ImmunoWELL ™

BORRELIA (LYME) TEST

CE

Product No. 3110

IVD **For *In Vitro* Diagnostic Use**

INTENDED USE

The ImmunoWELL Borrelia (Lyme) Test is an enzyme immunoassay (EIA) for the qualitative and/or semi-quantitative detection of *Borrelia burgdorferi* total antibodies (IgG, IgM, and IgA) in serum and is used as an aid in the diagnosis of Lyme disease.

SUMMARY AND EXPLANATION

Lyme borreliosis is reported to occur in North America, Europe, and Asia[1,2,3,4,5]. Lyme disease has been present in Europe for years, but was first recognized in the United States in 1975 at Lyme, Connecticut. The disease, transmitted through the bite of a tick infected with the spirochete, *Borrelia burgdorferi*, exhibits a variety of symptoms which may be confused with immune and inflammatory disorders. Inflammation around the tick bite eventually causing skin lesions, erythema chronicum migrans (ECM), is the first stage of disease. *B. burgdorferi* disease is also associated with neurologic or cardiac symptoms (stage 2) or arthritic symptoms (stage 3). A definitive distinction between stages is not always seen. In some cases, these secondary symptoms may occur even though the patient does not remember a tick bite or rash.

The criteria for the diagnosis of Lyme borreliosis are not clearly defined. Unless the typical ECM lesions are present, serological diagnosis is necessary to identify patients exposed to the agent. However, cross-reactions within the Borrelia genus and other cross-reactions (e.g., flagellin reactions with spirochetes and membrane reactions with bacterial membrane proteins) have limited the reliability of *B. burgdorferi* serology. ImmunoWELL Borrelia (Lyme) Test combines purified *Borrelia burgdorferi* cell lysate and the recombinant 39 kilodalton (P39) protein[6,7] as antigens. Therefore, human antibodies against any *Borrelia burgdorferi* antigen (i.e., flagellin [41Kd], outer surface proteins [OsA and OsB], etc.) are detected using this test kit.

Antibodies against P39, unlike antibodies to flagellin which cross-react with other spirochetal flagellins, are specific to *Borrelia burgdorferi* [7] and are conserved among North American and European isolates. Because P39 protein is highly antigenic, but constitutes only a small fraction of the protein in the organism, it may not be detected in other assay systems (e.g., western blots, EIA, IFA, etc.) which are not enriched with P39 protein.

ASSAY PRINCIPLE

The ImmunoWELL Borrelia (Lyme) Test utilizes an EIA microtiter plate technique for the detection of antibodies. Because the B. *burgdorferi*-specific P39 protein represents a small fraction of borrelia protein, assay sensitivity is improved by addition of recombinant P39 antigen to the whole borrelial cell extract. To improve assay specificity serum is absorbed in a blocking solution containing *E. coli* proteins. The blocked serum is added to antigen coated microtiter wells and allowed to react. After removal of unbound antibodies, horseradish peroxidase-conjugated antihuman antibodies are allowed to react with bound antibodies. The bound peroxidase reacts with 2-2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS®), the chromogenic substrate, developing a color. Finally, the substrate reaction is stopped and the optical density is read with a spectrophotometric microwell reader.

REAGENTS

Reaction Wells coated with *Borrelia burgdorferi* sensu strictu (strain B31) extract and P39 recombinant protein

Specimen Diluent consisting of 0.01 M phosphate buffered saline (PBS, pH 6.2-7.6) and carrier protein containing <0.1% NaN_3

Calibrator consisting of human anti-*B. burgdorferi* serum (prediluted to 1:20) in Specimen Diluent

Positive Control consisting of human anti-*B. burgdorferi* serum containing <0.1% NaN_3

Negative Control consisting of nonreactive human serum containing <0.1% NaN_3

Borrelia Blocker consisting of *E. coli* protein in 0.01 M PBS (pH 6.2-7.6) with carrier protein and <0.1% NaN_3

Wash Buffer Concentrate consisting of a 20X concentrate of 0.01 M PBS (pH 6.2-7.6) and 0.05% Tween

Conjugate consisting of peroxidase-conjugated goat antihuman antibodies (IgG, IgM and IgA) in PBS (pH 6.2-7.6) and carrier protein containing preservatives

Substrate Buffer consisting of 0.1 M sodium citrate (pH 4.4-4.6) and 0.01% hydrogen peroxide

Substrate Concentrate 2.19% 2-2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) in 0.1 M sodium citrate (pH 4.4-4.6)

Stop Solution 0.25 M Oxalic Acid

Warnings and Precautions

***For In Vitro Diagnostic Use*:** ImmunoWELL reagents have been optimized for use as a system. Do not substitute other manufacturers' reagents or other ImmunoWELL Microtiter Test reagents. Dilution or adulteration of these reagents may also affect the performance of the test. Do not use kit if evidence of microbial contamination (cloudiness) is present. Do not use any kits beyond the stated expiration date. Close adherence to the test procedure will assure optimal performance. Do not shorten or lengthen stated incubation times since this may result in poor assay performance.

Some reagents contain sodium azide which may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.

Warning - Potential Biohazardous Material: Human sera used in the preparation of this product were tested and found non-reactive for hepatitis B surface antigen and for antibodies to HIV-1, HIV-2, and hepatitis C virus. Because no test method can offer complete assurance that infectious agents are absent, handle reagents and specimens as if capable of transmitting infectious disease[8].

Reconstitution and Storage

Kit is stored at 2-8°C until stated expiration date.

Reaction wells are removed from the foil pouch and unused wells are resealed in the pouch using the integral zip-lock.

Wash Buffer (pH 6.2-7.6) is prepared by adding the contents of the Wash Buffer Concentrate (20X) bottle into 1 liter of distilled/deionized water. After reconstitution, the 1X solution is stored at 2-8°C. Discard when visibly turbid.

> Note: In some instances the Wash Buffer Concentrate (20X) may develop crystals upon storage at 2-8°C. It is important that these crystals are completely redissolved before dilution of the Concentrate. This can be accomplished by warming the Concentrate to 37°C in a water bath with occasional mixing.

Color Developer is prepared by adding one (1) drop of Substrate Concentrate to 1mL of Substrate Buffer. One mL of Color Developer is sufficient for one eight-well strip. **Use within one hour.**

SPECIMEN COLLECTION AND HANDLING

ImmunoWELL Borrelia (Lyme) Test is performed on serum. Hemolyzed or lipemic serum has not been shown to be an acceptable specimen. The test requires 10 µL of serum. Serum is collected according to standard practices and may be stored at 2-8°C for up to five days. Serum may be frozen below -20°C for extended periods.

PROCEDURE

Materials Provided

Microtiter Wells in Carrier	Specimen Diluent
Calibrator	Positive Control
Negative Control	Wash Buffer Concentrate (20X)
Borrelia Blocker	Substrate Buffer
Conjugate	Substrate Concentrate
Package Insert	Stop Solution

Materials Required But Not Provided

Distilled or deionized water	Pipets
Microwell washer	Test tubes
Microwell spectrophotometer (405 nm)	

Performance Considerations

Reproducibility of the assay is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the assay procedure.

Positive and Negative Control Sera (undiluted) are used to assure test performance.

Calibrator (prediluted to 1:20) is used to standardize between run values.

Substrate Blank - All reagents, except serum, are added to the substrate blank well. This blank well is intended to baseline (zero) the microwell spectrophotometer.

Assay Procedure

1. Allow all components including diluted Wash Buffer to warm to room temperature (22-27°C).
2. Determine the total number of specimens to be tested. Include one blank and duplicates of calibrator and controls in each run.
3. For each control and specimen, pipet 10 µL serum into a clean tube containing 200 µL Specimen Diluent and mix (1:20 dilution).

CAUTION: Calibrators are prediluted. Do not dilute further.

4. Pipet 10 µL of each 1:20 diluted control and specimen (step 3) into a clean tube containing 200 µL Borrelia Blocker and mix. Pipet 20 µL prediluted Calibrator into a clean tube containing 400 µL Borrelia Blocker and mix.
5. Determine the total number of wells to be run including blank, calibrators, controls, and specimens. Well strips can be broken to the exact number needed to conserve reagent wells. Strips need to be completed with used wells to facilitate washing procedures.
6. Add 100 µL of Borrelia Blocker into the first well as a Substrate Blank. Pipet 100 µL of blocked calibrator, controls, and specimens (step 4) into each assigned well.
7. Incubate at room temperature (22-27°C) for 30±5 minutes.
8. Aspirate the samples out of the wells. Do not allow the wells to dry.
9. Wash the wells three times by completely filling the wells with Wash Buffer (see Reconstitution and Storage) and aspirate the wells completely after washes.
10. Pipet 100 µL Conjugate into all wells.
11. Incubate the wells at room temperature (22-27°C) for 30±5 minutes.
12. Aspirate the conjugate out of the wells. Do not allow the wells to dry.
13. Wash the wells three times as described in step 9.
14. Prepare fresh Color Developer (see Reconstitution and Storage).
15. Pipet 100 µL of Color Developer into each well.
16. Incubate at room temperature (22-27°C) for 30±5 minutes.
17. Add 100 µL of Stop Solution to each well.
18. Inspect the outside bottom surface of the microwells for the presence of condensation, dried buffer salts or wash solution which might interfere with the spectrophotometric reading. Carefully clean the well bottoms with a soft tissue.
19. Using the Substrate Blank to zero the spectrophotometer, read the optical density of each well at 405 nm within 30 minutes of completion of step 17.

NOTE: It is recommended that dual wavelength spectrophotometers use only one wavelength, 405 nm.

Quality Control

Determine the activity of the positive and negative controls using the method described in the next section. Each control must be within the expected range given in the Package Insert Supplement included in this reagent kit.

INTERPRETATION

Procedure for Calculating Activity of Specimen

The assigned value (U/mL) of the calibrator used in the calculation below will vary by lot number. Please verify that the lot number on the vial matches the lot number on the Package Insert Supplement to assure the proper value is used in the calculation.

In order to eliminate the effects of washing variation, instrument variability, etc. specimen values are normalized according to the following calculation:

$$A_N = AV_C \times A_S / A_C$$

Where:

A_N	=	Normalized activity of the specimen (U/mL)
A_S	=	Absorbance of the specimen
A_C	=	Mean absorbance of the Calibrator obtained in the assay
AV_C	=	Assigned Value (U/mL) of the Calibrator given in the Supplement

The interpretive ranges are:

	Units/mL	Interpretation
Nonreactive	<120	Antibody to *B. burgdorferi* not detected. If symptomology is suggestive of Lyme disease, redraw in 2-4 weeks and retest.
Borderline	120 – 160	Repeat the test using the same sample. If result repeats as either reactive or non reactive, report the definitive result. If test repeats as borderline, report test as "borrelia antibody detected at borderline (equivocal) level." Suggest that another sample be tested 2-4 weeks later.
Reactive	>160	Antibody to *B. burgdorferi* detected.

This assay, like all tests which use borrelia proteins (flagellin, outer membrane antigens, etc.) has cross-reactions with antibodies against some other agents. Further testing with other methods (western blot analysis[9], specific recombinant proteins, RPR to rule out syphilis) may be indicated.

LIMITATIONS

1. Positive results should be interpreted with caution. Sera from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis, relapsing fever, peridontal disease, etc.) may also give positive results. The diagnosis of Lyme Disease should be based on interpretation of test results in combination with patient clinical signs and symptoms, other clinical and laboratory test results, and epidemiological data.
2. Patients with connective tissue autoimmune diseases (rheumatoid arthritis, ANA, SLE, etc.) may have antibodies which cross-react with *B. burgdorferi* antigens.
3. Patients with other bacterial and viral infections such as Rocky Mountain Spotted Fever, EBV, CMV, and HIV may also have antibodies which cross-react with *B. burgdorferi* antigens.
4. Negative results do not rule out the diagnosis of Lyme Disease. Early antibiotic therapy may suppress antibody response, and some individuals may not develop antibodies above detectable levels. Negative results in suspected early Lyme Disease should be repeated in 4-6 weeks.
5. The continued presence or absence of antibodies cannot be used to determine the success or failure of therapy.
6. Testing should not be performed as a screening procedure for the general population. The predictive accuracy of a positive or negative serologic result depends on the pretest likelihood of Lyme disease being present. Testing should only be done when clinical evidence suggests the diagnosis of Lyme disease.
7. A single positive result only indicates previous immunologic exposure; level of antibody response or class of antibody response may not be used to determine active infection or disease stage.

EXPECTED RESULTS

In general, three types (stages) of Lyme disease are recognized: erythema chronicum migrans (ECM), neurologic, and arthritic. Antibody levels are generally low or absent during early (ECM) infection. Most

symptomatic patients will have either no antibody or highly cross-reactive antibody during the first 1-2 weeks after tick bite and the antibody titer will rise and become more specific with time. Highest antibody levels are seen in chronic arthritis subjects.

The number of antibody positive subjects in a population depends on several factors: 1) prevalence of the causative agent, 2) assay used to detect antibody, and 3) clinical screening criteria to select tested subjects. Because early assays lacked accuracy[10], the number of antibody positive subjects in a population at present (1991) is highly dependent on the assay used. Whenever a suitably accurate test is used, few positives should be detected in a randomly screened population in a non-endemic area. On the other hand, if patients with typical ECM signs in an endemic region are tested, many positive results are expected.

Disagreement between assays which do not use an absorbent and those assays like ImmunoWELL which do use an absorbent are expected. Fawcett[11,12] has shown that assays using an absorbent were equally sensitive to those without an absorbent and that the absorbed assays were significantly more specific. Addition of recombinant P39 protein does not affect assay specificity, but does increase the absorbance reading for some samples. Cross reactions with other borrelia or closely related species (treponema and leptospira) are expected because of the common flagellin protein.

PERFORMANCE CHARACTERISTICS

Reproducibility

The within-run and between-run variability were determined using five different runs of reference samples tested in triplicate. The results are shown in Table 1.

Table 1: Borrelia (Lyme) Test Reproducibility

MEAN	WITHIN ASSAY VARIATION (%CV)	BETWEEN ASSAY VARIATION (%CV)
1140	4%	9%
800	3%	5%
580	5%	11%
440	4%	10%
300	5%	13%
150	7%	20%
20	21%	57%

Correlation

The specificity of ImmunoWELL Borrelia (Lyme) Test using 250 sera from asymptomatic blood donors collected from areas other than the upper Midwestern and Northeastern United States (hyperendemic regions) is 100% specific. Six samples (2.4%) tested borderline.

The specificity was also determined using sera from Lyme disease negative, symptomatic subjects (Table 2). Because of the high frequency of false positive reactions with samples from syphilis patients, subjects suspected to have syphilis or related disorders must also be tested with a non-treponemal test (e.g. RPR) to rule out this cause.

Table 2: Specificity - Lyme Disease Negative Patients

Sample Type	Nonreactive	Borderline	Reactive	Specificity
ANA +	37	1	0	100%
CMV IgM +	24	0	1	96%
Heterophile +	20	4	1	96%
RF +	23	1	1	96%
Syphilis +	0	1	11	8%
HIV +	15	0	0	100%

Sera from seventy-seven patients diagnosed with Lyme borreliosis were used to assess assay sensitivity. Diagnoses were based on epidemiological, clinical, and serological criteria. These studies were conducted by two outside laboratories. The results are summarized in Table 3, and site specific results follow.

Table 3: Sensitivity - Diagnosed Lyme Disease Patients

Sample Type	Nonreactive	Borderline	Reactive	Sensitivity
Stage 1 (Early) (Neurological)	0	1	7	88%
Stage 2	0	0	1	N/A
Stage 3 (Arthritis)	0	0	21	100%
Unknown Stage	0	0	47	100%

Each trial site used clinical, epidemiologic, and laboratory criteria to classify the patients as Lyme disease cases. Site 1 subdivided the patients into the three stages of disease which are indicated in Table 3. Four Site 1 patients are classified as unknown stage, and the balance of the other unknown stage patients are from Site 2. At Site 1, the laboratory performed an absorbed and unabsorbed EIA test as well as a western blot analysis. The mean absorbance values for the three EIA assays performed at Site 1 are shown in Table 4. These data further support that the use of *E. coli* absorption does not interfere with assay sensitivity, and show that higher antibody reactivity is associated with later stage disease.

Table 4: Site 1 Results

Assay Type	Stage 1	Stage 3
Standard EIA	560	1160
Absorbed EIA	530	1220
ImmunoWELL	550	1020

Site 1 also tested fifty sera from Lyme disease negative subjects. This control group was comprised of sera from normal volunteers and patients with diagnosed viral infections (Epstein-Barr virus) and rheumatic disorder (juvenile arthritis and systemic lupus erythematosus) and sera from patients seen at the rheumatology clinic with other unclassified disorders among whom past or current Lyme disease was ruled out. None of the sera reacted in the ImmunoWELL test (100% specificity). Two sera (4%) reacted as borderline.

Site 2 tested sera from 43 Lyme disease patients and all reacted positive in ImmunoWELL. This site also tested a select group of 55 sera from patients who had been originally classified as possibly having Lyme disease, but were subsequently determined to have western blot reactivity inconsistent with specific antibody. Forty-eight of these sera were nonreactive and seven sera reacted positive in ImmunoWELL. Because absolute identification of disease subjects is difficult to assess, the additional reactivity may indicate increased sensitivity or measurement of the cross-reactivity.

ImmunoWELL was compared with a commercial EIA test using 147 sera submitted to a national reference laboratory facility. Neither clinical nor epidemiologic history of the subjects were available, so resolution of discrepancies was not possible. Table 5 shows the results.

Table 5: Suspected Lyme Disease Subjects

	ImmunoWELL		
EIA	Nonreactive	Borderline	Reactive
Nonreactive	51	0	2
Reactive	49	11	34

ImmunoWELL identified two sera originally classified as negative by the alternate EIA as reactive and agreed with the negative classification of the remaining 51 samples. Agreement between samples classified as reactive by the alternate assay and ImmunoWELL was poor. This discrepancy is expected because the alternate EIA does not use an absorbent to remove cross-reactive antibody. Loss of test sensitivity is not anticipated by use of the absorbent[11,12].

Thirty-nine proficiency samples from two state public health laboratories and a national proficiency program were also used to evaluate ImmunoWELL performance. ImmunoWELL identified 17 out of 17 nonreactive samples correctly and 17 of 18 reactive samples correctly. One reactive sample was determined to be borderline by ImmunoWELL. In addition, three of four borderline proficiency samples were identified as nonreactive.

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QUICK REFERENCE PROCEDURE

ImmunoWELL *Borrelia (Lyme)*

- Prepare Wash Buffer from Wash Concentrate
- Pipet 10 µL of each control and specimen into 200 µL Specimen Diluent and mix (1:20 dilution).
- Add 10 µL of each 1:20 diluted control and specimen to 200 µL Borrelia Blocker and mix. Add 20 µL of prediluted calibrator to 400 µL Borrelia Blocker and mix.
- Add 100 µL of Borrelia Blocker into the first well as a substrate blank.
- Pipet 100 µL of the blocked calibrator, controls, and specimens to coated microwells and incubate 30 min at room temperature.
- Aspirate microwells and wash microwells three times with Wash Buffer.
- Pipet 100 µL of Conjugate to microwells and incubate 30 min at room temperature.
- Aspirate microwells and wash microwells three times with Wash Buffer.
- Prepare fresh Color Developer
- Pipet 100 µL of Color Developer to microwells and incubate 30 min at room temperature.
- Pipet 100 µL of Stop Solution to microwells and read results at 405 nm.

To place an order for ImmunoWELL products, contact your local distributor or call GenBio directly for the distributor nearest you and for additional product information.

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