LYME DISEASE IgM IFA TEST SYSTEM

INTENDED USE
These reagents are intended for use in the detection and quantitation of IgM antibody in human sera to Borrelia burgdorferi by the indirect fluorescent antibody (IFA) procedure. For In Vitro Diagnostic Use

SUMMARY AND PRINCIPLES
Lyme disease is a multisystem disease caused by a spirochete Borrelia burgdorferi (2,3,5). The disease has been documented in Europe since early this century. It was more recently documented in the United States during an epidemic in 1975 among children in Old Lyme, Connecticut who demonstrated arthritic symptoms. Steere, et al. recognized the disease as a separate clinical entity (18).

Since Lyme disease is newly recognized, its symptoms have been mistaken with many diseases including: juvenile rheumatoid arthritis, lupus erythematosus, multiple sclerosis, rheumatic fever, Reiter's syndrome, myocarditis and viral meningitis.

The infection is transmitted through an insect vector from an animal reservoir. B. burgdorferi, was first isolated from Ixodes dammini ticks, and was shown to be the etiologic agent of Lyme disease (1,4,16). Ixodes dammini ticks are principally responsible for transmission in Northeastern, and mid-Atlantic regions as well as in Minnesota and Wisconsin. Ixodes pacificus transmits Lyme disease in California coastal and mountain regions. Amblyomma americanum has been reported to be an insect vector in New Jersey. Ixodes scapularis has been reported in the Southeast and Gulf states. Ixodes ricinus has been reported as a vector in Switzerland.

Animal reservoirs include deer, wild mice, birds, raccoons, horses, dogs and cats. The ticks are commonly found on vegetation in epidemic areas especially in wooded areas common to the animal reservoir. The incidence of human infection coincides with the tick season from May through September (11,12).

Among the many symptoms of Lyme disease are:
1. A red lesion at or near the site of a tick bite. The lesion is called erythema migrans (EM).
2. Arthritic symptoms
3. Low grade "flu like" fever
4. Headaches
5. Dizziness
6. Stiff Neck
7. Fatigue and general malaise
8. Muscular aches and pains
9. Abdominal pain
10. Irregular pulse and heart beat

The clinical progression of the disease can be divided into three stages:

Stage I: EM may develop within a few days to weeks following a tick bite. The lesion typically starts at the site of the bite and radiates slowly in a circular pattern. It may reach 50 cm in diameter and generally clears centrally within a few weeks. The lesion, unfortunately, cannot be relied upon for the clinical diagnosis of Lyme disease. Additionally, because of the very small size of the tick (1 mm) as much as 80% of tick bites are unrecognized. During this period symptoms of headache, malaise, myalgia, fever, arthralgia fatigue and lymphadenopathy are usually present.

Stage II: Neurological, cardiac and musculoskeletal involvement. Generally these symptoms may appear from weeks to months following Stage I. This stage is characterized by symptoms of dizziness, weakness and irregular heartbeat, meningitis, inflamed nerve roots in the neck and Bell's palsy.

Stage III: Arthritic Symptoms. Generally the large joints are effected with pain and swelling. Other symptoms include: mood swings, loss of memory, inability to concentrate, poor motor coordination, and somnolence. The arthritic attacks may occur for months or years.

Direct culture from the site of EM has been reported, as well as isolation from blood and spinal fluid. However, these direct culture methods cannot be relied upon in the large scale diagnosis of Lyme disease. Overgrowth by the competing microflora, complicated growth medium requirements and the slow growth rate of the spirochete are all factors influencing the outcome of direct culture.

Serologic methods are the most common methods of choice. Both EIA and IFA have been employed. EIA is considered more sensitive than IFA (7,14,15).

Steere et al. reported that patients with Lyme disease produce antibodies to the IgM class during the first few weeks after onset of EM and produce antibodies of the IgG class more slowly (18). Both IgM and IgG titers can remain positive for many months or years (7).

Antibody cross-reactions have been reported with other pathogenic spirochetal diseases such as syphilis. Although the clinical picture is quite different between active syphilis and Lyme disease, an easy means of differentiating the two diseases is by the use of the VDRL or RPR tests. In active syphilis the VDRL or RPR are positive. In Lyme disease the VDRL and RPR are generally negative. Additionally, antibody cross-reactions with Lyme disease have been reported in leptospirosis, relapsing fever, mononucleositis, and systemic lupus erythematosus.

PRINCIPLES OF THE TEST
The indirect fluorescent antibody test is used for the detection of human IgM antibody to the antigens of B. burgdorferi (strain B31). Cultured organisms are washed and placed in the wells of specially prepared microscope slides.

Patient sera are pretreated with MarSorb G to remove IgG. This prevents false negative IgM reactions due to competitive binding by IgG and false positive IgM reactions due to the simultaneous presence of rheumatoid factor (RF) and immune IgG.

Dilutions of patient sera are placed on the antigen coated wells where antibody, if present, binds to the antigen. The reaction is visualized through the use of a conjugate. The conjugate is fluorescein isothiocyanate (FITC) labeled, anti-human IgM (mu chain specific). Excitation of the FITC by ultraviolet (UV) light causes this dye to emit longer, visible, wavelengths of light in the yellow-green portion of the color spectrum. The conjugate will bind with human IgM antibodies attached to the cells causing them to fluoresce when viewed through a microscope equipped with a UV light source. Since the antigen is composed of intact bacterial cells, the fluorescent image through the microscope consists of spirochetes.

The direct tetramethylrhodamine isothiocyanate (TRITC) counterstain contains specific spirochetal antibodies which will bind to the Borrelia cells following the indirect FITC procedure. Exclusion of the TRITC by UV light causes this dye to emit light in the red portion of the color spectrum. This counterstain allows for red fluorescent visualization of the cells in samples which are negative for IgM antibody to B. burgdorferi. A titer of antibody activity is determined by taking the reciprocal of the last dilution of patient sera showing FITC fluorescence.

PRECAUTIONS
1. Follow the procedure instructions exactly as they appear in this insert to ensure valid results.
2. Thimerosal (Merthiolate), used as a preservative in some of the reagents, may be toxic if ingested, inhaled or absorbed through the skin and is a reproductive hazard.
3. Some components contain sodium azide which is toxic if ingested and forms potentially explosive copper and lead azide compounds in waste plumbing lines. Should the reagents come in contact with copper or lead waste plumbing, flush the waste line with large quantities of water to prevent the formation of potentially explosive compounds.
4. WARNING - POTENTIAL BIOHAZARDOUS MATERIAL. Each donor unit used in the preparation of this material was tested by an FDA approved method for the presence of antibodies to HIV, as well as HBsAg and found to be negative (were not reactively reactive). Because no test method can offer complete assurance that human immunodeficiency virus (HIV), hepatitis B virus, or other infectious agents are absent, these human control reagents 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", 1988.
5. The phosphate buffered saline and mounting medium found in this kit are irritating to the eyes, respiratory system and skin.
6. Some components in this kit contain 0.1% Proclin 300. At full strength Proclin 300 is corrosive and will cause burns and possibly sensitization by skin contact.
7. Slides and reagents should be stored at +2 to +8°C until used.
8. Do not use components beyond their expiration date.
9. Handle slides by the edge since direct pressure on the antigen wells may damage the antigen.
10. Once the procedure has started, do not allow the wells to dry.

Materials provided:

<table>
<thead>
<tr>
<th>Prod #</th>
<th>Description</th>
<th>Qty</th>
</tr>
</thead>
<tbody>
<tr>
<td>32-5010</td>
<td>B. burgdorferi 10 Well Slides</td>
<td>10 ea</td>
</tr>
<tr>
<td>32-5003</td>
<td>Lyme IgM Positive Control</td>
<td>250 µL</td>
</tr>
<tr>
<td>32-5001</td>
<td>Lyme Negative Control</td>
<td>250 µL</td>
</tr>
<tr>
<td>32-1515M</td>
<td>Lyme FITC IgM Conjugate (Rodent)</td>
<td>3.0 mL</td>
</tr>
<tr>
<td>32-5004</td>
<td>Lyme TRITC Counterstain</td>
<td>3.0 mL</td>
</tr>
<tr>
<td>41-9000</td>
<td>MarSorb G</td>
<td>2x2.0 mL</td>
</tr>
<tr>
<td>90-1607</td>
<td>FITC Mounting Medium (pH 7.5)</td>
<td>3.0 mL</td>
</tr>
<tr>
<td>90-1607</td>
<td>Phosphate Buffered Saline (pH 7.5)</td>
<td>2x10 gm</td>
</tr>
<tr>
<td>90-1700</td>
<td>Coverslips, 70x22 mm</td>
<td>12 ea</td>
</tr>
<tr>
<td>90-1710</td>
<td>Bioters, 10 Well</td>
<td>10 ea</td>
</tr>
</tbody>
</table>
PREPARATION OF REAGENTS

1. Allow all reagents to come to room temperature before use.
2. B. burgdorferi slides (Prod #32-5010), should be brought to room temperature prior to breaking the package seal. Peel back the top portion of the package and remove the slide without touching the antigen wells. The slide is now ready to use.
3. Reconstitute each 10 gram vial of PBS (Prod #90-1607) with 1.0 L distilled water.
4. Lyme FITC IgM conjugate (Prod #30-1515M) is provided at the recommended working dilution.
   Note: The conjugate may require reactivation. Variations in absolute fluorescence between microscopes can be expected due to the variation in the optical sensitivity of the microscope components including light source, objective lenses, ocular lenses, total magnification, etc. If the controls consistently result yields higher or lower than expected, the conjugate may require reactivation. This is accomplished by retreating the controls at appropriate two-fold dilutions of the conjugate using PBS as a conjugate diluent. If reactivation of conjugate is required, please contact the MarOx technical support department for assistance.
5. Lyme TRITC counterstain (Prod #32-5004) is provided at the recommended working dilution.
6. MarSorb G (Prod #41-9000) is provided at the recommended working dilution. See instructions below for usage.
7. The mounting medium (Prod #90-1610) is used at the concentration provided.

ADDITIONAL MATERIALS REQUIRED BUT NOT SUPPLIED

1. Test tubes, test tube rack, pipettes, or a microtiter system for preparing dilutions.
2. Volumetric flask (1 liter) for PBS.
3. Moist incubation chamber.
4. Slide washing chamber.
5. Epifluorescent microscope with 40X objective lens and 10X ocular lenses. FITC filter assemblies at an excitation of 490 nm and emission of 520 nm and TRITC filter assemblies at an excitation of 550 nm and emission of 620 nm.
7. Distilled water.
8. Microcentrifuge tubes, 1.5 mL
9. Microcentrifuge or centrifuge capable of centrifuging microcentrifuge tubes.

STORAGE AND STABILITY

1. B. burgdorferi 10 well Slides (Prod #32-5010): Store at +2 to +8°C or lower. Slides are stable until their expiration date on the product label.
2. Lyme IgM Positive Control (Prod #32-5003): Stored at +2 to +8°C. Refer to expiration date on label.
3. Lyme Negative Control (Prod #32-5001): Store at +2 to +6°C. Refer to expiration date on label.
4. Lyme FITC Labelled Anti-Human IgM Conjugate (Prod #32-1515M) and Lyme TRITC Counterstain (Prod #32-5004): Store at +2 to +8°C. Refer to expiration date on label.
5. MarSorb G (Prod #41-9000): Stored at +2 to +8°C. Refer to expiration date on label.
6. Phosphate Buffered Saline, pH 7.5 (Prod #90-1607). PBS is stable at room temperature in its uncentrifuged form. Refer to label for expiration date. PBS contains no preservative and should be stored at +2 to +8°C after it is reconstituted. Discard if turbidity develops.
7. Mounting Medium (Prod #90-1610): Store at +2 to +8°C. Refer to the expiration date on label.

SPECIMEN COLLECTION

Serum should be collected using aseptic technique. Hemolysis is avoided through prompt separation of the serum from the clot. Serum should be stored at +2 to +8°C if it is to be analyzed within a few days, and may be held for months at -20°C or lower. Lipemic and strongly hemolytic serum should be avoided.

PREPARATION OF CONTROLS

Include the positive and negative controls in each run.

The Lyme IgM Positive Control (Prod #32-5003) and Lyme Negative Control (Prod #32-5001) are supplied as a ready to use liquid. No reconstitution is necessary. Mix well by vortex or inversion prior to use. Prepare dilutions using PBS. Do not pretreat control sera with MarSorb G (see below).

1. The positive control is standardized to give end-point activity within one two-fold dilution above or below the dilution stated on the vial. Include in the test procedure all dilutions from one two-fold dilution below to one two-fold dilution above the expected end-point dilution. Refer to the vial label for the specified dilution end-point of each lot.
2. The negative control is standardized to demonstrate a negative reaction at the dilution of 1:16. Include in the test the negative control at the dilution of 1:16.

PREPARATION OF SPECIMENS

For an accurate measurement of IgM specific antibody to B. burgdorferi, the IgG component of each sample is removed.

1. Place 5µL of test serum sample into the bottom of a microcentrifuge tube or micro-test tube capable of centrifugation.
2. Add 75µL of MarSorb G and mix. Allow to incubate at room temperature for 15minutes.
3. Centrifuge samples at 2000 RPM or greater for 10 minutes.
4. Remove samples from the centrifuge.
5. Prepare the screening dilutions of patient sera to final dilutions of 1:16 and 1:128. Samples pretreated with MarSorb G are already at a 1:16 dilution. To prepare a 1:64 dilution from the MarSorb G pretreated sample, pipet 50µL of treated serum to 150µL of PBS and mix well. To prepare the 1:128 dilution, pipet 100 µL of the 1:64 dilution to 100 µL of PBS. The sample is now free of IgG and RF interference and is at the proper dilutions of 1:64 and 1:128 for screening. Note: Samples screening positive at 1:128 should be titrated to end-point by preparing two-fold serial dilutions starting at 1:128. Mix equal volumes of diluted serum and PBS for subsequent two-fold dilutions.

TEST PROCEDURE

1. Remove the number of slides needed from the sealed pouches and mark them with a marking pen as necessary.
2. Add controls and diluted serum (15-20 µL) to antigen wells.
3. Incubate slides in a moist chamber at room temperature for 30 minutes.
4. After incubation with sera the slides should be tapped onto a piece of paper towel in such a way as to prevent the serum of one well coming into contact with any of the other wells. Direct a gentle stream of PBS over the slide using a washing bottle. Do not aim the stream of PBS directly onto the wells.
5. Place the slides in a wash chamber filled with PBS for 5 minutes. Replace wash chamber with fresh PBS and wash slides for another 5 minutes.
6. Remove the slides from the PBS and place, antigen side up, on a dry paper towel. Carefully place the 10 well blower over the slide, positioned so as not to come into contact with the reaction wells. Hold one edge of the blower with one hand to keep the blower in place and apply sufficient gentle pressure with the microscope slide roller to remove the moisture surrounding antigen wells. DO NOT ALLOW THE ANTIGEN WELLS TO DRY.
7. Using dispenser provided, deliver 1 drop of conjugate per antigen well. The conjugate dispenser is provided with a calibrated tip and allows quantitative delivery (20 µL) of reagents from the storage bottle. To use, wipe the tip with a paper towel, invert the bottle and squeeze gently to release one drop. If the tip contains an air bubble, tap the bottle gently to remove air bubble which will ensure precise drop delivery.
8. Incubate slides as described above (#3).
9. Rinse, wash and blot slides as described above (#4, #5, #6). DO NOT ALLOW THE ANTIGEN WELLS TO DRY.
10. Dispense 1 drop (20µL) of TRITC counterstain to each antigen well.
11. Incubate for 5 minutes at room temperature.
12. Rinse, wash and blot slides as described above (#4, #5, #6). DO NOT ALLOW THE ANTIGEN WELLS TO DRY.
13. Place 2 to 3 drops of mounting medium on slide and cover with a coverslip avoiding air bubbles.
14. Read slides with a fluorescence microscope.

READING SLIDES

1. Do not attempt to read the slides before the microscope has been switched on for at least 5 minutes.
2. Read slides within one hour. Slides may be read within 24 hours if stored refrigerated in a moist chamber. Allow refrigerated slides to warm to room temperature before reading.
3. The slides should be examined at a total magnification of 400X.
4. Drying may disturb the most peripherally situated organisms in the well, therefore disregard the reactions seen with these organisms.
5. The reaction should be considered positive only if a majority (>80%) of the organisms in the well show a distinct fluorescence.
6. The staining intensity of individual cells may vary. However, the degree of staining is based on the overall appearance of the antigen smear.
7. Record reaction intensity at each dilution using the following criteria:
   - 2+ to 4+ = moderate to strong yellow-green fluorescence
   - 1+ = Weak but definite yellow-green fluorescence
   - Negative = Vaguely visible or no fluorescence

8. Negative samples may be difficult to read. On these samples, the TRITC filter block should be used to visualize the cells. The birefringent cells will fluoresce red. This confirms that cells are actually present in the reaction well. After focusing on the cells in the microscopic field of view, shift to the FITC filter to verify negativity.
9. Read the controls before proceeding to the test sera.

QUALITY CONTROL

1. The positive control must demonstrate positive fluorescence within one dilution of the endpoint dilution stated on the vial or the test is invalid.
2. The negative control must demonstrate the absence of yellow-green specific fluorescence at the stated dilution on the vial or the test is invalid.
3. Reading of test serum end-points with each microscope assembly must be made with reference to the reactivities of the control sera with the antigen slides and conjugate provided.

INTERPRETATION OF RESULTS

1. Titer of 1:128 or greater. Positive. IgM class antibodies to B. burgdorferi present. This usually indicates early active Lyme disease.
2. Titer of 1:94 or less: Negative. No IgM antibodies to B. burgdorferi present. If Lyme disease is still suspected, test sample for IgG and/or test a second sample taken 2 to 4 weeks after the first sample with a test sensitive for IgM and IgG.

LIMITATIONS OF PROCEDURE

1. Light sources, total magnification, objective lenses, and ocular lenses influence intensity of staining. Variations in intensities may be observed when different microscope assemblies are used. Testing of sera should not be attempted unless the positive control gives the expected titer with one two-fold dilution and the negative control yields negative results.
2. The accuracy in the test often depends on the competency of the operator.
3. The patient clinical data and other laboratory tests should be carefully reviewed by a medical authority before a diagnosis is made.
LIMITATIONS OF PROCEDURE (continued)

4. Sera from patients with other pathogenic spirochetal diseases such as syphilis, yaws, pinta, leptospirosis, and relapsing fever may give false positive results. Sera from patients with mononucleosis (EBV), lupus erythematosus, and rheumatoid arthritis, may also give false positive results. In cases of potential false positive results, clinical epidemiologic and laboratory worksup should be carried out.

5. Although the clinical picture of Lyme disease is quite different from that of active syphilis, an easy means of differentiating these two diseases is by the use of the VDRL or RPR tests. In active syphilis the VDRL or RPR are positive. In Lyme disease the VDRL and RPR are generally negative.

6. Patients with very early stage Lyme disease may test negative with this procedure, because IgM antibodies may not have reached detectable levels. If a sample tests negative and Lyme disease is still suspected, a second sample taken 2 to 4 weeks later should be evaluated.

7. Patients with late Lyme disease may test negative with this procedure, because IgM levels tend to drop as IgG levels rise. If Lyme disease is still suspected, the sample should be tested for IgG. If the sample is negative for IgG, a second sample, taken 2-4 weeks after the first, should be tested for IgM and IgG. IgM antibodies may also be present late in infection.

8. Antibiotic therapy given early in the disease may prevent the development of an antibody response.

9. All test results must be considered in conjunction with the clinical picture presented by the patient, the patient exposure history in areas endemic for Lyme disease, and the potential of other spirochetal diseases.

EXPECTED VALUES

In patients without infection with B. burgdorferi, test results should be negative except for patients with cross reacting antibodies (See Limitations of Procedure).

In patients with Lyme disease, the test results are dependent on the stage of the disease. It has been reported that patients reporting within 1-3 weeks of onset of EM will have a positive test 94% of the time. The chances of a positive IgM test drop rapidly if the sample is taken more than 3 weeks after onset of EM (14). IgM testing is most useful for detecting early Lyme disease.

For detecting all cases, a test which detects IgG is also necessary. IgM antibodies rise quickly after infection, reaching their peak within 3 weeks. IgG antibodies start rising 2 to 3 weeks after infection and persist while symptoms are present and drop slowly during recovery.

SPECIFIC PERFORMANCE CHARACTERISTICS

Study 1:
A total of 70 specimens received by a midwestern reference laboratory were utilized. Sixty of the 70 specimens were from patients with Lyme disease as defined by clinical case history (presence of EM and plus two additional symptoms of Lyme disease). Ten samples were from patients without clinical symptoms in a non-endemic area. The samples were tested by the reference laboratory's IFA (IgM) and with MarDx Lyme Disease IFA (IgM) Test. The MarDx results compared to the reference results are presented in Table 1. The results of both tests compared to clinical diagnosis is presented in Table 1.

Table 1: MarDx and Reference Results Compared to Clinical Diagnosis

<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>Total #</th>
<th># MarDx Pos</th>
<th># Reference Pos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyme Disease</td>
<td>60</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>Negative Population</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Study 2:
A total of 133 specimens testing negative by a commercial ELISA test which detects both IgG and IgM, and a total of 21 positive samples by a reference IFA IgM method were utilized in this study. Specimens were collected from 5 areas of the country including endemic and non-endemic areas. All specimens were run on the MarDx Lyme Disease IFA (IgM) Test. Results for these samples and the samples from study 1 are summarized in Table 2.

Table 2: Results, MarDx vs Reference Results

<table>
<thead>
<tr>
<th>MarDx Positive</th>
<th>MarDx Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref +</td>
<td>Ref -</td>
</tr>
<tr>
<td>Ref +</td>
<td>Ref -</td>
</tr>
</tbody>
</table>

Study 1
20 6 1 43
Study 2
20 0 9 0 124
Total
20 15 1 167

*These 6 specimens were from clinically defined Lyme disease cases.

Sensitivity 95% (20/21)
Specificity 92% (167/182)
Correlation 92% (187/203)

REFERENCES

11. Lyme Disease in California and the USA (1985) California Morbidity #39

TABLE 3: Results MarDx IFA (IgM) vs MarDx EIA (IgM)

<table>
<thead>
<tr>
<th>IFA Positive</th>
<th>EIA Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>2</td>
</tr>
</tbody>
</table>

IFA Positive
Sensitivity 97% (28/29)
Specificity 99% (137/139)
Correlation 98% (165/168)