfection may occur. Therefore, seroconversions (negative to positive) are unusual.
11. The performance characteristics of the *Mycoplasma pneumoniae* IgG Test Kit with automated equipment other than Diamedix Automated EIA Systems have not been established.

References

1. E. Jacobs.1993. Serological Diagnosis of *Mycoplasma pneumoniae* Infections: A Critical Review of Current Procedures. Clinical Infectious Diseases. 17 (Suppl 1) 79-82.
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5. Vikerfors, T., Brodin, G., Grandien, M., Hirshberg, L., Krook, A., and C. A. Pettersson. 1988. Detection of specific IgM antibodies for the diagnosis of *Mycoplasma pneumoniae* infections: a clinical evaluation. Scand. J. Infect. Dis. 20(6): 601-610.
6. Procedures for the Handling and Processing of Blood Specimens: Approved Guidelines – Second Edition NCCLS Document H18-A2, Vol. 19, No. 21. 1999.