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IVD	See external label	2°C-8°C	Σ=96 tests	REF #1423-2

Borrelia burgdorferi Antibody ELISA Test System

An Enzyme-Linked Immunosorbent Assay (ELISA) for
the detection of antibodies to *Borrelia burgdorferi*

Cat. No. 1423-2

INTENDED USE

The Diagnostic Automation, Inc. *Borrelia burgdorferi* IgG/IgM IFA test system is designed for the qualitative and semi-quantitative presumptive detection of total (IgG and IgM) antibodies to *Borrelia burgdorferi* in human serum. This test should only be used for patients with signs and symptoms that are consistent with Lyme disease. Equivocal or positive results must be supplemented by testing with a standardized Western blot procedure. Positive supplemental results are supportive evidence of exposure to *B. burgdorferi* and can be used to support a clinical diagnosis of Lyme disease.

SIGNIFICANCE AND BACKGROUND

Borrelia burgdorferi is a spirochete that causes Lyme disease. The organism is transmitted by ticks of the genus *Ixodes*. In endemic areas, these ticks are commonly found on vegetation and animals such as deer, mice, dogs, horses, and birds.

B. burgdorferi infection shares features with other spirochetal infections (diseases caused by three genera in humans: *Treponema, Borrelia,* and *Leptospira*). Skin is the portal of entry for *B. burgdorferi* and the tick bite often causes a characteristic rash called *erythema migrans* (EM). EM develops around the tick bite in 60% to 80% of patients. Spirochetemia occurs early with wide spread dissemination through tissue and body fluids. Lyme disease occurs in stages, often with intervening latent periods and with different clinical manifestations.

In Lyme disease there are generally three stages of disease, often with overlapping symptoms. Symptoms vary according to the sites affected by the infection such as joints, skin, central nervous system, heart, eye, bone, spleen, and kidney. Late disease is most often associated with arthritis or CNS syndromes. Asymptomatic subclinical infection is possible and infection may not become clinically evident until the later stages.

Patients with early infection produce IgM antibodies during the first few weeks after onset of EM and product IgG antibodies more slowly (1). Although IgM only may be detected during the first month after onset of illness, the majority of patients develop IgG antibodies within one month. Both IgG and IgM antibodies can remain detectable for years.

Isolation of *B. burgdorferi* from skin biopsy, blood, and spinal fluid has been reported (2). However, these direct culture detection methods may not be practical in the large scale diagnosis of Lyme borreliosis. Serological testing methods for antibodies to *B. burgdorferi* include indirect fluorescent antibody (IFA) staining, immunoblotting, and enzyme immunoassay (EIA).

B. burgdorferi is antigenically complex with strains that vary considerably. Early antibody responses often are to flagellin which has cross reactive components. Patients in early stages of infection may not produce detectable levels of antibody. Also, early antibiotic therapy after EM may diminish or abrogate good antibody response. Some patients may never generate detectable antibody levels. Thus, serological tests for antibodies to *B. burgdorferi* are known to have low sensitivity and specificity and because of such inaccuracy, these tests cannot be relied upon for establishing a diagnosis of Lyme disease (3,4).

In 1994, the Second National Conference on Serological diagnosis of Lyme disease recommended a two-step testing system toward standardizing laboratory serologic testing for *B. burgdorferi*. Because EIA and IFA methods were not sufficiently specific to support clinical diagnosis, it was recommended that positive or equivocal results from a sensitive EIA or IFA (first step) should be further tested, or supplemented, by using a standardized Western Blot method (second step) for detecting antibodies to *B. burgdorferi* (Western Blot assays for antibodies to *B. burgdorferi* are supplemental rather than confirmatory because their specificity is less than optimal, particularly for detecting IgM). Two-step positive results provide supportive evidence of exposure to *B. burgdorferi*, which could support a clinical diagnosis of Lyme disease but should not be used as a sole criterion for diagnosis.

PRINCIPLE OF THE IFA ASSAY

The Diagnostic Automation, Inc. *Borrelia burgdorferi* IgG/IgM IFA Test System is designed to detect circulating antibodies to the Lyme disease spirochete in human sera. The test is particularly useful for, but not limited to, the diagnosis of Lyme disease in its later stages. The system employs the Lyme disease spirochetes (*Borrrelia burgdorferi*) immobilized on a glass slide and fluorescein-labeled anti-human immunoglobulin. The test procedure involves two steps:

1. In the first step, human sera are reacted with the spirochetes immobilized on the slides. Antibodies in the sera will bind to the spirochetes and remain attached after rinsing.
2. Fluorescein-labeled anti-human immunoglobulin is added in the second step and will bind to the antibodies causing the spirochetes to fluoresce. The intensity of staining is graded on a scale of 1+ to 4+ or as negative. Negative sera lacking antibodies will not show fluorescence.

KIT COMPONENTS

Reactive Reagents

1. Antigen slides: Ten-well substrate slides containing fixed *Borrelia burgdorferi* (strain B31) organisms. Each slide is individually packaged in an envelope with a desiccant.
2. Goat anti-human immunoglobulin labeled with FITC: One 3.0mL vial, lyophilized.
3. Human positive 4+ control sera: One 1.0mL vial, lyophilized, composed of human sera.
4. Human negative control sera: One 1.0mL vial, lyophilized, composed of human sera.

Non-reactive Reagents

1. Phosphate buffered saline (PBS): Supplied as a powder. Sufficient to make 4 liters, pH 7.6 ± 0.1.
2. Mounting Media: Phosphate buffered glycerol, 3.0mL, pH 8.9 ± 0.1.

NOTE: All reactive reagents, as well as buffered glycerol contain a preservative which may be toxic if ingested. (Thimerosal, mercury derivative 1:10,000.)

PRECAUTIONS

1. For *in vitro* diagnostic use.
2. The preservative may be toxic if ingested.
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 (5).
4. Do not mix reagents from different lots.
5. Do not apply pressure to slide envelope, this may damage the substrate.
6. The components of this kit are matched for optimum sensitivity and reproducibility. Reagents from other kits or sources should not be interchanged. Follow test procedures carefully.
7. Reconstitute reagents gently but thoroughly. Reagents should be free of particulate matter. If reagents become cloudy, bacterial contamination should be suspected.

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

1. Small serological, Pasteur, capillary, or automatic pipettes.
2. Small test tubes, 13 x 100mm or comparable.
3. Test tube racks.
4. Staining dish. A large staining dish with a small magnetic mixing set-up provides an ideal mechanism for washing slides between incubation steps.
5. Cover slips: 24 x 60mm, thickness No. 1.
6. Distilled water.
7. Properly equipped fluorescence microscope assembly.

The following filter systems or their equivalents have been found to be satisfactory for routine use with transmitted or incident light darkfield assemblies:

TRANSMITTED LIGHT		
Light Source: Mercury vapor 200W or 50W		
Excitation Filter	Barrier Filter	Red Suppression Filter
KP490	K510 or K530	BG38
BG12	K510 or K530	BG38
FITC	K520	BG38
Light Source: Tungsten – Halogen 100W		
KP490	K510 or K530	BG38

INCIDENT LIGHT			
Light Source: Mercury Vapor 200, 100, 50 W			
Excitation Filter	Dichroic Mirror	Barrier Filter	Red Suppression Filter
KP500	TK510	K510 or K530	BG38
FITC	TK510	K530	BG38
Light Source: Tungsten – Halogen 50 and 100 W			
KP500	TK510	K510 or K530	BG38

FITC	TK510	K530	BG38

SPECIMEN COLLECTION

1. It is recommended that specimen collection be carried out in accordance with NCCLS document M29: Protection of Laboratory Workers from Infectious Disease.
2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.
3. Only freshly drawn and properly refrigerated sera obtained by approved aseptic venipuncture procedures should be used in this assay (6, 7). No anticoagulants or preservatives should be added. Avoid using hemolyzed, lipemic, or bacterially contaminated sera.
4. Store sample at room temperature for no longer than 8 hours. If testing is not performed within 8 hours, sera may be stored between 2° and 8°C for no longer than 48 hours. If delay in testing is anticipated, store test sera at –20°C or lower. Avoid multiple freeze/thaw cycles that may cause loss of antibody activity and give erroneous results.

STORAGE CONDITIONS

1. Substrate slides containing *B. burgdorferi* organisms: Store at ≤ -20ºC.
2. Goat anti-human immunoglobulin labeled with FITC: Store at 2-8ºC. Stable for 90 days after reconstitution. Frozen aliquots are stable for 6 months at ≤ -20ºC or lower.
3. Human positive and negative control serum: Store at 2-8ºC. Stable for 90 days after reconstitution. Frozen aliquots are stable for 6 months at ≤ -20ºC.
4. Phosphate-buffered-saline: Store at room temperature. Store reconstituted buffer at 2-8ºC.
5. Buffered glycerol mounting media: Store at 2-8ºC.

NOTE:

1. All kit components are stable until the expiration date printed on the label provided the recommended storage conditions are strictly followed.
2. Do not freeze and thaw reagents and sera more than once. Repeated freezing and thawing destroys antibody activity. Do not store in self-defrosting freezers.

PROCEDURE

Preparation of Reagents

1. Phosphate buffered saline, pH 7.6 ± 0.1: Empty contents of one buffer pack into one liter of distilled water. Mix until all salts are thoroughly dissolved.
2. Human negative and positive control sera: Reconstitute each vial with 1.0mL distilled water.
3. Goat anti-human immunoglobulin FITC-labeled conjugate: Reconstitute with 3.0mL distilled water. Alternatively, aliquot on 0.5mL amounts and store at -20ºC or lower in small capped tubes.

Test Procedure

1. Remove substrate slides from the freezer and allow slides to reach room temperature (20-25ºC). Tear open the protective envelope and remove slides containing the Lyme spirochete. DO NOT APPLY PRESSURE TO FLAT SIDES OF PROTECTIVE ENVELOPE.
2. Prepare the 1+ dilution of the positive control using PBS to dilute to the titer indicated on the positive control vial (example: 1+ equals 1:16). Prepare a 1:16 dilution by adding 0.1mL of positive control to 1.5mL of PBS.
3. Dilute each test serum for screening to dilutions of 1:128 and 1:256 in microtiter wells or test tubes. (example: PBS 0.05mL per well (or tube), serum 0.05mL to first well (or tube), and serially dilute).
4. Add 20µL of diluted test or control serum to the appropriate wells on the slide. Care must be taken to completely cover the wells and to avoid disturbing the antigen smear.
5. Incubate slides in a moist chamber at room temperature (20-25ºC) for 30 minutes. DO NOT ALLOW WELLS TO DRY.
6. Remove slides from moist chamber and wash with a stream of PBS. DIRECT THE STREAM OF PBS DOWN THE CENTER OF THE SLIDE. DO NOT DIRECT THE STREAM INTO THE TEST WELLS.
7. Place slides in staining dish for two, five minute washes with a change of PBS. For optimal washing, a magnetic stirrer is recommended.
8. Rinse slides in a gentle stream of distilled water for five to ten seconds. Allow to air dry for approximately five minutes. Slides must be completely dry before adding conjugate.
9. Place slides in a moist chamber and add 20µL of conjugate to each well.
10. Incubate slides for 30 minutes at room temperature. DO NOT ALLOW SLIDES TO DRY.
11. Repeat steps 6,7, and 8.
12. Add 3-4 drops of buffered glycerol to the mask area of each slide and coverslip. Examine slides immediately. View at 400X.
13. Reading:

2+ to 4+	=	Moderate to strong fluorescence
1+	=	Weak but definite fluorescence
Negative	=	Vaguely visible or no fluorescence

Fluorescence at the 1+ level is considered positive. A serum showing fluorescence equal to or greater than 1+ at the 1:256 dilution should be titered to an end-point. This can be accomplished by making serial dilutions in the microtiter plate.

QUALITY CONTROL

1. A positive and negative control should be run with each assay.
2. It is recommended that the positive and negtive controls be read prior to evaluating test results. This will assist in establishing the references required to interpret the test sample. If controls do not appear as described, test results are invalid.

3. The negative control is characterized by the absence of fluorescence.
4. The positive control will exhibit a 4+ to 3+ apple-green fluorescence staining intensity.
5. The intensity of the observed fluorescence may vary with the microscope and filter used.
6. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

INTERPRETATION OF RESULTS

Negative: No detectable antibody; result does not exclude *B. burgdorferi* infection. An additional sample should be tested within 4-6 weels if an early infection is suspected (8). For the IFA test system, a negative reaction is equal to or less than 1+ fluorescence of a 1:128 dilution.

Positive: Antibody to *B. burgdorferi* presumptively detected.

Per current recommendations, the result cannot be further interpreted without supplemental Western-blot testing. (Western-blot assays for antibodies to *B. burgdorferi* are supplemental rather than confirmtory because their specificity is less than optimal, particularly for detecting IgM.) Results should not be reported until the supplemental testing is complete. For the IFA test system, a positive reaction will demonstrate 1+ or greater fluorescence staining at a dilution of 1:256 or greater.

Borderline: Current recommendations state that borderline results should be followed by supplemental Western-blot testing. The borderline result should be reported with the results from Western-blot testing. Results should not be reported until the supplemental testing is complete. For the IFA test system, a borderline specimen will demonstrate a 1+ or greater fluorescence at 1:128 but less than 1+ fluorescence at 1:256.

LIMITATION OF THE ASSAY

1. Sera from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis, and relapsing fever), mononucleosis, and occasionally systemic lupus erythematosus may give false positive results. In cases where false positive reactions are observed, extensive clinical, epidemiologic and laboratory workups should be carried out to determine the specific diagnosis. False positive sera from syphilis patients can be distinguished from true Lyme disease positive patients by running an RPR or MHATP assay on such specimens (9). Lyme sera will be negative in these tests (9, 10). Also, when titered in the FTA-ABS test, syphilis sera will show higher titers against the *T. pallidum* antigen than against the *B. burgdorferi* antigen (9).
2. Patients with early Lyme disease may have undetectable antibody titers with this assay. In early Lyme disease IgG/IgM antibodies may not reach a level of diagnostic significance and may never do so when ECM is the sole manifestation (11). If Lyme disease is suspected early on, additional serum samples at varying intervals should be taken to demonstrate a rise in IgG titer.
3. An alternative method for determining IgM antibodies may be more useful for diagnosing early Lyme disease or reinfection.
4. Early antibiotic therapy may abort an antibody response to the spirochete.
5. All data should be interpreted in conjunction with clinical symptoms of disease, epidemiologic data, and exposure in endemic areas.
6. Screening of the general population should not be performed. The positive predictive value depends on the pretest likelihood of infection. Testing should only be performed when clinical symptoms are present or exposure is suspected.
7. The performance characteristics of the DIA *B. burgdorferi* IgG/IgM IFA test are not established with samples from individuals vaccinated with *B. burgdorferi* antigens.

EXPECTED VALUES

Most investigators agree that a cut-off dilution of 1:256 differentiates positive and negative results with a high degree of specificity, particularly in the later stages of Lyme disease (12). The 1:256 cut-off dilution was established in a study of 267 serum specimens. Of the 267 specimens, 177 were obtained from blood bank donors and 90 from non-Lyme disease patients (12 dermatologic, 2 cardiac, 26 neurologic, and 50 patients with rheumatic diseases). One of the normal serum samples showed a titer >1:256, while 9 produced titers $\geq$1:256 for a total of 10 out of 177 specimens. One out of 90 specimens obtained from subjects with non-Lyme disease disorders was $\geq$1:256; therefore, of the 267 sera tested, <5% (13) produced values >1:256.

PERFORMANCE CHARACTERISTICS

A. The Diagnostic Automation, Inc. IFA test system for Lyme disease IgG/IgM (*B. burgdorferi*) was compared to an ELISA assay in a double blind study at a large reference laboratory. The results of this study are summarized in Table 1. The relative sensitivity and specificity of the IFA and ELISA assays in this study are based on specific diagnosis determined by clinical features and extensive laboratory workups of patients in each disease category, *i.e.,* all suspected Lyme disease patients demonstrated specific clinical features that were confirmed by positive assays for Lyme disease antibodies.

TABLE 1.
Comparison of the DIA IFA Test System and a reference ELISA assay for serological detection of antibodies in *B. burgdorferi*

DISEASE CATEGORY	**NUMBER TESTED**	NUMBER POSITIVE **IFA**	**ELISA**
B. burgdorferi ECM	10	1	6
Neurologic	9	8	9
Arthritic	10	10	10
Autoimmune	112	5	2
Other Infectious Disease	106	23	16
The overall sensitivity for both methods was:*		66%	79%
The overall specificity for each method was:*		87%	92%
*As compared to clinical diagnosis and other clinical laboratory			

data.			

B. These results show that the Diagnostic Automation, Inc. IFA procedure is less sensitive in the early stages of Lyme disease (*B. burgdorferi*), particularly in those cases associated with erythema chronicum migrans. However, in complicated cases of Lyme disease, the sensitivity was virtually 100% for both methods. These results are consistent with the lower amount of specific IgG antibodies present in the early stages of Lyme disease. These results are consistent with routine referral specimens submitted for analysis at the reference laboratory. The study was conducted in a double-blind manner. The results of this study are shown in Table 2.

Table 2.
Comparison of the DIA IFA test system and a reference IFA procedure for serological detection of antibodies in *B. burgdorferi*

NUMBER	NUMBER NEGATIVE		NUMBER POSITIVE	
TESTED	**DIAIFA**	**REF. IFA**	**DIAIFA**	**REF. IFA**
50	38	38	12	12

The results of this study show 100% agreement between the two procedures in detecting positive and negative sera using a 1:256 dilution as a cut-off point.

C. In a study conducted by the CDC (10), the IFA and ELISA methods for detecting antibodies in serum against *B. burgdorferi* were compared employing serum specimens obtained during different stages of Lyme disease. The results are shown in Table 3.

Table 3.

DISEASE STAGE	**IFA POSITIVE**	**ELISA POSITIVE**
ECM	50	50
Carditis	100	100
Neuritis	92	100
Arthritis	100	97
More than one complication	71	80

These results are similar to those reported in Table 1 above and further substantiates that the IFA assay for detection of antibodies associated with Lyme disease is a reliable, sensitive method, particularly in cases of complicated late stage Lyme disease.

D. The following information is from a serum panel obtained from the CDC and tested in-house at Diagnostic Automation, Inc. on the *B. burgdorferi* IgG/IgM IFA test system. The results are presented as a means to convey further information on the performance of this assay with a masked characterized serum panel. This does not imply and endorsement of the assay by CDC. Table 4 shows the results of this study.

Table 4.
The CDC *B. burgdorferi* Disease Serum Panel Stratified by Time After Onset

Time from onset	Pos	+/-	Neg	Total	% agreement with clinical diagnosis
Normals	2	1	1	4	33%
< 1 month	5	0	1	6	83%
1-2 months	7	0	2	9	78%
3-12 months	13	2	4	19	76%
> 1 year	1	4	3	8	25%
Total	28	7	11	46	69%

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