These titrations will have the following dilutions:

Fourfold	Twofold
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. **COVERSLIP**
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 titered 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]