

SUMMARY OF PROCEDURE

1. Prepare 1:21 dilutions of Negative Control, Calibrator, and Positive Control, and each patient sample in Sample Diluent.
2. Add 100 µl of diluted controls, calibrator and patient sample into the antigen wells.
3. Add 100 µl of Sample Diluent to well A1 as a reagent blank.
4. Incubate at 20-25°C for 25 ± 5 min.
5. Discard the contents of the wells. Wash the wells 5 times with Wash Solution.
6. Add 100 µl of Conjugate to each well.
7. Incubate at 20-25°C for 25 ± 5 min.
8. Wash the wells as in #5 above.
9. Add 100 µl of Substrate to each well.
10. Incubate at 20-25°C for 10 to 15 min.
11. Add 50 µl of Stop Solution to each well.
12. Read the absorbance at 450/630 nm.

INTENDED USE

For the qualitative detection of IgG antibodies to *Mycoplasma pneumoniae* in human sera. The test may aid in the determination of the patient's serological status, or in the diagnosis of disease associated with *Mycoplasma pneumoniae*. Potential cross-reactivity with *M. genitalium* has not been assessed, nor were studies performed on very young and/or elderly patients. The test can be performed manually or in conjunction with the MAGO[®]Plus Automated EIA Processor.

SUMMARY AND EXPLANATION

Mycoplasma pneumoniae is the most common cause of pneumonia and febrile upper-respiratory tract infections in the general population (except for influenza A).¹⁻⁵ Other nonrespiratory complications may also develop with this disease in virtually any organ system, with insult ranging from mild to life-threatening.^{6,8}

Mycoplasma pneumoniae, a prokaryote, is the smallest (10 x 200nm), and simplest self-replicating microorganism known, and more closely resembles a bacterium rather than a virus. However, because it lacks a cell-wall, a resistance to cell-wall-active antibiotics is obvious (*i.e.*, penicillin, cephalosporins¹). This concern for diagnostic, or at least therapeutic accuracy in the early management of community-acquired infections is particularly critical in very young or elderly patients where very little temporal margin of error exists.

Until recently, the routine laboratory diagnosis of this infection has been limited to insensitive and/or non-specific assays (*i.e.*, cold agglutinins, complement-fixation, culture isolation). Species-specific antibodies to surface antigens are now known to exist. They are protective, and are readily detected by ELISA, even in the early stages of the disease. The diagnosis therefore, is best achieved serologically.⁹

The DiaMedix **immunosimplicity**[®] *Is*-Mycoplasma IgG Test Kit is an EIA procedure intended for the qualitative detection of IgG antibodies to Mycoplasma antigen. The results are reported in Index Values or OD Ratios determined by comparison to a Cut-off Calibrator.

PRINCIPLE OF THE PROCEDURE

Partially purified Mycoplasma antigen is bound to microwells. Diluted patient sera, Calibrator and controls are placed in the microwells and incubated. Anti-Mycoplasma IgG antibodies, if present, will bind to the antigen in the microwells. After washing the microwells to remove unbound antibodies, a second incubation with anti-human IgG conjugated to horseradish peroxidase is carried out. The conjugate will bind to human anti-Mycoplasma IgG antibodies, if present, forming an immunocomplex. The microwells are then

washed again to remove unbound components and the enzyme substrate is 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 630 nm) and is directly proportional to the concentration of IgG antibodies to Mycoplasma antigen present in the sample.

REAGENTS

Each *Is*[®] - Mycoplasma IgG Test Kit contains reagents for 96 tests.

Antigen Wells	Twelve 8-well strips coated with partially purified inactivated <i>M. pneumoniae</i> (strain FN) antigen. The strips are packaged in a strip holder and sealed in an envelope with desiccant.
Conjugate	One bottle with red cap containing 30 ml of (horseradish peroxidase) goat anti-human IgG (γ chain specific). Ready to use. Preservative added.
Positive Control	One vial with white cap containing 0.5 ml human serum. Preservative added.
Cut-Off Calibrator	One vial with blue cap containing 0.5 ml human serum. Preservative added.
Negative Control	One vial with black cap containing 0.5 ml human serum. Preservative added.
Sample Diluent	One bottle with green cap containing 50 ml of Tween-20, bovine serum albumin and phosphate-buffered-saline, (pH 7.2 ± 0.2). Green solution, ready to use.
	NOTE: Shake well before use. Preservative added.
TMB Substrate	One amber bottle with amber cap containing 30 ml of 3,3',5,5'-tetramethylbenzidine (TMB). Ready to use.
Stop Solution	One bottle with white cap containing 30 ml 1M H ₂ SO ₄ , 0.7M HCl. Ready to use.
Wash Concentrate (10X)	Two bottles with clear caps, each containing 50 ml of 10X concentrated phosphate-buffered-saline and Tween-20 solution (blue solution). Contains preservative.

Store these reagents at 2 to 8°C

OTHER MATERIALS REQUIRED

- ELISA microwell reader capable of reading at a wavelength of 450nm (reference 630 nm).
- Pipettes capable of accurately delivering 10 to 200µl.
- Multichannel pipette capable of accurately delivering 50-200µl.
- Reagent reservoirs for multichannel pipettes.
- Wash bottle or microwell washing system.
- Distilled or deionized water.
- One liter graduated cylinder.
- Serological pipettes.
- Disposable pipette tips.
- Paper towels.
- Laboratory timer to monitor incubation steps.
- Disposal basin and disinfectant. (example: 10% household bleach, 0.5% sodium hypochlorite.)

PRECAUTIONS

REAGENTS: For *In Vitro* Diagnostic Use.

1. Normal precautions exercised in handling laboratory reagents should be followed. In case of contact with eyes, rinse immediately with a large quantity of water and seek medical advice. Wear suitable protective clothing, gloves, and eye/face protection. Do not breathe vapor. Dispose of waste observing all local, state, and federal laws.

2. The wells of the ELISA plate do not contain viable organisms. However, the strips should be considered **POTENTIALLY BIOHAZARDOUS MATERIALS** and handled accordingly.
3. The human serum controls are **POTENTIALLY BIOHAZARDOUS MATERIALS**. Source materials from which these products were derived were found negative for HIV-1 antigen, HBsAg, and for antibodies against HCV and HIV by approved test methods. However, since no test method can offer complete assurance that infectious agents are absent, these products should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories": current edition; and OSHA's Standard for Bloodborne Pathogens (14).
4. Adherence to the specified time and temperature of incubations is essential for accurate results. **All reagents must be allowed to reach room temperature (20-25°C) before starting the assay.** Return unused reagents to refrigerated temperature immediately after use.
5. Improper washing could cause false positive or false negative results. Be sure to minimize the amount of any residual wash solution; (e.g., by vigorously blotting or aspiration) before adding Conjugate or Substrate. Do not allow the wells to dry out between incubations.
6. The human serum controls, Sample Diluent, Conjugate, and Wash Buffer concentrate contain a preservative (thimerosal, 0.04% (w/v)) which may be toxic if ingested.
7. The Stop Solution is TOXIC. Causes burns. Toxic by inhalation, in contact with skin and if swallowed. In case of accident or if you feel unwell, seek medical advice immediately.
8. The TMB Solution is HARMFUL. Irritating to eyes, respiratory system and skin.
9. The Wash Concentrate is an IRRITANT. Irritating to eyes, respiratory system and skin.
10. Wipe bottom of plate free of residual liquid and/or fingerprints that can alter optical density (OD) readings.
11. Dilution or adulteration of these reagents may generate erroneous results.
12. Reagents from other sources or manufacturers should not be used.
13. The following components are not kit lot number dependent and may be used interchangeably with other Diamedix ELISA assays providing the Reagent Catalog numbers (on the bottle) are the same: TMB Substrate, Stop Solution, Wash Concentrate and, in some cases, Sample Diluent.
14. TMB Solution should be colorless, very pale yellow, very pale green, or very pale blue when used. Contamination of the TMB with conjugate or other oxidants will cause the solution to change color prematurely. Do not use the TMB if it is noticeably blue in color.
15. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.
16. Avoid microbial contamination of reagents. Incorrect results may occur.
17. Cross contamination of reagents and/or samples could cause erroneous results.
18. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
19. Avoid splashing or generation of aerosols.
20. Do not expose reagents to strong light during storage or incubation.
21. Allowing the microwell strips and holder to equilibrate to room temperature prior to opening the protective envelope will protect the wells from condensation. Extra strips should be immediately resealed with desiccant and returned to proper storage. Strips are stable for 60 days after the envelope has been opened and properly resealed and the indicator strip on the desiccant pouch remains blue.
22. Wash solution should be collected in a disposal basin. Treat the waste solution with 10% household bleach (0.5% sodium hypochlorite). Avoid exposure of reagents to bleach fumes.
23. Caution: Liquid waste at acid pH should be neutralized before adding to bleach solution.
24. Do not use ELISA plate if the indicator strip on the desiccant pouch has turned from blue to pink.
25. Do not allow the conjugate to come in contact with containers or instruments that may have previously contained a solution utilizing sodium azide as a preservative. Residual amounts of sodium azide may destroy the conjugate's enzymatic activity.
26. Do not expose any of the reactive reagents to bleach-containing solutions or to any strong odors from bleach-containing solutions. Trace amounts of bleach (sodium hypochlorite) may destroy the biological activity of many of the reactive reagents within this kit.

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 or lower. 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.^{10,11}

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

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.

Wash Concentrate (10X): Store at 2° to 25°C. Diluted Wash Solution (1X) is stable at room temperature (20° to 25° C) for up to 7 days or for 30 days at 2° to 8°C. Prepare Wash Solution by adding 2 X 50 ml Wash Concentrate (10X) to 900 ml deionized or distilled water.

MANUAL USERS:

1. Determine the number of microwells needed. Allow six wells for Control/Calibrator determinations (one Blank, one Negative Control, three Calibrators and one Positive Control) per run. A Reagent Blank should be run on each assay. Check software and reader requirements for the correct Controls/Calibrator configurations. Return unused strips to the resealable pouch with desiccant, seal, and return to storage between 2° and 8°C.
2. Prepare 1:21 dilutions (e.g.: 10µl of serum + 200µl of Sample Diluent) of the Negative Control, Calibrator, Positive Control, and each patient serum.
Note: Shake Sample Diluent Well Before Use.
3. Mix sample dilutions gently by withdrawing and expelling in a pipette 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100µl of each diluted control, calibrator and patient sample to the wells. Avoid formation of bubbles when transferring diluted samples. Use a different pipette tip for each sample.
Note: Include one well which contains 100µl of Sample Diluent as a reagent blank. This will ultimately be used to "zero" the photometer before reading test results.
4. Allow the wells to incubate uncovered at room temperature (20-25°C) for 25 ± 5 minutes.
5. Wash the microwell strips five times as follows:

Vigorously shake out the liquid from the wells. Invert the plate over a paper towel and tap firmly to remove any residual solution from the wells. Visually inspect the plate to ensure that no residual solution remains. Fill each microwell with Wash Buffer. Make sure no air bubbles are trapped in the wells. Repeat these steps for a total of 5 washes.

If using an automated microwell wash system, set the dispensing volume to 300-350 µl/well. Set the wash cycle for 5 washes with no delay between washes. If necessary, the microwell plate may be removed from the washer, inverted over a paper towel and tapped firmly to remove any residual wash solution from the microwells.

6. Add 100µl of the Conjugate to each well, including reagent blank well, at the same rate and in the same order as the specimens were added.
7. Incubate the plate at room temperature (20-25°C) for 25 ± 5 minutes
8. Wash the microwells by following the procedure as described in step 5.
9. Add 100µl of TMB to each well, including reagent blank well, at the same rate and in the same order as the specimens were added.
10. Incubate the plate at room temperature (20-25°C) for 10 to 15 minutes.
11. Stop the reaction by adding 50µl of Stop Solution to each well, including reagent blank well, at the same rate and in the same order as the TMB was added. Positive samples will turn from blue to yellow. After adding the Stop Solution, tap the plate from side to side several times to ensure the well contents are thoroughly mixed.
12. Set the microwell reader to read at a wavelength of 450nm (reference 630) and measure the absorbance, e.g. optical density (OD), of each well against the reagent blank. The plate should be read within 30 minutes after the addition of the Stop Solution.

MAGO PLUS AUTOMATED EIA PROCESSOR USERS:

When using the MAGO Plus Automated EIA Processor, refer to the MAGO Plus Operating Manual for the test setup and procedures.

QUALITY CONTROL

1. Each time the assay is run the Calibrator must be run in triplicate. A reagent blank, Negative Control, and Positive Control must also be included in each assay.
2. Calculate the mean of the three Calibrator wells. If any one of the three values differs by more than 15% from the mean, discard that value and calculate the mean using the remaining two values.
3. The mean absorbance (OD) value for the Calibrator and the OD values for the Positive and Negative Controls must fall within the following ranges:

	OD Range
Negative Control	≤ 0.250
Calibrator	≥ 0.300
Positive Control	≥ 0.500

- a. The OD of the Negative Control divided by the mean OD of the Calibrator should be ≤ 0.9.
- b. The OD of the Positive Control divided by the mean OD of the Calibrator should be ≥ 1.25.

If any of the above conditions are not met the test should be considered invalid and should be repeated.

The Positive and Negative Controls are intended to monitor for substantial reagent failure and will not ensure precision at the assay cut-off.

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

RESULTS

A. Calculation

1. Correction Factor

A Cut-Off OD value for positive samples has been determined by the manufacturer and correlated to the Cut-Off Calibrator. The correction factor (CF) will allow you to determine the cutoff value for positive samples and to correct for slight day-to-day variations in test results. The correction factor is determined for each lot of kit components and is printed on the data label located on the inside lid of the kit box.

2. Cut-Off OD Value

To obtain the Cut-Off OD value, multiply the CF by the mean OD of the Calibrator determined above.

$$(CF \times \text{mean OD of Calibrator} = \text{Cut-Off OD value})$$

3. Index Values or OD Ratios

Calculate the Index Value or OD Ratio for each specimen by dividing its OD value by the Cut-Off OD from step 2.

Example:

Mean OD of Calibrator	= 0.793
Correction Factor (CF)	= 0.25
Cut-Off OD	= 0.793 x 0.25 = 0.198
Unknown Specimen OD	= 0.432
Specimen Index Value or OD Ratio	= 0.432 / 0.198 = 2.18

B. Interpretation:

Index Values or OD Ratios are interpreted as follows:

Index Value or OD Ratio	Interpretation
≤ 0.90	No detectable IgG antibodies to <i>M. pneumoniae</i> . Indicates absence of current or previous infection.
0.91 to 1.09*	* Equivocal for IgG antibodies to <i>M. pneumoniae</i> .
≥ 1.10	Reactive for IgG antibodies to <i>M. pneumoniae</i> . Indicates a past or recent infection.

* When Equivocal results are obtained, specimens should be retested in duplicate. Any two of the three results which agree should be reported. Specimens that remain equivocal after repeat testing should be tested by an alternate serologic procedure.

NOTE: Performance of this assay has not been established with specimens known to be positive for antibodies to organisms which are associated with lower respiratory illness (i.e., Influenza A & B, CMV, *C. pneumoniae*, parainfluenza), and closely related Mycoplasma serovars known to cross-react with *M. pneumoniae*. **Cross-reactivity studies with such organisms have not been performed with this test system.**

LIMITATIONS

1. A diagnosis should not be made on the basis of anti-Mycoplasma results alone. Test results for anti-Mycoplasma should be interpreted in conjunction with the clinical evaluation and the results of other diagnostic procedures.
2. If testing 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 fourteen days later and tested in parallel with the original sample.
3. The use of hemolytic, lipemic, bacterially contaminated or heat inactivated specimens should be avoided. Erroneous results may occur.
4. Assay performance characteristics have not been established for matrices other than serum.
5. A single positive result only indicates previous immunologic exposure. The level of antibody response or class of antibody response may both be required to determine active infection or disease stage.
6. 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.
7. The continued presence or absence of antibodies cannot be used to determine the success or failure of therapy.
8. Testing should not be performed as a screening procedure for the general population. The predictive value of a positive or negative serologic result depends on the pretest likelihood of *M. pneumoniae* being present. Testing should only be done when clinical evidence suggests the diagnosis of *M. pneumoniae* associated disease.
9. The performance of this device has not been established on neonates or immunocompromised patients.

EXPECTED VALUES

Symptomatic infections attributable to this organism most commonly occur in children and young adults (ages 2-19 years).¹² One report demonstrated that 97-98% of sera from a healthy adult population were non-reactive for *M. pneumoniae* antibody by CF and IFA.¹³ Each laboratory should establish their own expected results based upon the population type typically evaluated.

The clinical study for this product included 205 random specimens which were sent to a reference laboratory in the Northeastern United States for routine Mycoplasma serological analysis. With respect to this population, 92/205 (45%) were negative, 21/205 (10%) were equivocal, and 92/205 (45%) were reactive.

PERFORMANCE CHARACTERISTICS

A. Comparison Testing

The performance of the Diamedix *Is*[®]-Mycoplasma IgG ELISA test system was evaluated in a two site clinical investigation. There were a total of 194 specimens tested; 109 at site one, and 85 at site two. Most of the specimens (192/194) were obtained from a reference laboratory in Northeastern United States. These specimens were sent to the lab for routine Mycoplasma serological analysis. The remaining two specimens were repository specimens which had been previously tested for Mycoplasma IgG antibody, and were found to be positive. All specimens were frozen and maintained according to the guidelines indicated under the Specimen Collection section of this insert.

Specimens were tested on the ELISA test system at the clinical sites, and were then tested in-house by IFA. Table 1 below shows the results of this comparison. These results represent those from single patient samples and not from multiple draws from the same patient.

TABLE 1

Calculation of Relative Sensitivity, Specificity, and Agreement

IFA Test System

		≥1:64 Positive	<1:32 Negative	1:32 Equivocal	Totals
Diamedix Mycoplasma IgG ELISA	+	69	12	17	98
	-	4	84	0	88
Results		±	2	6	0
Totals		75	102	17	194

95% CI*

Relative Sensitivity = 69/73 = 94.5% = 89.3 to 99.7%

Relative Specificity = 84/96 = 87.5% = 80.9 to 94.1%

Relative Agreement = 153/169 = 90.5% = 86.1 to 94.9%

* 95% confidence intervals calculated by the Exact Method.¹⁵

NOTE: Be advised that relative refers to the comparison of this assay's results to that of a similar assay. There was not an attempt to correlate the assay's results with disease presence or absence. No judgment can be made on the comparison assay's accuracy to predict disease.

In addition to the two-site clinical study, the Diamedix *Is*[®]-Mycoplasma IgG ELISA test was used to evaluate 35 pairs of acute and convalescent specimens which were previously characterized by complement fixation (CF). Of the 35 pairs, 29 pairs demonstrated a four-fold or greater increase in the CF endpoint titer. Of the 29 pairs, 16 pairs were ELISA negative at the acute stage, and positive at the convalescent stage; eight pairs were positive at both the acute and convalescent stage; and five pairs were negative at both the acute and convalescent stage.

B. Precision

Precision was evaluated as outlined in document number EP5: Evaluation of Precision Performance of Clinical Chemistry Devices, Current Edition, as published by the National Committee for Clinical Laboratory Standards (NCCLS), Villanova, PA. Precision studies were conducted at both clinical sites using the same specimens.

Briefly, six specimens were tested, two relatively strong positive specimens, two specimens near the cut-off, and two which were clearly negative. The kit Negative Control and High Positive Control were included as additional panel members, for a total of eight specimens. Each of the eight specimens were assayed in duplicate, on each day of testing, once in the morning and once in the afternoon, for a total of four replicates for each specimen daily. The precision study continued for a twenty day period, for a total of 80 observations for each of the eight panel members. A summary of this investigation appears in Table 2.

TABLE 2

Summary of Precision Testing Conducted at Clinical Sites 1 and 2

Specimen	Site	Mean Ratio	Result	SWR*	ST**	Days	Total Observations	Overall % CV
M-1	1	6.056	High Positive	0.682	1.016	20	80	16.75
	2	6.124		0.349		0.683	20	80
M-2	1	3.084	High Positive	0.220	0.449	20	80	14.55
	2	3.295		0.185		0.397	20	80
M-3	1	1.089	Near Cut-off	0.117	0.127	20	80	11.68
	2	0.896		0.087		0.124	20	80
M-4	1	0.881	Near Cut-off	0.056	0.073	20	80	8.32
	2	0.611		0.056		0.094	20	80
M-5	1	0.475	Negative	0.024	0.076	20	80	16.03
	2	0.093		0.045		0.077	20	80
M-6	1	0.443	Negative	0.026	0.072	20	80	16.24
	2	0.049		0.051		0.067	20	80
High Positive Control	1	3.611	Positive	0.210	0.275	20	80	7.61
	2	3.680		0.257		0.311	20	80
Negative Control	1	0.415	Negative	0.013	0.068	20	80	16.42
	2	0.111		0.062		0.119	20	80

* Point estimate of within run precision standard deviation.

** Point estimate of total precision standard deviation.

NOTE: The precision results depicted in Table 2 are presented only as an example of those results obtained during the clinical study, using ideal conditions of environment, equipment, and technique. Precision should be evaluated at each laboratory, and may vary, depending upon the conditions at the laboratory.

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