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.

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:[24]

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 BORRELIA BURGDORFERI SUBSTRATE SLIDES, the Borrelia burgdorferi microorganisms should exhibit less than 2+ fluorescence.

Positive Control

Using a positive control serum on Bion BORRELIA BURGDORFERI SUBSTRATE SLIDES, the Borrelia burgdorferi microorganisms should exhibit apple-green fluorescence at an intensity of 2+ or greater. 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 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 Borrelia burgdorferi antibodies if the organisms exhibit less than 2+ fluorescence.

A sample is considered negative for significant IgG Borrelia burgdorferi antibodies if the organisms exhibit less than 2+ fluorescence at a serum dilution of 1:80 and all greater dilutions.

A sample is considered negative for IgM Borrelia burgdorferi antibodies if the organisms exhibit less than 2+ fluorescence at a serum dilution of 1:10 and all greater dilutions.

Negative samples may exhibit fluorescent staining of the Borrelia burgdorferi organisms slightly greater than the negative control, but less than 2+ fluorescence.

POSITIVE

A serum dilution is considered positive for Borrelia burgdorferi antibodies if well defined specific fluorescent staining of the complete spirochetal microorganism is observed at an intensity of 2+ or greater.

A sample is considered positive for Borrelia burgdorferi IgG antibodies if well defined smooth, homogeneous fluorescent staining of the complete spirochetal microorganism is observed at an intensity of 2+ or greater at a serum dilution of 1:80 or greater.

A sample is considered positive for Borrelia burgdorferi IgM antibodies if well defined specific heterogeneous (rough) fluorescent staining of the spirochetal microorganism is observed at an intensity of 2+ or greater at a serum dilution of 1:10 or greater.

TITRATION

If a semi-quantitative titration is performed, the result should be reported as the reciprocal of the last dilution in which 2+ apple-green fluorescent intensity is detected in the stained B. burgdorferi organisms. When reading fourfold serial dilutions, endpoints can be extrapolated where necessary.

EXAMPLE OF ENDPOINT EXTRAPOLATION:

1:80 = 4+
1:320 = 3+
1:1280 = 1+
1:5120 = +/-

The extrapolated endpoint is reported as 640.

TROUBLESHOOTING

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

INTERPRETATION OF RESULTS

Most clinical investigators agree that a serum specimen should be reported as positive serological evidence of B. burgdorferi antibodies if it demonstrates an IgG titer of 1:320 or greater in a single serum sample, or a fourfold change in titer between two samples.[12,15] Titers of 1:40 to 1:160 have been seen in patients without detectable disease. IgG titers less than 1:80 are considered negative for B. burgdorferi antibodies.

Positive results from a single assay should only be interpreted as a presumptive determination of antibodies to B. burgdorferi. A second assay method (Western Blot) should be performed and results from both assays reported to the clinician. When reporting positive results from only one assay before or in the absence of confirming results by a second assay, the likelihood of misdiagnosis of Lyme disease may increase. This may mislead the clinician from the true cause of illness and potentially expose the patient to inappropriate and/or toxic therapy.

Negative results should be reported as "No detectable antibodies to B. burgdorferi." However, this may represent primary infection with the humoral response not yet developed to detectable levels. If infection with B. burgdorferi is still suspected, a second specimen should be obtained 14-28 days later and the paired specimens tested simultaneously, looking for a seroconversion.

LIMITATIONS OF THE PROCEDURE

1. Borrelia burgdorferi antibody test results do not provide definitive results for establishing or ruling out a diagnosis of Lyme disease and should always be used in conjunction with information available from clinical evaluation and other diagnostic information (history of exposure, symptoms, signs, etc.).
2. A single serological IgG antibody titer to B. burgdorferi should not be used as the only criterion for diagnosis. Paired serum samples (acute and convalescent) and testing for IgM specific B. burgdorferi 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. Positive test results from cord blood or neonates should be interpreted with caution. The presence of B. burgdorferi 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.[26]
9. 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.
10. 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.
11. Antigen 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.
12. 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.[27] Therefore, all sera should be treated by ion exchange chromatography[21] or IgG immunoprecipitation[22,23] before testing to eliminate possible RF interference.
13. 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 chromatography[21] or IgG immunoprecipitation[22,23] before testing to avoid this possible problem.
14. 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. Also, only a portion of the IgM antibodies can be recovered.[25]
15. 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 relation ship 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.[25]

SPECIFIC LIMITATIONS OF THE B. BURGORFERI ASSAY

1. False-positive IFA reactions have been reported from patients with other spirochetal diseases (syphilis, yaws, pinta, relapsing fever and leptospirosis).[5,6] Lyme borreliosis patients occasionally have reactive FTA-ABS and treponemal agglutination tests for syphilis.[5,6] False positive sera from syphilis cases may be distinguished from true Lyme positive sera by performing reagin antibody assays like the RPR card and VDRL assay.[6] Lyme disease sera will be negative.[6]
2. Some patients with infectious mononucleosis and rheumatic diseases such as rheumatoid arthritis and systemic lupus erythematosus have false-positive test results to B. burgdorferi.[5,13]
3. In the later stages of Lyme Disease, IgM antibody determinations tend to be less specific than those for IgG antibodies, but may be useful in early disease or when reactivation or reinfection is suspected.[5,10]
4. Patients with early disease often have serum antibody titers below the diagnostic threshold for six weeks or more after onset.[5,10,11] Only about 50 to 60% of patients with early disease (appearance of EM) have diagnostic titers as measured by either IFA or ELISA.[5,10,11]
5. Antibiotic therapy early in first-stage disease may blunt antibody response to the point that diagnostic threshold levels are never attained.[4]
6. In cases of reinfection, antibody titers to Borrelia burgdorferi may show a fourfold rise from previous convalescent values.[14]
7. A positive result from one (first-step) serological test is only presumptive evidence of the presence of B. burgdorferi antibodies. Another assay (second-step) should be performed to increase specificity.
8. Screening of the general population should not be performed. The positive predictive value depends on the pretest likelihood of infection.