# *B. burgdorferi* IgG/IgM Test System

IVD

REF 3Z9651/SM3Z9651 3Z9651B

Σ96 Σ480

CE

Rx Only

## INTENDED USE

The ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System is an enzyme-linked immunosorbent assay (ELISA) for the qualitative detection of IgG and IgM class antibodies to *Borrelia burgdorferi* in human serum. The assay is intended for testing serum samples from symptomatic patients or those suspected of Lyme Disease.

Positive and equivocal test results with the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System for the presence of *Borrelia burgdorferi* antibodies must be confirmed through additional testing by one of the following approaches:

(1) Standard two-tier test methodology (STTT) using IgG or IgM Western blot testing following current guidelines;

or

(2) Modified two-tier test methodology (MTTT) using the ZEUS ELISA *Borrelia* VlsE1/pepC10 IgG/IgM Test System.

Positive test results by either the STTT or MTTT methodology are supportive evidence for the presence of antibodies and exposure to *Borrelia burgdorferi, the cause of Lyme disease. A diagnosis* of Lyme disease should be made based on the presence of *Borrelia burgdorferi* antibodies, history, symptoms, and other laboratory data.

## SIGNIFICANCE AND BACKGROUND

*Borrelia burgdorferi* is a spirochete that causes Lyme disease. Ticks of the genus *Ixodes* transmit the organism. In endemic areas, these ticks reside 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 three 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 produce IgG antibodies more slowly (1). 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 (ELISA).

*B. burgdorferi* is antigenically complex with strains that vary considerably. Early antibody responses often are to flagellin that has cross-reactive components. Patients in early stages of infection may not produce detectable levels of antibody. In addition, 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* have low sensitivity and specificity and because of such inaccuracies, health care professionals do not rely exclusively on these tests to establish 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 ELISA and IFA methods were not sufficiently specific to support clinical diagnosis, it was recommended that positive or equivocal results from a sensitive ELISA 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*. These results could support a clinical diagnosis of Lyme disease, but scientists suggest avoiding their use as a sole criterion for diagnosis. This scenario is commonly referred-to as the standard two-tier testing (STTT) protocol. Recent studies (18, 19, 20) have demonstrated that using a second ELISA test in place of the *Borrelia* immunoblot can result in a modified two-tier testing (MTTT) protocol with performance that is comparable to the STTT protocol.

## PRINCIPLE OF THE ASSAY

The ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test Systemis designed to detect IgM and IgG class antibodies to *Borrelia burgdorferi* in human sera. The sensitized wells of plastic microwell strips are prepared by passive adsorption with *Borrelia burgdorferi* whole cell antigen. The test procedure involves three incubation steps:

1. Test sera (properly diluted) are incubated in antigen coated microwells. Any antigen specific antibody in the sample will bind to the immobilized antigen. The plate is washed to remove unbound antibody and other serum components.
2. Peroxidase Conjugated goat anti-human IgM/IgG is added to the wells and the plate is incubated. The Conjugate will react with IgM and/or IgG antibody immobilized on the solid phase in step 1. The wells are washed to remove unreacted Conjugate.
3. The microwells containing immobilized peroxidase Conjugate are incubated with peroxidase Substrate Solution. Hydrolysis of the Substrate by peroxidase produces a color change. After a period of time the reaction is stopped and the color intensity of the solution is measured photometrically. The color intensity of the solution depends upon the antibody concentration in the original test sample.

## TEST SYSTEM COMPONENTS

**Materials Provided:**

Each Test System contains the following components in sufficient quantities to perform the number of tests indicated on the packaging label. **NOTE: The following components contain Sodium Azide as a preservative at a concentration of <0.1% (w/v): Controls, Calibrator and Sample Diluent.**

| Component | Σ96 | Σ480 | Description |
|---|---|---|---|
| PLATE | 1 | 5 | Plate: 96 wells configured in twelve, 1x8-well, strips coated with inactivated *B. burgdorferi* (B31 Strain) antigen. The strips are packaged in a strip holder and sealed in an envelope with desiccant. |
| CONJ | 1 | 5 | Conjugate: Conjugated (horseradish peroxidase) goat anti-human IgG/IgM in 15mL, white-capped bottle(s). Ready to use. |
| CONTROL + | 1 | 2 | Positive Control (Human Serum): 0.35mL, red-capped vial(s). |
| CAL | 1 | 4 | Calibrator (Human Serum): 0.5mL, blue-capped vial(s). |
| CONTROL - | 1 | 2 | Negative Control (Human Serum): 0.35mL, green-capped vial(s). |
| DIL SPE | 1 | 4 | Sample Diluent: 30mL, green-capped, bottle(s) containing Tween-20, bovine serum albumin and phosphate-buffered-saline. Green solution. Ready to use. |
| SOLN TMB | 1 | 5 | TMB: 15mL, amber-capped, amber bottle(s) containing 3, 3', 5, 5' - tetramethylbenzidine (TMB). Ready to use. |

| | | | | |
|---|---|---|---|---|
| SOLN | STOP | 1 | 3 | Stop Solution: 15mL, red-capped, bottle(s) containing 1M $H_2SO_4$, 0.7M HCl. Ready to use. |
| WASHBUF | 10X | 1 | 5 | Wash Buffer Concentrate (10X): Dilute 1 part concentrate + 9 parts deionized or distilled water. 100mL, clear-capped, bottle(s) containing a 10X concentrated phosphate-buffered-saline and Tween-20 solution (blue solution). **NOTE: 1X solution will have a pH of 7.2 ± 0.2.** |

**NOTES:**

1. **The following components are not Test System Lot Number dependent and may be used interchangeably with the ZEUS ELISA Test Systems: TMB, Stop Solution, and Wash Buffer.**
2. **Test System also contains a Component Label containing lot specific information inside the Test System box.**

## PRECAUTIONS

1. For *In Vitro* diagnostic use.
2. Follow normal precautions exercised in handling laboratory reagents. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves, and eye/face protection. Do not breathe vapor. Dispose of waste observing all local, state, and federal laws.
3. The wells of the ELISA plate do not contain viable organisms. However, consider the strips **potentially biohazardous materials** and handle accordingly.
4. The 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, handle these products 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 (16).
5. Adherence to the specified time and temperature of incubations is essential for accurate results. **All reagents must be allowed to reach room temperature (20 - 25°C) before starting the assay**. Return unused reagents to refrigerated temperature immediately after use.
6. Improper washing could cause false positive or false negative results. Be sure to minimize the amount of any residual wash solution; (e.g., by blotting or aspiration) before adding Conjugate or Substrate. Do not allow the wells to dry out between incubations.
7. The Sample Diluent, Controls, and Calibrator contain Sodium Azide at a concentration of <0.1% (w/v). Sodium Azide has been reported to form lead or copper azides in laboratory plumbing which may cause explosions upon hammering. To prevent, rinse sink thoroughly with water after disposing of solution containing Sodium Azide.
8. The Stop Solution is TOXIC if inhaled, has contact with skin or if swallowed. It can cause burns. In case of accident or ill feelings, seek medical advice immediately.
9. The TMB Solution is HARMFUL. It is irritating to eyes, respiratory system and skin.
10. The Wash Buffer concentrate is an IRRITANT. It is irritating to eyes, respiratory system and skin.
11. Wipe the bottom of the plate free of residual liquid and/or fingerprints that can alter optical density (OD) readings.
12. Dilution or adulteration of these reagents may generate erroneous results.
13. Do not use reagents from other sources or manufacturers.
14. TMB Solution should be colorless, very pale yellow, very pale green, or very pale blue when used. Contamination of the TMB with Conjugate or other oxidants will cause the solution to change color prematurely. Do not use the TMB if it is noticeably blue in color.
15. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.
16. Avoid microbial contamination of reagents. Incorrect results may occur.
17. Cross contamination of reagents and/or samples could cause erroneous results.
18. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
19. Avoid splashing or generation of aerosols.
20. Do not expose reagents to strong light during storage or incubation.
21. Allow the microwell strips and holder to equilibrate to room temperature prior to opening. The protective envelope will protect the wells from condensation.
22. Collect the wash solution in a disposal basin. Treat the waste solution with disinfectant (i.e.: 10% household bleach - 0.5% Sodium Hypochlorite). Avoid exposure of reagents to bleach fumes.
23. Caution: Neutralize any liquid waste at an acidic pH before adding to a bleach solution.
24. Do not use ELISA plate if the indicator strip on the desiccant pouch has turned from blue to pink.
25. Do not allow the Conjugate to come in contact with containers or instruments that may have previously contained a solution utilizing Sodium Azide as a preservative. Residual amounts of Sodium Azide may destroy the Conjugate's enzymatic activity.
26. Do not expose any of the reactive reagents to bleach-containing solutions or to any strong odors from bleach-containing solutions. Trace amounts of bleach (sodium hypochlorite) may destroy the biological activity of many of the reactive reagents within this Test System.

## MATERIALS REQUIRED BUT NOT PROVIDED

1. ELISA microwell reader capable of reading at a wavelength of 450nm. **NOTE: Use of a single (450nm), or dual (450/620 - 650nm), wavelength reader is acceptable. Dual wavelength is preferred, as the additional reference filter has been determined to reduce potential interference from anomalies that may absorb light.**
2. Pipettes capable of accurately delivering 10 - 200µL.
3. Multichannel pipette capable of accurately delivering 50 - 200µL.
4. Reagent reservoirs for multichannel pipettes.
5. Wash bottle or microwell washing system.
6. Distilled or deionized water.
7. One liter graduated cylinder.
8. Serological pipettes.
9. Disposable pipette tips.
10. Paper towels.
11. Laboratory timer to monitor incubation steps.
12. Disposal basin and disinfectant (i.e.: 10% household bleach - 0.5% Sodium Hypochlorite).

## STORAGE CONDITIONS

| | |
|---|---|
| 2°C – 8°C | Coated Microwell Strips: Immediately reseal extra strips with desiccant and return to proper storage. After opening - strips are stable for 60 days, as long as the indicator strips on the desiccant pouch remains blue. |
| | Conjugate – DO NOT FREEZE. |
| | Unopened Test System, Calibrator, Positive Control, Negative Control, TMB, Sample Diluent |
| 2°C – 25°C | Stop Solution: 2 - 25°C<br>Wash Buffer (1X): 20 - 25°C for up to 7 days, 2 - 8°C for 30 days.<br>Wash Buffer (10X): 2 - 25°C |

## SPECIMEN COLLECTION

1. ZEUS Scientific recommends that the user carry out specimen collection in accordance with CLSI document M29: Protection of Laboratory Workers from Infectious Disease (Current Edition).
2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, consider all blood derivatives potentially infectious.
3. Use only freshly drawn and properly refrigerated sera obtained by approved aseptic venipuncture procedures in this assay (14, 15). Do not use if there are any added anticoagulants or preservatives. 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 - 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 which may cause loss of antibody activity and give erroneous results. It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine stability criteria for its laboratory (17).

## ASSAY PROCEDURE

1. Remove the individual components from storage and allow them to warm to room temperature (20 - 25°C).
2. Determine the number of microwells needed. Allow for six Control/Calibrator determinations (one Reagent Blank, one Negative Control, three Calibrators and one Positive Control) per run. Run a Reagent Blank on each assay. Check software and reader requirements for the correct Controls/Calibrator configurations. Return unused strips to the resealable pouch with desiccant, seal, and return to storage between 2 - 8°C.

| EXAMPLE PLATE SET-UP | | |
|---|---|---|
| | 1 | 2 |
| A | Blank | Patient 3 |
| B | Negative Control | Patient 4 |
| C | Calibrator | Etc. |
| D | Calibrator | |
| E | Calibrator | |
| F | Positive Control | |
| G | Patient 1 | |
| H | Patient 2 | |

3. Prepare a 1:21 dilution (e.g.: 10µL of serum + 200µL of Sample Diluent) of the Negative Control, Calibrator, Positive Control, and each patient serum. Ensure that the samples are properly mixed.
4. To individual wells, add 100µL of each diluted Control, Calibrator and patient specimen. Use a different pipette tip for each sample.
5. Add 100µL of Sample Diluent to well A1 as a Reagent Blank. Check software and reader requirements for the correct Reagent Blank well configuration.
6. Incubate the plate at room temperature (20 - 25°C) for 25 ± 5 minutes.
7. Wash the microwell strips 5 times.
   a. **Manual Wash Procedure**:
      1. Vigorously shake out the liquid from the wells.
      2. Fill each microwell with Wash Buffer. Make sure no air bubbles are trapped in the wells.
      3. Repeat steps 1. and 2. for a total of 5 washes.
      4. Shake out the wash solution from all the wells. Invert the plate over a paper towel and tap firmly to remove any residual wash solution from the wells. Visually inspect the plate to ensure that no residual wash solution remains. Collect wash solution in a disposable basin and treat with disinfectant at the end of the day's run.
   b. **Automated Wash Procedure**:
      If using an automated microwell wash system, set the dispensing volume to 300 - 350µL/well. Set the wash cycle for 5 washes with no delay between washes. If necessary, the microwell plate may be removed from the washer, inverted over a paper towel and tapped firmly to remove any residual wash solution from the microwells.
8. Add 100µL of the Conjugate to each well, including the Reagent Blank well, at the same rate and in the same order as the specimens.
9. Incubate the plate at room temperature (20 - 25°C) for 25 ± 5 minutes.
10. Wash the microwells by following the procedure as described in step 7.
11. Add 100µL of TMB to each well, including the Reagent Blank well, at the same rate and in the same order as the specimens.
12. Incubate the plate at room temperature (20 - 25°C) for 10 - 15 minutes.
13. Stop the reaction by adding 50µL of Stop Solution to each well, including the Reagent Blank well, at the same rate and in the same order as the TMB. Positive samples will turn from blue to yellow. After adding the Stop Solution, tap the plate several times to ensure that the samples are thoroughly mixed.
14. Set the microwell reader to read at a wavelength of 450nm and measure the optical density (OD) of each well against the Reagent Blank. Read the plate within 30 minutes of the addition of the Stop Solution.

### ABBREVIATED TEST PROCEDURE

1. Dilute Serum 1:21.
2. Add diluted sample to microwell - 100µL/well.
3. ⟶ *Incubate 25 ± 5 minutes.*
4. Wash.
5. Add Conjugate - 100µL/well.
6. ⟶ *Incubate 25 ± 5 minutes.*
7. Wash.
8. Add TMB - 100µL/well.
9. ⟶ *Incubate 10 - 15 minutes.*
10. Add Stop Solution - 50µL/well - Mix.
11. READ within 30 minutes.

## QUALITY CONTROL

1. Each time the assay is performed, the Calibrator must be run in triplicate. A Reagent Blank, Negative Control, and Positive Control must also be included.
2. Calculate the mean of the three Calibrator wells. If any of the three values differ by more than 15% from the mean, discard that value and calculate the mean using the remaining two wells.
3. The mean OD value for the Calibrator, Positive Control, and Negative Control should fall within the following ranges:

| | OD Range |
|---|---|
| Negative Control | ≤0.250 |
| Calibrator | ≥0.300 |
| Positive Control | ≥0.500 |

a. The OD of the Negative Control divided by the mean OD of the Calibrator should be ≤0.9.
b. The OD of the Positive Control divided by the mean OD of the Calibrator should be ≥1.25.
c. If the above conditions are not met the test should be considered invalid and should be repeated.

4. The Positive Control and Negative Control are intended to monitor for substantial reagent failure, but will not ensure precision at the assay Cutoff.
5. Additional Controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.
6. Refer to CLSI document C24: Statistical Quality Control for Quantitative Measurement Procedures for guidance on appropriate QC practices.

## INTERPRETATION OF RESULTS

1. **Calculations:**
   a. *Correction Factor:* The manufacturer determined a Cutoff OD Value for positive samples and correlated it to the Calibrator. The Correction Factor (CF) allows for the determination of the Cutoff Value for positive samples. It will also correct for slight day-to-day variations in test results. The Correction Factor is determined for each lot of components and is printed on the Component Label located in the Test System box.
   b. *Cutoff OD Value:* To obtain the Cutoff OD Value, multiply the CF by the mean OD of the Calibrator determined above.
   *(CF x Mean OD of Calibrator = Cutoff OD Value)*
   c. *Index Values/OD Ratios:* Calculate the Index Value/OD Ratio for each specimen by dividing its OD Value by the Cutoff OD from step b.

| | | | |
|---|---|---|---|
| Example: | Mean OD of Calibrator | = | 0.793 |
| | Correction Factor (CF) | = | 0.25 |
| | Cutoff OD | = | 0.793 x 0.25 = 0.198 |
| | Unknown Specimen OD | = | 0.432 |
| | Specimen Index Value/OD Ratio | = | 0.432 /0.198 = 2.18 |

2. **Interpretations:** Index Values/OD Ratios are interpreted as follows.

| | Index Value/OD Ratio |
|---|---|
| Negative Specimens | ≤0.90 |
| Equivocal Specimens | 0.91 - 1.09 |
| Positive Specimens | ≥1.10 |

   a. An OD ratio ≤0.90 indicates no significant amount of IgM and/or IgG antibodies to *B. burgdorferi* detected. An additional sample should be tested within four to six weeks if early infection is suspected (5).
   b. An OD ratio ≥1.10 is presumptively positive for IgG/M antibody to *B. burgdorferi*. 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 confirmatory because their specificity is less than optimal, particularly for detecting IgM. Results should not be reported until the supplemental testing is completed.
3. **MTTT (2-EIA) Use and Interpretation for IgG/IgM Antibody Detection:**
   In addition to being used as the first-tier immunoassay in the standard two-tier testing (STTT) method, this device may be used as a second-tier assay in the 2-EIA or modified two-tier testing (MTTT) protocol in the following way.
   a. The samples must be tested first with the ZEUS ELISA *Borrelia* VlsE1/pepC10 IgG/IgM Test System.
   b. All the positive and equivocal samples must then be tested with this ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System.
   c. Positive and equivocal results from the second-EIA testing should be reported as positive and interpreted as supportive evidence for the presence of IgG/IgM antibodies and exposure to *B. burgdorferi.*

## LIMITATIONS OF THE ASSAY

1. The MTTT study was conducted using the ZEUS ELISA *Borrelia* VlsE1/pepC10 IgG/IgM Test System as the first-tier assay and the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System as the second-tier assay with testing performed in that order. The performance characteristics of the device have not been established for the alternate order of testing or for the use of other EIA assays in the MTTT (2-EIA) procedure.
2. Sera from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis, and relapsing fever), infectious mononucleosis, or systemic lupus erythematosus may give false positive results (6). Observations of false positive reactions require extensive clinical epidemiologic and additional laboratory workups to determine the specific diagnosis. Technicians can distinguish false positive sera from syphilis patients from true *B. burgdorferi* disease positive sera by running an RPR and a treponemal antibody assay on such specimens (7).
3. Drawing serum samples too early after onset of disease, before antibody levels have reached significant levels, results in false negative results (8). In addition, early antibiotic therapy may abort an antibody response to the spirochete (9).
4. Interpret all data in conjunction with clinical symptoms of disease, epidemiologic data, exposure in endemic areas, and results of other laboratory tests.
5. Do not perform screening of the general population. The positive predictive value depends on the pretest likelihood of infection. Only perform testing when clinical symptoms are present or exposure suspected.
6. ZEUS Scientific did not establish performance characteristics of the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System for samples from individuals vaccinated with *B. burgdorferi* antigens.

## EXPECTED RESULTS

Titers of IgM antibodies to *B. burgdorferi* peak three to six weeks after onset of EM and gradually decline thereafter (10). Titers of IgG antibodies are low during EM but increase in titer during the course of the disease, reaching peak titers when arthritis is present (10). IgG antibodies may remain elevated for years (11). Studies have shown that 90% or more of patients with EM alone develop elevated titers of IgM antibodies (10, 12). In the absence of EM, a positive ELISA test may distinguish early *B. burgdorferi* disease from other febrile illnesses (10). However, a much lower percentage of patients have elevated IgM antibodies when tested during the first three weeks after onset of EM (6, 13). In these patients, obtaining a more complete serological picture by testing acute and convalescent sera is necessary. Most patients (94 - 97%) with neurological complications and essentially all patients with arthritis have elevated IgG titers to the spirochete (6, 12). In later stages, a positive antibody test may help distinguish *B. burgdorferi* disease from viral meningitis or unexplained nerve palsies. A positive antibody test may be particularly useful in differentiating *B. burgdorferi* arthritis from rheumatoid arthritis, juvenile arthritis, and Reiter's Syndrome (10). Patients without signs or clinical features of *B. burgdorferi* disease should test negative with the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System.

## PERFORMANCE CHARACTERISTICS

1. **Comparative Study**
   The ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System was compared with a commercially available ELISA, and a reference IFA *B. burgdorferi* assay for the detection of antibodies in two, double blind clinical studies. The first study compared the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System to a commercially available IFA *B. burgdorferi* test system for the detection of antibodies in 199 serum samples randomly processed at a large medical center on the east coast. Depicted in Table 1 are the results of this double blind study.

**Table 1:** ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System vs. Commercially Available IFA Test

| | | **ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System** | |
|---|---|---|---|
| | | Positive | Negative |
| ***B. burgdorferi* IFA Procedure** | Positive | 58 | 5 |
| | Negative | 7 | 129 |

Analysis of the data in Table 1 reveals a sensitivity of 92%, a specificity of 95%, and an overall concordance of 94%.

The second study compared the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System to a reference IFA *B. burgdorferi* test procedure for the detection of IgG and IgM antibodies in 263 serum samples randomly processed at a large reference laboratory. The results of this double blind study are depicted in Table 2.

**Table 2:**

| | | **ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System** | |
|---|---|---|---|
| | | Positive | Negative |
| **Reference *B. burgdorferi* IFA for IgG/IgM** | Positive | 11 | 2 |
| | Negative | 8 | 242 |

Statistical analysis of the data in Table 2 show a sensitivity of 85%, and a specificity of 97%. The overall concordance was 96%.

In both clinical studies, repeating all discrepant results yielded identical results. In addition, results showed that IgM positive/IgG negative serum samples (7) tested positive with the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System, and pooled IgM positive/IgG negative reference sera tested positive with the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System. These results indicate that the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System is capable of detecting both IgG and IgM class specific antibodies against *B. burgdorferi* in individual microtiter wells.

Table 3 shows test results obtained using a serum panel from the CDC. Presented are the results to convey further information on the performance of this assay with a masked, characterized serum panel. This does not imply an endorsement of the assay by the CDC.

**Table 3: CDC *B. burgdorferi* Disease Serum Panel; Stratified by Time After Onset**

| Time from Onset | Positive | Equivocal | Negative | Total | % Agreement with Clinical Diagnosis |
|---|---|---|---|---|---|
| Normals | 1 | 1 | 3 | 5 | 75; 3/4 |
| <1 Month | 6 | 0 | 0 | 6 | 100; 6/6 |
| 1 - 2 Months | 7 | 0 | 1 | 8 | 88; 7/8 |
| 3 - 12 Months | 18 | 0 | 2 | 20 | 90; 18/20 |
| >1 Year | 8 | 0 | 0 | 8 | 100; 8/8 |
| **Total** | 40 | 1 | 6 | 47 | 93 (39/42 Positive)<br>(3/4 Negative) |

2. **Reproducibility**

The intra-assay and inter-assay variation was determined by running eight replicates of positive, borderline, and negative samples on three consecutive days. The results of these assays are as follows:

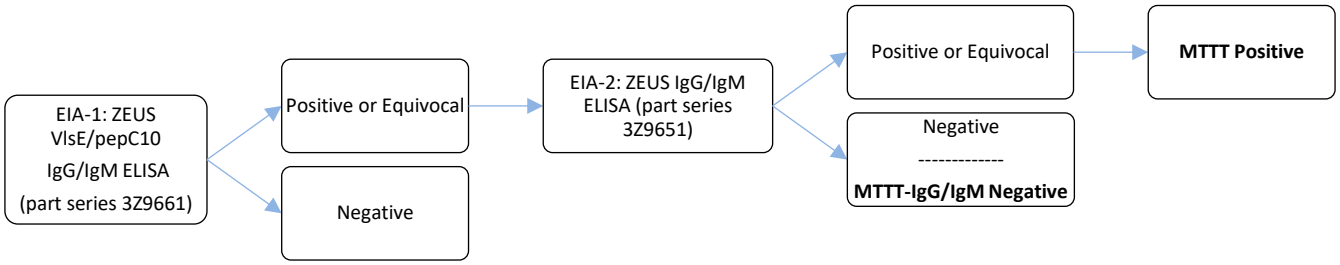
| | **Intra-Assay** | | | | | | **Inter-Assay** | |
|---|---|---|---|---|---|---|---|---|
| | Run 1 | | Run 2 | | Run 3 | | | |
| | Mean | % CV | Mean | % CV | Mean | % CV | Mean | % CV |
| Negative | 0.42 | 16.6 | 0.49 | 5.7 | 0.49 | 5.7 | 0.47 | 7.0 |
| Positive | 1.65 | 6.8 | 1.63 | 3.1 | 1.64 | 3.7 | 1.64 | 0.01 |
| Positive | 1.20 | 2.5 | 1.02 | 7.8 | 1.30 | 6.1 | 1.20 | 2.20 |
| Borderline | 0.76 | 15.4 | 0.77 | 5.5 | 0.93 | 2.9 | 0.82 | 9.5 |

3. **MTTT (2-EIA) Performance Characteristics**

The following studies were conducted to determine the performanceof the ZEUS ELISA *Borrelia* burgdorferi IgG/IgM Test System as a second-tier assay in the modified two-tier testing (MTTT) or the 2-EIA protocol.

a. **MTTT-IgG/IgM Method Comparison**: The ZEUS ELISA *Borrelia* burgdorferi IgG/IgM Test System was utilized as the second-tier assay in a MTTT protocol as depicted in the flow chart below. The EIA used in the first-tier was ZEUS ELISA *Borrelia* VlsE1/pepC10 IgG/IgM Test System. Performance of MTTT-IgG/IgM versus STTT was assessed using two separate cohorts; a retrospective cohort and a prospective cohort.

**Flow Chart: MTTT-IgG/IgM Algorithm**




b. **Retrospective Cohort Testing:** The 356-sample retrospective cohort consisted of the 280 member CDC Premarketing Panel that was supplemented with an additional 46 Stage 2 Lyme Disease (LD) specimens and an additional 30 Stage 3 LD specimens. Therefore, the retrospective panel consisted of 166 cases of LD (60 Stage 1, 56 Stage 2 and 50 Stage 3), 90 specimens from diseases other than LD and 100 healthy controls (50 endemic and 50 non-endemic).

Initially, the 356 retrospective samples were tested with the first-tier assay, ZEUS ELISA *Borrelia* VlsE1/pepC10 IgG/IgM Test System. There were 160 positive and 6 equivocal results. In the STTT protocol the samples that were positive or equivocal (n=166) were tested with *B. burgdorferi* IgM and/or IgG Western blots. In the MTTT-IgG/IgM protocol the samples (n=166) were tested on a second EIA, the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System. The second-tier EIA equivocal and positive results were considered positive. The equivocal and positive results were added together, and the results compared with the STTT positive results. Table 4 shows the outcome of MTTT-IgG/IgM as compared to the STTT protocol.

**Table 4: Comparison of MTTT-IgG/IgM and STTT (IgG and/or IgM) Results for Retrospective Cohort**

| | **Stage I (n=60)** | | **Stage II (n=56)** | | **Stage III (n=50)** | | **Healthy Controls (n=100)** | | **Disease Controls (n=90)** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **STTT-IgG/IgM** | **MTTT-IgG/IgM** | **STTT-IgG/IgM** | **MTTT-IgG/IgM** | **STTT-IgG/IgM** | **MTTT-IgG/IgM** | **STTT-IgG/IgM** | **MTTT-IgG/IgM** | **STTT-IgG/IgM** | **MTTT-IgG/IgM** |
| **Positive** | 38 | 47 | 34 | 37 | 50 | 50 | 0 | 0 | 0 | 2 |
| **Negative** | 22 | 13 | 22 | 19 | 0 | 0 | 100 | 100 | 90 | 88 |
| **Sensitivity or PPA** | **63.3%** | **78.3%** | **60.7%** | **66.1%** | **100%** | **100%** | N/A | N/A | N/A | N/A |
| **Specificity or NPA** | N/A | N/A | N/A | N/A | N/A | N/A | **100%** | **100%** | **100%** | **97.8%** |

c. **Prospective Cohort Testing:** A prospective cohort of serum samples sent to a laboratory for routine *Borrelia* serology was assembled. These specimens were collected from three different geographical locations in the US, all from areas endemic to LD. Two of the three sites (Massachusetts and Minnesota) collected the specimens and performed the respective ELISA testing. One site (Wisconsin) collected the specimens and sent them to the manufacturer for the respective ELISA testing. The three sites and their corresponding number of specimens have been summarized in Table 5 below:

**Table 5: Summary of the Prospective Specimen Cohort.**

| Geographic Location | Sample Size (n) |
|---|---|
| Massachusetts | 900 |
| Wisconsin | 990 |
| Minnesota | 1042 |
| **Total** | **2932** |

Initially, the 2,932 prospective samples were tested with the first-tier assay, ZEUS ELISA *Borrelia* VlsE1/pepC10 IgG/IgM Test System. There were 363 positive and 58 equivocal results. In the STTT protocol the samples that are positive or equivocal (n=421) are tested with B. burgdorferi IgM and/or IgG Western blots. In the MTTT-IgG/IgM protocol the samples (n=421) were tested on a second ELISA, the ZEUS ELISA *Borrelia burgdorferi* IgG/IgM Test System. The second-tier EIA equivocal and positive results were considered positive. The equivocal and positive results were added together, and the results compared with the STTT positive results. A summary of the outcome of STTT versus MTTT-IgG/IgM appears in Table 6 below:

**Table 7: MTTT-IgG/IgM Method compared to STTT (IgG and/or IgM) Method in the Prospective Cohort**

| | | STTT (IgG and/or IgM) | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| **MTTT-IgG/IgM** | Positive | 167 | 63** | 230 |
| | Negative | 12* | 2690 | 2702 |
| | Total | 179 | 2753 | 2932 |

Positive Agreement: 93.3% (167/179) 95% CI: 88.6 – 96.12%
Negative Agreement: 97.7% (2690/2753) 95% CI: 97.1 – 98.2%

*Of the 12 samples that were STTT positive/MTTT negative, one of the 12 was confirmed to be a case of Stage 1 Lyme Disease. One sample had no clinical information available and the remaining ten did not have clinical information consistent with Lyme disease.

**Of the 63 samples that were MTTT positive/STTT negative, four samples were from confirmed cases of Lyme Disease (three Stage 1 and one late disease). Thirty two samples had no clinical information available and the remaining twenty seven specimens did not have clinical information consistent with Lyme disease.

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