

MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES CE

NOTE: Changes highlighted

PRODUCT AVAILABILITY

The following Organism Antigen Substrate Slides are available individually from Bion Enterprises, Ltd. (Bion):

Antigen Substrate Slide	Code No.	REF
Mycoplasma pneumoniae	MP-1212	
Number of Tests	12-Well	

INTENDED USE

The Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES may be used as the antigenic substrate in indirect fluorescent antibody assays for the qualitative and/or semi-quantitative determination of Mycoplasma pneumoniae antibodies in human serum. The Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES are intended for use as an aid in the diagnosis of active infection and as a determination of immunological experience with Mycoplasma pneumoniae.

SUMMARY AND EXPLANATION

Mycoplasma pneumoniae was the first mycoplasma for which an etiologic role in human disease was demonstrated. The first descriptions of Primary Atypical Pneumonia (PAP) as a clinical syndrome appeared in the late 1930's, with the recognition that some cases of pneumonia failed to respond to therapy with sulfonamides or penicillin. The infectious nature of PAP was demonstrated by Eaton and colleagues and the offending organism came to be called Eaton's agent.¹

Most bacterial pneumonias (typical pneumonias) have an abrupt, often rigorous onset. Mycoplasma (atypical) pneumonia is characterized by flu-like symptoms, including a dry cough. Symptoms are typically chronic in both onset and recovery. The nondescript and somewhat chronic nature of the disease prompted the nickname "walking pneumonia". M. pneumoniae is the leading cause of pneumonia in older children and young adults, causing up to 20% of all cases of pneumonia.² It is a common cause of a wide range of upper and lower respiratory tract infections. While the majority of these infections appear to be relatively mild cases of pneumonia, more severe infections, such as pneumonia requiring hospitalization or lung abscess can also occur.³ This disease may also be found with non-respiratory complications in other organ systems, and the subsequent infection may be life-threatening.⁴

Mycoplasma pneumoniae and viral pneumonia, pharyngitis, and tracheobronchitis are often clinically indistinguishable. It is important to differentiate mycoplasma and viral infections because mycoplasma infections are readily treatable with tetracycline and its derivatives and with erythromycin, while viral infections do not respond to antibiotics.² The organism remains in the respiratory tract for prolonged periods, even after symptoms subside, and this feature may contribute to the endemic nature of the disease. Unlike other respiratory diseases, M. pneumoniae infections are not seasonal.⁵ The disease spreads slowly in families and transmission seems to require close contact between family members.⁵

Diagnosis of mycoplasma infections by isolation of the organism is difficult because mycoplasmas grow slowly and require complex media for isolation. Therefore, diagnosis of Mycoplasma pneumoniae infections has relied heavily upon serological methods such as complement fixation (CF) tests. However, due to the fact that the lipid antigen of the organism is found broadly distributed in nature, this has resulted in clearly false positive assay results using this test method.⁷ For this reason, other assays have been developed in an attempt to overcome the problem of nonspecific lipid antigen. Enzyme linked immunosorbent assays (ELISA)⁸ and whole organism immunofluorescence assays (IFA)^{9,10,11} appear suitable for the detection of infection caused by Mycoplasma organisms. In IFA, the original problem of fixing the organism to glass slides was alleviated by use of cell cultures, which provide both a plane of focus and a matrix for adherence of mycoplasmas. Both ELISA and IFA can be used to detect IgG, IgA or IgM antibodies in serum and IgA antibodies in secretions.⁶

PRINCIPLE OF THE IFA PROCEDURE

The Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES may be utilized in the indirect fluorescent antibody assay method first described by Weller and Coons¹² and further developed by Riggs, et al.¹³ The procedure is carried out in two basic reaction steps:

Step 1 - Human serum is reacted with the antigen substrate.

Antibodies, if present, will bind to the antigen forming stable antigen-antibody complexes. If no antibodies are present, the complexes will not be formed and serum components will be washed away.

Step 2 - Fluorescein labeled antihuman IgG (or IgM) antibody is added to the reaction site which binds with the complexes formed in step one. This results in a positive reaction of bright apple-green fluorescence when viewed with a properly equipped fluorescence microscope. If no complexes are formed in step one, the fluorescein labeled antibody will be washed away, exhibiting a negative result.

REAGENTS

Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES are individually foil-wrapped twelve well slides with Mycoplasma pneumoniae (FH Strain) infected HEP-2 cells fixed onto each well. Each reaction well will contain a cell monolayer along with microorganisms and colonies of the Mycoplasma pneumoniae antigen.

STORAGE AND STABILITY

The Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES are stable in sealed foil pouches at 8°C or lower until labeled expiration date.

WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use. Thus, only staff trained in methods of *in vitro* diagnostics may perform the test. **IVD**
- Substrate slides are for single use only and must not be used more than once. **ⓧ**
- Use with non-Bion reagents could result in erroneous results.
- Care should be taken when handling substrate slides due to sharp edges.
- The antigenic substrates have been fixed and contain no detectable live mycoplasma agents. However, they should be handled and disposed of as any potentially biohazardous laboratory material.
- Do not remove slides from pouches until ready for testing. Do not use if pouch has been punctured, as indicated by a flat pouch.
- Antigen substrate slides should be brought to room temperature (20-25°C) prior to use.
- Abnormal test results may be seen if the antigen substrate slides are allowed to dry during the staining procedure.
- Refrigeration (2-8°C) of antigen substrate slides immediately upon arrival will insure stability until labeled expiration date.
- Antigen substrate slides should not be used beyond stated expiration date.
- Avoid microbial contamination of all reagents involved in the testing procedure or incorrect results may occur.
- Incubation times or temperatures other than those specified may give erroneous results.
- Reusable glassware must be washed and thoroughly rinsed free of detergents.
- Care should be taken to avoid splashing or generation of aerosols.
- Previously frozen specimens after thawing should be thoroughly mixed prior to testing. It is recommended that sera is freeze thawed no more than one time. If repeated testing is required, it is suggested that specimen be aliquoted.
- Patient samples, as well as all materials coming into contact with them, should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/NIH manual "Biosafety in Microbiological and Biomedical Laboratories", 1984 Edition. Never pipette by mouth. Avoid contact with skin and mucous membrane.

SPECIMEN COLLECTION

Blood should be collected fasting or at least one hour after meals to avoid lipemic serum, as excess lipids may produce a "film" over the substrate. Aseptically collect 5-8 ml of blood by venipuncture. Allow the blood to clot at room temperature (20-25°C) before separating serum to avoid hemolysis which could interfere with test results. Specimens should be stored refrigerated at 2-8°C and tested within one week of collection. Long term storage should be at -20°C in aliquots to avoid repeated freezing and thawing. Do not store in self-defrosting freezer.

Avoid using contaminated sera as they may contain proteolytic enzymes which will digest the substrate. It is unnecessary to heat inactivate serum specimens prior to testing; however, sera that have been heat inactivated may be used.

When testing paired samples to look for evidence of recent infection, the acute specimen should be obtained as soon as possible after onset of illness and the convalescent specimen obtained 7-14 days later. Acute and convalescent specimens must be tested simultaneously, in the same assay, looking for a significant change in antibody titer between the paired sera. If the first specimen is obtained too late during the course of the infection, a significant rise in the antibody titer may not be detected.

PROCEDURE

Detailed descriptions of indirect immunofluorescence techniques may be found in the references listed in the bibliography.^{14,15,16}

MATERIALS PROVIDED

Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES. Lot Number provided on label. **LOT**

MATERIALS AVAILABLE FROM Bion

1. Fluorescent Antibody Conjugate with 0.01% Evans Blue counterstain
2. Mycoplasma pneumoniae Positive Human Control Serum
3. Mycoplasma pneumoniae Negative Human Control Serum
4. Phosphate Buffered Saline (PBS)
5. Mounting Medium
6. IgG Binding Reagent

MATERIALS REQUIRED BUT NOT PROVIDED

1. Disposable test tubes (12 x 75 mm or comparable) and rack
2. Disposable serological pipettes
3. Calibrated pipettes to deliver 50 µl, 100 µl and 200 µl with disposable pipette tips
4. Pasteur pipettes and bulbs
5. Moist chambers
6. Plastic squeeze wash bottle
7. Coplin jars or staining dishes with slide racks
8. 24 x 60 mm #1 coverslips
9. Felt tip marking pen
10. Fluorescence microscope equipped with a mercury or tungsten-halogen light source, a 390-490 nm excitation filter and 515-520 nm barrier filter, and optics to give a total magnification of 200X or 250X. The excitation wavelength of FITC is 490 nm and the emission wavelength is 520 nm.

TEST PROCEDURE

1. SPECIMEN PREPARATION

Screening:

Each laboratory should establish its own protocol for the preparation of serum screening dilutions. Most indirect fluorescent antibody staining procedures utilize a 1:10 dilution of each patient's serum which is prepared by adding 0.05 ml (50 µl) of the patient's serum to 0.45 ml of PBS. However, due to the large number of low titers seen in the population, it is suggested that a 1:160 IgG screening dilution is utilized.

NOTE: If testing for IgM specific antibodies using an IgM specific fluorochrome conjugate, each patient serum specimen must be pre-treated to remove any IgG interference by separating the IgM from the IgG, and then running the screening test on the IgM eluate. Suggested methodologies are ion exchange chromatography¹⁷ or IgG immunoprecipitation.^{18,19}

IgG Binding Reagent Catalog No. GBR-9982 available from BION Enterprises, Ltd.

Semi-quantitation:

Serum dilutions are utilized to measure antibody titer. Each laboratory should establish its own titrating protocol. The selection of either twofold or fourfold dilution procedures depends upon the experience level and training of the individual(s) reading the fluorescent antibody assay.

The following fourfold serial titration is suggested for IgG testing:

- a. Prepare a 1:10 dilution of each patient's serum by adding 0.05 ml (50 µl) of patient's serum to 0.45 ml of PBS in tube #1.
- b. Add 0.3 ml PBS to tubes #2, #3, #4, #5 and #6.
- c. Using a 100 µl pipette, transfer 0.1 ml (100 µl) from tube #1 to tube #2. Mix. Using a new tip for each dilution, transfer 0.1 ml (100 µl) from the second tube to the third, from the third tube to the fourth, from the fourth tube to the fifth, and from the fifth tube to the sixth mixing after each transfer.
- d. Do not use tube #1 & #2. Starting with tube #3, apply to substrate slide.

The following twofold titration is suggested for IgM testing:

- a. Prepare a 1:10 dilution of each patient's serum using one of the treatment methodologies mentioned in the "Screening NOTE" above. This will be designated as tube #1.
- b. Add 0.2 ml PBS to tubes #2, #3, #4, and #5.
- c. Using a 200 µl pipette, transfer 0.2 ml (200 µl) from tube #1 to tube #2. Mix. Using a new tip for each dilution, transfer 0.2 ml (200 µl) from the second tube to the third, from the third tube to the fourth, and from the fourth tube to the fifth, mixing after each transfer.
- d. Do not use tube #1. Starting with tube #2, apply to substrate slide.

These titrations will have the following dilutions:

<u>Fourfold</u>	<u>Twofold</u>
Tube #1 = 1:10	Tube #1 = 1:10
Tube #2 = 1:40	Tube #2 = 1:20
Tube #3 = 1:160	Tube #3 = 1:40
Tube #4 = 1:640	Tube #4 = 1:80
Tube #5 = 1:2560	Tube #5 = 1:160
Tube #6 = 1:10,240	

2. SLIDE PREPARATION

Remove reagents and as many slides as are required from the refrigerator or freezer and allow to equilibrate to room temperature (20-25°C) for at least five minutes. Remove slides from sealed foil pouches being careful not to touch the antigen surface. Identify each slide using a felt tip marking pen.

3. SPECIMEN APPLICATION

Using separate Pasteur pipettes, apply one drop (20-30 µl) of the positive control, one drop (20-30 µl) of the negative control and one drop (20-30 µl) of each patient serum dilution to individual wells of the slide. Do not touch the antigen surface with the pipette while dropping. Do not allow drops to mix, as cross contamination of samples between wells could cause erroneous results.

4. INCUBATION 1

Incubate in a moist chamber at room temperature (20-25°C) for 30 minutes. THE ANTIGEN MUST NOT BE ALLOWED TO DRY DURING ANY OF THE FOLLOWING STEPS. Nonspecific binding may occur if the reagent is allowed to dry on the slide.

NOTE: For IgM testing, incubate the substrate slides in a moist chamber at 35-37°C for 90 minutes.

5. RINSE 1

Remove slides from moist chamber and rinse GENTLY with PBS using a squeeze wash bottle. Do not focus the PBS stream directly onto the wells. To prevent cross contamination tilt slide first toward wells 1-6 and, running a PBS stream along the midline of the slide, allow the PBS to run off the top edge of the slide. Then, tilt the slide toward wells 7-12 and repeat this procedure, allowing the PBS to run off the bottom edge of the slide. For six well slides, tilt slide down and run the PBS stream across the slide above the wells, allowing the PBS to run off the bottom edge of the slide.

6. WASH 1

Place slides in Coplin jars or staining dishes and wash in two changes of PBS for not less than five minutes or more than ten minutes each, agitating gently at entry and prior to removal.

7. CONJUGATE APPLICATION

Remove slides from the wash one at a time, shake off excess PBS, dry around outside edges if necessary and return each slide to the moist chamber. Apply one drop of an appropriate fluorescent antibody (IgG or IgM) conjugate with counterstain (diluted to its predetermined proper working dilution) to each well of each slide, making sure that each well is completely covered.

8. INCUBATION 2

Incubate in a moist chamber at room temperature (20-25°C) for 30 minutes. Protect slides from excessive light.

NOTE: For IgM testing, incubate in a moist chamber at 35-37°C for 60 minutes.

9. RINSE 2

Remove slides from moist chamber and rinse GENTLY with PBS using a squeeze wash bottle. As suggested in step 5., do not focus PBS stream directly onto the wells.

10. WASH 2

Place slides in Coplin jars or staining dishes and wash in two changes of PBS for not less than five minutes or more than ten minutes each, agitating gently at entry and prior to removal.

11. COVERSGLIP

Remove slides one at a time from the last PBS wash, shake off excess PBS and immediately add two to four drops of mounting medium across the slide. Tilt slide and rest the edge of the coverslip against the bottom of the slide allowing the mounting medium to form a continuous bead between the coverslip and slide. Gently lower the coverslip from the bottom of the slide to the top, being careful to avoid air bubbles. Drain excess mounting medium by holding the edge of the slide against absorbent paper. Wipe off back of slide.

12. READ

Examine stained slides as soon as possible using a properly equipped fluorescence microscope. It is recommended that slides be examined on the same day they are stained. If any delay is anticipated, store slides in the refrigerator (2-8°C) away from direct light and read the following day. Do not allow mounting medium to dry between slide and coverslip. If drying should occur, add additional mounting medium or recoverslip slide.

FLUORESCENT INTENSITY GRADING

Fluorescent intensity may be semi-quantitated by following the guidelines established by the Centers for Disease Control, Atlanta, Georgia:²⁰

- 4+ = Maximal fluorescence; brilliant yellow-green.
- 3+ = Less brilliant yellow-green fluorescence.
- 2+ = Definite but dull yellow-green fluorescence.
- 1+ = Very dim subdued fluorescence.

The degree of fluorescent intensity is not clinically relevant and has only limited value as an indicator of titer. Differences in fluorescence microscope optics, filters and light sources may result in differences of 1+ or more fluorescent intensity when observing the same slide using different microscopes.

QUALITY CONTROL

SPECIFICITY CONTROL

Both a positive and negative antibody control must be included with each run. These controls must be examined prior to reading test samples and should demonstrate the following results:

Negative Control

Using a negative control serum on Bion MYCOPLASMA PNEUMONIAE SUBSTRATE SLIDES, the Mycoplasma pneumoniae microorganisms and colonies should exhibit less than 1+ fluorescence with the background cells appearing reddish-orange due to the counterstain.

Positive Control

Using a positive control serum on Bion MYCOPLASMA PNEUMONIAE SUBSTRATE SLIDES, the Mycoplasma pneumoniae infected cells should exhibit well defined specific fluorescent staining at an intensity of 3+ or greater. The Mycoplasma staining pattern consists of both solid colonies and glass rod-like forms. The rod-like forms are never seen alone; however, colony staining alone may be seen with low-titered sera. In addition to colonies and rods, ring-like subunits around the colony rims may be seen with high-titered IgM sera. Each well should exhibit this specific staining pattern with the background cells staining reddish-orange due to the counterstain.

Each control must demonstrate the expected reaction in order to validate the test. If the controls fail to appear as described above, the test results should not be reported and the test should be repeated. If upon repeat testing the controls still fail to show the proper reaction, do not report the test results.

SENSITIVITY CONTROL

A titrated control included with each run tests substrate sensitivity, as well as, checks technique, conjugate quality and the microscope optical system. The endpoint titer of this control must be determined and there must not be more than a twofold difference (+/-) in titer from this determined endpoint. Each run should include the endpoint dilution, one twofold or fourfold dilution above and one twofold or fourfold dilution below the endpoint dilution. The more concentrated dilution should be positive and the less concentrated dilution negative. If the control does not behave as described, the test results are invalid and the tests should be repeated. If the control again fails to show the proper reaction upon repeat testing, do not report the test results.

READING OF TEST RESULTS

NEGATIVE

A serum dilution is considered to be negative for *Mycoplasma pneumoniae* antibodies if the *Mycoplasma* microorganisms and colonies exhibit less than 1+ fluorescence, or if the fluorescence observed is not the specific staining pattern of *Mycoplasma pneumoniae*.

A sample is considered negative for *Mycoplasma pneumoniae* antibodies if it exhibits less than 1+ fluorescence at a serum dilution of 1:10 and all greater dilutions, or if the fluorescence observed is not the specific staining pattern of *Mycoplasma pneumoniae*.

- ... Negative samples may exhibit fluorescent staining slightly greater than the negative control, but less than 1+.
- ... Nonspecific staining of all the background tissue culture cells observed with some sera at low dilutions is most likely due to the presence of autoantibodies against cellular components in either the nucleus or cytoplasm.
- ... Staining of areas other than colonies or microorganisms should be interpreted as negative, and attention should be directed to specific steps in the staining method (e.g., RINSE and WASH steps).

POSITIVE

A serum dilution is considered positive for *Mycoplasma pneumoniae* antibodies if well defined specific fluorescent staining is observed at an intensity of 1+ or greater. The *Mycoplasma* staining pattern consists of both solid colonies and glass rod-like forms. The rod-like forms are never seen alone; however, colony staining alone may be seen with low-titered sera. In addition to colonies and rods, ring-like subunits around the colony rims may be seen with high-titered IgM sera. The number of microorganisms and colonies of the positive staining reaction and the type of fluorescent staining should closely approximate that seen with the positive control.

A sample is considered positive for *Mycoplasma pneumoniae* antibodies if it exhibits the characteristic staining pattern with a fluorescent intensity of 1+ or greater at a serum dilution of 1:160 IgG or 1:20 IgM or greater.

NOTE: Each field should contain cells that exhibit no apple-green fluorescence. Should most of the cells in the patient test wells fluoresce apple-green in the nucleus and/or cytoplasm, an autoimmune staining reaction due to the presence of autoantibodies should be considered.^{21,22} It is recommended that such samples be diluted beyond the interference for better interpretation. It is possible that autoantibody staining may mask specific staining such that an interpretation cannot be made. Should this occur, results should be reported as "Unable to interpret due to the presence of interfering antibodies."

TITRATION

If a semi-quantitative titration is performed, the result should be reported as the reciprocal of the last dilution in which 1+ apple-green fluorescent intensity of the specific staining pattern is detected. When reading fourfold serial dilutions, endpoints may be extrapolated where necessary.

EXAMPLES OF ENDPOINT EXTRAPOLATION:

1:160 = 3+

1:640 = 2+

1:2560 = +/-

The extrapolated endpoint is reported as 1:1280.

TROUBLESHOOTING

Possible solutions to problems that may occur in immunofluorescent assays are discussed in an accompanying brochure entitled "TROUBLESHOOTING IN IMMUNOFLUORESCENCE".

INTERPRETATION OF RESULTS

Detection of the presence of *Mycoplasma pneumoniae* antibodies indicates a current or past infection with the organism. A fourfold or greater increase in titer between the acute and convalescent serum samples and/or a positive test for IgM specific antibody usually indicates evidence of an active or recent infection.^{10,11}

LIMITATIONS OF THE PROCEDURE

1. *Mycoplasma pneumoniae* antibody test results should be used in conjunction with information available from clinical evaluation and other diagnostic information.
2. A single serological IgG antibody titer to *M. pneumoniae* should not be used as the only criterion for diagnosis. Paired serum samples (acute and convalescent) and testing for IgM specific *M. pneumoniae* antibodies may provide more meaningful data.
3. A negative test result does not necessarily rule out current or recent infection. The specimen may have been collected too early in the disease before demonstrable antibody is present.
4. Lack of significant rise in titer does not exclude the possibility of recent infection but may indicate an acute phase specimen was obtained too late.
5. In some instances, high IgG or IgM antibody levels in the first of paired specimens may prevent the detection of increases in total antibody, resulting in apparently stationary total antibody titer.
6. Test results on specimens from immunosuppressed patients and pregnant women may be difficult to interpret.
7. Positive test results may not be valid in persons who have received blood transfusions or various blood products within the past several months.
8. Antinuclear antibodies (ANA) present in serum may interfere with the *M. pneumoniae* IFA test. They can be differentiated from *Mycoplasma* staining in that ANAs stain the nuclei in all cells; whereas, *Mycoplasma* antibodies exhibit staining only *Mycoplasma* microorganisms and colonies.²¹
9. Cytoplasmic fluorescence in the majority of the cells may be due to the presence of antimitochondrial antibodies (AMA) from the specific antigen staining in that AMA will stain the cytoplasm of all cells; whereas, *Mycoplasma* antibodies exhibit staining in only the *Mycoplasma* microorganisms and colonies.
10. Positive test results from cord blood or neonates should be interpreted with caution. The presence of *M. pneumoniae* IgG antibodies in cord blood is usually the result of passive transfer from mother to the fetus. A negative test, however, may be useful in excluding possible infection.²³
11. Endpoint reactions may vary between laboratories due to differences in type or condition of fluorescence microscope employed, diluting apparatus, IgG/IgM separation methods, as well as the experience level of personnel performing the assay.
12. If both the positive and negative control substrate cells are not visible when viewed using the fluorescence microscope, it may be necessary to replace or realign the light source and check the specific filters.
13. Cell culture substrate slides may exhibit nonspecific fluorescence due to contamination of antibodies or PBS rinse-wash solutions with bacteria or fungi. It is very important that personnel reading the staining results have experience in fluorescence microscopy.

LIMITATIONS OF THE PROCEDURE (continued)

14. If testing for IgM specific antibodies, the presence of Rheumatoid Factor (RF) in serum may cause a false positive reaction if pathogen specific IgG is also present. Routine RF tests may not be sensitive enough to detect small amounts of RF which exist within the normal range, but are sufficient to cause a false positive reaction in the more sensitive IFA technique.²⁴ Therefore, all sera should be treated by ion exchange chromatography¹⁷ or IgG immunoprecipitation^{18,19} before testing to eliminate possible RF interference.
15. If testing for IgM specific antibodies, high titers of specific IgG when present in the patient serum may compete with the pathogen specific IgM for the antigen sites resulting in a false negative IgM reaction.²⁴ Therefore, all sera should be treated by ion exchange chromatography¹⁷ or IgG immunoprecipitation^{18,19} before testing to avoid this possible problem.
16. Two methods such as immunoprecipitation and ion exchange chromatography have been commonly used for neutralizing or removing possibly interfering IgG antibodies prior to testing for specific IgM antibodies in IFA tests. Immunoprecipitation neutralizes all classes of IgG while not affecting the IgM levels; however, high levels of IgG may need to be treated with proportionally increased amounts of the precipitating reagent. Ion exchange chromatography will only eliminate IgG subclasses 1, 2 and 3 with subclass 4 (usually less than 5% of the total IgG) remaining in the fraction with the IgM.
17. IgG removal reagents may lower the total amount of IgM present in serum after treatment. The amount removed can be as high as 25% of total IgM present²⁹. The effects of IgG removal on the sample may vary by assay type performed (IFA, ELISA, etc.). The specific IgM may vary by assay specificity of related organism as well. Inherent cross-reactivity between related viruses, previous infections with related viral species, and bacterial infections is also possible³⁰. It is suggested that paired serums be treated and processed for antibody titers at the same time to minimize processing differences. Serums tested for acute infection should be checked for the patient's clinical history and repeated if indeterminate. Paired samples are recommended for analysis.
18. IgM serology performed by IFA is very attractive since it combines specificity with sensitivity and in most cases only a single serum sample is required. However, the need for careful interpretation of the significance of positive IgM tests in relationship to patient's clinical situations must be emphasized. It is essential to have an awareness of understanding of the many problems associated with IgM testing to avoid the many pitfalls that can trap the most experienced of workers. IgM results must always be interpreted with caution.²⁷

SPECIFIC LIMITATIONS OF THE M. PNEUMONIAE ASSAY

1. One specific Mycoplasma organism, *Mycoplasma genitalium*, is known to resemble *Mycoplasma pneumoniae*. It has demonstrated serological cross-reactivity with *Mycoplasma pneumoniae*.²⁵
2. A fourfold rise in IgG titer is diagnostic; however, because the symptoms of *M. pneumoniae* are so insidious and mild, many patients do not initially seek medical care but wait until the disease fails to improve. Thus, the acute phase sera are not readily available. Testing for specific IgM antibodies can be beneficial in these cases.
3. The level of IgG antibodies normally rises much later than the IgM level. Therefore, early in the disease, serology tests will be negative if only testing for IgG.²⁸
4. Many older patients demonstrate high titers of IgG but do not produce IgM antibodies. This is probably a result of reinfection.²⁸
5. Because antibodies persist long after infection, the presence of IgG antibody cannot be used as an indicator of recent infection; however, high levels of antibody are suggestive and indicate the need to test for IgM and/or look for a rise in IgG titer between acute and convalescent sera.⁶

EXPECTED VALUES

Mycoplasma-specific immunoglobulin titers are known to peak during, or shortly after acute infection, and then decline. The systemic antibody response to *M. pneumoniae* demonstrates an initial rise of IgM titer, usually seven to ten days following infection with the microorganism, and then the development of a Mycoplasma IgG titer.¹¹ The time interval between the development of IgM and IgG antibodies is quite brief, and it is not common to find a serum specimen from an acutely ill patient that is positive for only IgM immunoglobulins.¹¹ During convalescence the Mycoplasma IgM titer drops and eventually becomes undetectable by IFA. The time period of this antibody drop varies with the individual patient and, in a low percentage of patients, IgM may persist at low levels for an extended period of time. For this reason, it is suggested that IgM antibody levels be titrated out to look for a drop in titer as evidence of convalescence. IgG persists indefinitely. It has been established that immunity to *M. pneumoniae* is not complete, and recurrent infections have been reported.²⁶

A study was performed on 60 normal blood donors from the Midwestern United States using the Bion MYCOPLASMA PNEUMONIAE-G ANTIBODY TEST SYSTEM to determine the range of normal adult IgG antibody titers to *Mycoplasma pneumoniae*. In this study, titers range from 1:40 to 1:2560 with a mean titer of 1:160 (63%).²⁷

SPECIFIC PERFORMANCE CHARACTERISTICS

Relative specificity and sensitivity evaluations using Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES were conducted in comparison with two other commercially available IFA test systems for the presence of IgG antibodies to *Mycoplasma pneumoniae*. A panel consisting of 62 specimens with titers ranging from less than 1:20 to 1:2560 was compared qualitatively and semi-quantitatively. Qualitatively there was an overall agreement in 20 of 62 specimens (32%) and 34 of 62 specimens (55%) between the BION Test System and the other two commercially available test systems respectively. The relative sensitivity was 100% and 93.3% respectively; whereas, the relative specificity was 16% and 19% respectively. This appeared to be due to the fact that when compared semi-quantitatively for IgG antibodies, BION titers were consistently several fold higher than the two other commercially available test systems. **TABLE 1** summarizes comparative results from 12 serum specimens ranging in titer from less than 1:20 to 1:5120 indicating an increase in relative sensitivity with the BION Test System. As further evidence of sensitivity, for the 60 normal blood donors tested, BION's calculated IgG mean titer was 1:218, whereas the other test systems' calculated mean titers were 1:68 and 1:37 respectively.²⁷

Relative specificity and sensitivity evaluations using the Bion MYCOPLASMA ANTIGEN SUBSTRATE SLIDES were conducted in comparison with two other commercially available IFA test systems for the presence of IgM antibodies to *Mycoplasma pneumoniae*. A panel consisting of 58 specimens was compared qualitatively with the two other commercially available test systems. Positive Complement Fixation titer ($\geq 1:64$) were present in 46 specimens with positive cold agglutinin results ($\geq 1:32$) present in 30 specimens. There was an overall agreement in 57 of 58 specimens (98%) on the three systems. *One specimen was negative in one test system but positive in the other two. Twelve serum specimens ranging in titer from less than 1:20 to 1:2560 were compared semi-quantitatively. Three were negative for IgG and IgM, four were positive for IgG and negative for IgM and five were positive for both IgG and IgM. Included are Complement Fixation (CF) and Cold Agglutinin (CA) results if available. Whereas the IgG test results indicate an increase in relative sensitivity with the BION System, the IgM test results showed that the three systems were essentially equivalent. These results are summarized in **TABLE 2**.²⁷

TABLE 1 - Summary of Relative IgG Sensitivity Testing

Spec #	BION	1st Other	2nd Other
1	<1:20	<1:20	<1:20
2	<1:20	<1:20	<1:20
3	<1:20	<1:20	<1:20
4	1:20	1:20	<1:20
5	1:80	1:40	1:40
6	1:80	1:40	<1:40 QNS
7	1:160	1:40	1:40
8	1:160	1:40	1:40
9	1:160	1:40	1:20
10	1:640	1:80	1:40
11	1:2560-5120	1:1280	1:1280
12	1:2560	1:160-1:320	1:80

TABLE 2- Summary of Relative IgM Sensitivity Testing

Spec #	BION	1st Other	2nd Other	CF Titers	Cold Agg. Titers
1	<1:20	<1:20	<1:20	ND	ND
2	<1:20	<1:20	<1:20	ND	ND
3	<1:20	<1:20	<1:20	ND	ND
4	<1:20	<1:20	<1:20	ND	ND
5	<1:20	<1:20	<1:20	ND	ND
6	<1:20	<1:20	<1:20	ND	ND
7 Acute	1:40	1:40	1:20	ND	ND
8 Conc.	1:2560	1:2560	1:1280	1:2048	1:16
9 Acute	<1:20	<1:20	<1:20	<1:8	1:64
10 Conc.	1:2560	1:640	1:640	1:512	1:64
11 Acute	1:40	1:40	1:40	1:256	1:32
12 Conc.	1:2560	1:640	1:640	≥1:1024	1:256

Interlot and intralot precision using Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES was evaluated by testing 12 serum specimens for Mycoplasma pneumoniae IgG antibodies; 3 negative and 9 positive sera ranging in titer from less than 1:20 to 1:5120. They were tested on three different lots of substrate slides, and three times on one lot. In each instance there was no more than a twofold difference (+/-) in titer between any of the comparison testings, which is within the confidence limits of this methodology. None of the sera vacillated between a positive or negative result.²⁷

Interlot and intralot precision of the Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES was evaluated by testing 12 serum specimens for Mycoplasma pneumoniae IgM antibodies; 7 negative and 5 positive sera ranging in titer from less than 1:20 to 1:2560. They were tested on three different lots, and three times on one lot. In each instance there was no more than a twofold difference (+/-) in titer between any of the comparison testings, which is within the confidence limits of this methodology. None of the sera vacillated between a positive or negative result.²⁷

In addition, 20 specimens from 13 patients with serological diagnosis of Mycoplasma pneumoniae infection based on Complement Fixation (CF) and Cold Agglutinin (CA) test results were compared on all three IFA Test Systems. Eighteen specimens were positive on all three IFA systems; five had either the CF or the CA not done; and for two the CF and CA results weren't available. *One acute specimen was negative on all systems except the Cold Agglutinin test and ** one specimen was positive on all test systems except the second other commercially available IFA system. Positive IgM IFA test results may allow diagnosis to be made immediately on results from acute specimens without waiting for convalescent specimens to show a significant rise in titer.

TABLE 3 - Comparison of IgM Test Results for IFA, CF and CA

Patient No.	Specimens	BION Test System	1st Other Test System	2nd Other Test System	CF Titers	CA Titers
1	Acute Convalescent	=/≥1:160 =/>1:160	1:80 =/>1:160	1:20 =/>1:160	1:32 1:1024	1:256 1:1024
2	Acute Convalescent	=/≥1:160 =/>1:160	1:80 =/>1:160	1:20 =/>1:160	1:16 1:2048	Not Done
3	Acute Convalescent	=/≥1:160 =/>1:160	1:80 =/>1:160	1:80 =/>1:160	1:256 =/>1:1024	1:32 1:256
4	Acute Convalescent	=/≥1:160 =/>1:160	=/>1:160 =/>1:160	=/>1:160 =/>1:160	1:256 =/>1:1024	Not Done
5	Acute Convalescent	=/≥1:160 =/>1:160	=/>1:160 =/>1:160	1:80 =/>1:160	1:128 1:1024	1:128 1:128
6 *	Acute Convalescent	<1:20 =/>1:160	<1:20 =/>1:160	<1:20 =/>1:160	<1:8 1:512	1:64 * 1:64
7	Acute Convalescent	=/>1:160 =/>1:160	=/>1:160 =/>1:160	=/>1:160 =/>1:160	Not Available	Not Available
8 **	Single Spec.	1:40	1:40	<1:20 **	1:512	1:256
9	Single Spec.	=/>1:160	=/>1:160	=/>1:160	=/>1:512	=/>1:512
10	Single Spec.	=/>1:160	=/>1:160	=/>1:160	1:64	1:128
11	Single Spec.	=/>1:160	=/>1:160	=/>1:160	1:64	1:256
12	Single Spec.	=/>1:160	=/>1:160	=/>1:160	1:128	1:64
13	Single Spec.	=/>1:160	=/>1:160	=/>1:160	Not Done	1:512

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