EPITOGEN *Epitogen Lyme* IgG Test

RUO

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REF **EGX96LD1**

1. SIGNIFICANCE AND BACKGROUND

Lyme disease is a vector-borne disease transmitted by tick bite, which facilitates the entry of the infectious agent, the spirochete *Borrelia burgdorferi* sensu lato (s.l.) into the bloodstream. The classic skin rash, called *erythema migrans* (EM), develops around the bite area with *Borrelia* infection in 60 - 80% of patients.
The course of multisystemic Lyme disease is often divided into three stages. This division is based on the progression from an initial local manifestation to further systemic symptoms. However, asymptomatic subclinical infection is still possible and infection may not become clinically evident until a later stage. Furthermore, the clinical manifestations attributed to Lyme disease overlap with other common infections making diagnosis of the disease even more difficult.

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

B. burgdorferi s.l. are a diverse group of bacteria, distributed worldwide, that include 20 spirochete species. Out of the 20 genospecies, several of them infect humans, causing LB, a disease often referred to as the 'great imitator' due to its diversity of clinical manifestations. The genospecies that commonly infect people include *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, and *B. mayoni*. Due to the development of new progressive diagnostic methods throughout the last decades, there have been dramatic increases in the number of diagnosed cases of LB across Europe and the USA. Yet as, *B. burgdorferi* genospecies are antigenically complex, serological testing is known to have low sensitivity and specificity, thus meaning that these tests cannot be relied upon exclusively for establishing a Lyme disease diagnosis.

Therefore, a two-step serology testing system is recommended to support the clinical diagnosis of Lyme disease. This current two-step approach provides positive evidence of exposure to *B. burgdorferi*, which could support a clinical diagnosis of Lyme disease, however it still should not be used as the sole criterion for diagnosis.

2. PRINCIPAL OF THE EPITOGEN LYME IgG TEST

The Epitogen Lyme IgG Test is a peptide-based assay designed to overcome the inherent limitations found in existing commercial serological tests. As mentioned above, *B. burgdorferi* are a diverse group of bacteria with differing antigenic profiles. Furthermore, there is variability in the expression profiles of *Borrelia* antigens during different disease stages and among different species. Therefore, an ideal serological test needs to consider the diversity of *Borrelia* pathogenic species to ensure its maximal performance. Moreover, at the population level, the humoral response to *B. burgdorferi* is heterogeneous meaning that an individual's antibody response will vary depending on the *Borrelia* antigens. In this way, traditional serological assays using few antigens or whole extracts of *B. burgdorferi* s.l will always be limited in their sensitivity.

The Epitogen Lyme IgG Test adopts **four principals** designed for maximum performance:

1. The Epitogen Lyme IgG Test focuses on **immunodominant B-cell hotspots**. This approach enriches positive signals and improves sensitivity.
2. The Epitogen Lyme IgG Test **combines 120 immunodominant epitopes** in mosaic antigens thereby further boosting assay sensitivity.
3. The Epitogen Lyme IgG Test overcomes population immune system heterogeneity by covering **37 *Borrelia* antigens** (OspC, OspA, flagellin, VlsE) which correspond to the major pathogenic species.
4. The Epitogen Lyme IgG Test **specificity is high**. This is because cross-reactive epitopes are identified and eliminated early on during the screening process which subsequently improves specificity.

3. INTENDED USE

The Epitogen Lyme IgG Test uses indirect-ELISA to qualitatively detect IgG antibody against *Borrelia* proteins in human serum or plasma. The test is intended to facilitate the identification of individuals with an adaptive immune response to *B. burgdorferi s.l.*, indicating a recent or prior infection. Due to high specificity, the test may be used as a screening procedure for the general population.

Positive test results with the Epitogen Lyme IgG Test provides evidence for the presence of antibodies and exposure to *B. burgdorferi s.l.*, the cause of Lyme disease. However, a negative result does not preclude *B. burgdorferi* infection since patients in the early stages of infection may not produce detectable levels of antibody. Similarly, early antibiotic therapy after EM display may diminish or abrogate good antibody response. In fact, some exposed patients may never generate detectable antibody levels.

Patients with early infection produce IgM antibodies during the first few weeks after the onset of EM and produce IgG antibodies at a later stage. Importantly, the presence of *B. burgdorferi* antibodies may be detected through additional testing by employing our **Epitogen Lyme IgM ELISA Test** and/or re-testing using a second sample collected two to four weeks later. In fact, both IgG and IgM antibodies can remain detectable for many years. It should also be noted that, false positive results for the Epitogen Lyme IgG Test assay may occur due to cross reactivity from pre-existing antibodies or other possible causes.

4. 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 **ProClin™ 300** as a preservative at a concentration of <0.01% (w/v): For positive and negative controls and sample diluent.

REAGENT	DESCRIPTION	QUANTITY
96-well plate	96-microplate coated with proprietary Epitogen Lyme antigens	1
Diluent additive (30X)	A protein additive to block interference effects	1 x 1 mL (# 1)
Positive control (P)	Pooled human Lyme disease positive serum	1 x 0.5 mL (# 2)
Negative control (NEG)	Pooled human serum	1 x 0.5 mL (# 3)
Conjugate	Goat anti-human IgG conjugated (horseradish peroxidase)	1 x 120 µl (# 4)
10X wash buffer	10X concentrated phosphate-buffered-saline and Tween®20	1 x 100 mL (# 5)
Sample diluent	1% casein in phosphate-buffered-saline	1 x 14.5mL (# 6)
Secondary diluent	1% casein in phosphate buffered-saline	1 x 11.5 mL (# 7)
TMB substrate	3, 3', 5, 5' -tetramethylbenzidine	1 x 12 mL (# 8)
Stop solution	1M sulfuric acid (H_2SO_4)	1 x 12 mL (# 9)

5. PREPARATION AND STABILITY OF THE REAGENTS

Note: All reagents must be brought to room temperature (+18 °C to 25°C) 30 minutes before use.

- **Coated Wells**: Ready for use. Tear open the protective wrapping of the plate immediately before use.
- **Wash Buffer**: The wash buffer is a 10X concentrate. Dilute 1 part reagent plus 9 parts deionized or distilled water. If crystallization occurs in the concentrated buffer, warm it to 37°C and mix well before diluting.
- **Sample diluent**: Mix thoroughly with diluent additive before use.
- **Diluent additive**:
    1. Mix thoroughly with sample diluent before use.
    2. Mix thoroughly with secondary diluent before use.
- **Secondary diluent**: Mix thoroughly with diluent additive and conjugate before use to prepare the **conjugate cocktail**.
- **Conjugate**: Mix thoroughly with diluent additive and secondary diluent before use.
- **Controls**: Ready for use. Mix thoroughly before use.
- **TMB substrate**: Ready for use. Light sensitive. Close the bottle immediately after use. The TMB substrate solution must be clear on use. Do not use the solution if it is coloured blue.
- **Stop solution**: Ready for use.

Storage and stability: Store kit between +2°C and +8°C. Do not freeze. Unopened, all the test components are stable until the indicated expiry date.

Waste Disposal: Patient samples, controls and microplates should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

6. MATERIALS REQUIRED BUT NOT PROVIDED

1. ELISA microwell reader capable of reading at a wavelength of 450nm.
2. Pipettes capable of accurately delivering 10 - 200µL.
3. Multichannel pipettes capable of accurately delivering 50 - 200µL.
4. Reagent reservoirs for multichannel pipettes.
5. Plate rocker for all incubation steps.
6. Wash bottle or microwell washing system.
7. Distilled or deionized water.
8. One litre graduated cylinder.
9. Serological pipettes.
10. Disposable pipette tips.
11. Paper towels.
12. Laboratory timer to monitor incubation steps.
13. Disposal basin and disinfectant (i.e. 10% household bleach - 0.5% Sodium Hypochlorite).

7. 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 remain blue.
	Conjugate – DO NOT FREEZE.
	Unopened Test System, Positive Control, Negative Control, TMB, Sample Diluent
2°C – 25°C	Stop Solution: 2 – 25°C Wash Buffer (1X): 20 - 25°C for up to 7 days, 2 - 8°C for 30 days. Wash Buffer (10X): 2 - 25°C

8. PREPARATION AND STABILITY OF SPECIMEN

Sample material: Human serum or EDTA, heparin or citrate plasma.

Stability: Samples to be investigated must be properly stored. Avoid multiple freeze/thaw cycles which may cause loss of antibody activity and provide erroneous results. Do not use if there are any other added anticoagulants/preservatives. Avoid using haemolyzed, lipemic, or bacterially contaminated sera.

Sample dilution: Samples are diluted 1:101 in diluent buffer.

9. PLATE LAYOUT AND ASSAY PROCEDURE

Legend – Antigen coating

Lyme-antigen coating: A set of composite IgG immunodominant peptides comprising 120 epitopes from 37 *Borrelia* antigens.

Scaffold-control coating: The Epitogen® scaffold protein (control).

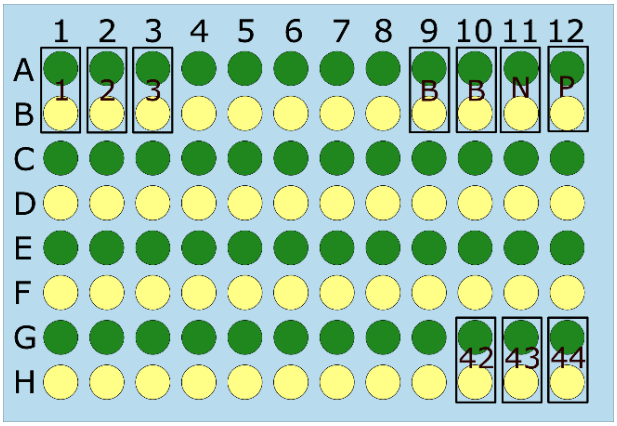
1 **Test Sample (1 – 44) –** 44 single tests.

B **Blank (B) –** Sample diluent buffer.

N **Negative serum (N) –** Lyme disease negative sera.

P **Positive serum (P) –** Lyme disease positive sera.

Epitogen Lyme IgG Test Layout



Remove the individual components from storage and allow them to warm to room temperature (20 – 25°C).

1. Pipette 0.5 mL of the diluent additive into the sample diluent and mix thoroughly.
2. Prepare the **conjugate cocktail**: pipette 0.4 mL of the diluent additive and 100 µL conjugate into the secondary diluent and mix gently.
3. Each plate can test 44 samples. Allow for four wells, two for the Positive Control and two for the Negative. Also, allow for four Blanks per plate (see plate layout).
4. Prepare a 1:101 dilution (e.g.: 3 µL of test sample + 300 µL of sample diluent with diluent additive) for each patient sample. Controls are pre-diluted and ready for use.

5. Wash the microwell plate once with 300 µL/well Wash Buffer. 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.
6. To a pair set of Lyme-antigen and scaffold-control wells (see above layout), add 100 µL to each well of diluted patient specimen, positive and negative controls (provided). Add sample diluent (with diluent additive) to the Blank wells. Ensure that the samples are properly mixed. Use a different pipette tip for each patient sample.
7. Incubate the plate on a plate rocker at room temperature (20 - 25°C) for 60 ± 1 minutes.
8. Wash the microplate 5 times.
   a. **Manual Wash Procedure**:
      1. Vigorously shake out the liquid from the wells.
      2. Fill each microwell with 300 µL 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 µL/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.
9. Add 100 µL of the **conjugate cocktail** to each well, including the sample Blank wells, at the same rate and in the same order as the specimens.
10. Incubate the plate on a plate rocker at room temperature (20 - 25°C) for 30 ± 1 minutes.
11. Wash the microplate 5 times following the same procedure as described in step **7**.
12. Add 100 µL of TMB to each well, including the sample Blank wells, at the same rate and in the same order as the specimens.
13. Incubate the plate at room temperature (20 - 25°C) for 5 minutes.
14. Stop the reaction by adding 100 µL of Stop Solution to each well, including the sample Blank wells, at the same rate and in the same order as the TMB. Positive samples will turn from blue to yellow.
15. Set the microwell reader to read at a wavelength of 450nm and measure the optical density (OD). Read the plate within 10 minutes after the addition of the Stop Solution.

Abbreviated Test Procedure

1. Remove plate from sealed bag.
2. Add diluent additive to sample diluent and mix thoroughly.
3. Add diluent additive and conjugate to secondary diluent and mix thoroughly (**conjugate cocktail**).
4. Dilute samples 1:101 (e.g.: 3 µL of sample + 300 µL of sample diluent and additive)
5. Wash plate once then add 100 µL of each diluted sample and controls to each microwell.
6. Incubate at RT for 60 ± 1 minutes.
7. Wash 5 times – 300 µL/well.
8. Add Conjugate – 100 µL/well.
9. Incubate at RT for 30 ± 1 minutes.
10. Wash 5 times – 300 µL/well.
11. Add TMB – 100 µL/well.
12. Incubate for 5 minutes at RT.
13. Add Stop Solution – 100 µL/well.
14. Read OD at 450nm within 10 minutes.

10. QUALITY CONTROL

Each time the assay is performed, the Blank sample, Negative Control and Positive Control must also be included.
The OD value for the Negative Control and Positive Control should fall within the following ranges:

	OD Range
Negative Control	≤ 0.15
Positive Control	≥ 0.5

Note: If the above conditions are not met the test should be considered invalid!

11. INTERPRETATION OF RESULTS

Calculations:
Signal to noise ratio (S/N): The result of each sample is calculated as the signal to noise (S/N) ratio of the OD readout from the Lyme-antigen (Ag) coated well, subtracted from the scaffold coated control (Ctl) of the same sample, then divided by the mean absorbance of the four Blank sample wells (B):

$$S/N = (OD_{Ag} - OD_{Ctl}) / OD_{B}$$

Example: The measured absorbance at 450 nm with a serum or plasma sample in the Ag coated well is 1.00 and the same sample in the Ctl coated well is 0.10, and the mean of the four Blanks (B) readout is 0.09.

Therefore, the S/N = (1.00 – 0.10) / 0.09 = 10.

Interpretations: The S/N Ratios are interpreted as follows.

	S/N Ratio	Interpretation
Negative	< 2.50	No significant amount of IgG antibodies to *B. burgdorferi s.l.* detected.
Borderline	≥ 2.50 to < 3.00	Antibodies specific to *B. burgdorferi s.l.* were detected. This indicates presumptive evidence of probable exposure. The specimen should be tested by a second step IgG and/or IgM western blot, or by testing a fresh sample 7 days later and re-tested in parallel with the first sample.
Positive	≥ 3.00	Antibodies specific to *B. burgdorferi s.l.* were detected - supportive evidence of probable exposure.

12. TEST CHARACTERISTICS

Antigen: The antigen source is a collection of highly specific **120 immunodominant epitopes** corresponding to **37 bacterial Borrelia antigens** which include OspC, OspA, flagellin, VlsE. These peptides cover the major pathogenic species thereby creating a Universal Reference antigen.

Detection Limit: The detection limit is defined as a value of three times the standard deviation of an analyte-free sample.

Precision: The reproducibility of the test was investigated by determining the intra- and inter- assay coefficients of variation (CV) using three sera. The Inter- and intra- assay CVs were less than 10%.

Cross reactivity: The quality of the antigen used ensures high specificity of the ELISA. Serum samples from healthy individuals and patients with infections caused by agents known to have cross-reactivity with Lyme serology tests were investigated using the Epitogen Lyme IgG Test

Table 1. Specificity of the Epitogen Lyme IgG Test.

Sample type & size	Number of non-reactive	Specificity; 95% CI
Cytomegalovirus (n = 20)	20	**100**; 83.2 – 100
Epstein–Barr virus (n = 20)	19	**95.0**; 75.1 – 99.9
Syphilis (n = 30)	27	**90.0**; 73.5 – 97.9
Rheumatoid arthritis (n = 17)	16	**94.1**; 71.3 – 99.9
Multiple sclerosis (n = 11)	11	**100**; 71.5 – 100
Healthy (n = 100)	96	**96.0**; 90.1 – 98.9
Total (n = 198)	189	**95.5**; 91.6 – 97.9

Table 2. Sensitivity of the Epitogen Lyme IgG Test. 171 clinically characterised patient samples were examined with the Epitogen Lyme IgG Test.

Sample type & size	Number of non-reactive	Sensitivity; 95% CI
Lyme neuroborreliosis (n = 30)	30	**100**; 88.4 – 100
ACA (n = 6)	6	**100**; 54.1 – 100
Lyme arthritis (n = 24)	24	**100**; 85.8 – 100
Erythema migrans (n = 111)	98	**88.3**; 80.8 – 93.6
↳ Acute EM (n = 49)	43	**87.8**; 75.2 – 95.4
↳ Early EM (n = 39)	32	**92.2**; 66.5 – 92.5
↳ Late EM (n = 23)	23	**100**; 85.2 – 100
Total (n = 171)	158	**93.3**; 87.4 – 95.9

[a] Acrodermatitis chronica atrophicans (ACA); *Erythema migrans* (EM).

13. LIMITATIONS OF THE ASSAY

1. Serum samples from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis, and relapsing fever), or infectious mononucleosis or 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.
2. False negative results may be obtained if serum samples are drawn too early after the onset of the disease, that is, if taken before antibody levels reached significant levels. Also, early antibiotic therapy may abort an antibody response to the spirochetes.
3. All data must be interpreted in conjunction with the clinical symptoms of the disease, epidemiologic data, exposure in endemic areas, and results of other laboratory tests.
4. The performance characteristics of the Epitogen Lyme IgG Test have not been established with samples from individuals vaccinated with *B. Burgdorferi* antigens.

14. PRECAUTIONS

1. For Research Use only.
2. Follow normal precautions exercised in handling laboratory reagents. In case of contact with eyes, rinse immediately with plenty of water then seek medical advice. Wear suitable protective clothing, gloves, and eye/face protection.
3. All materials of human origin should be handled as being potentially infectious. Handle these products at the **Biosafety Level 2** as recommended for any potentially infectious human serum or blood specimen.
4. Adherence to the specified time and temperature of incubations is essential for accurate results.
5. Improper washing may cause false positive or false negative results. Be sure to minimize the amount of any residual wash solution before adding Conjugate or Substrate and do not allow the wells to dry out between incubations.
6. The Stop Solution is TOXIC, if inhaled, comes into contact with skin or if swallowed. It can also cause burns. If there is an accident or someone feels ill, immediately seek medical advice.
7. The TMB Solution is HARMFUL. It is an irritant to the eyes, the respiratory system, and the skin.
8. The Wash Buffer concentrate is also an irritant to the eyes, the respiratory system, and the skin.
9. Wipe the bottom of the plate free of any residual liquid and/or fingerprints that can alter optical density (OD) readings.
10. Do not use reagents from any other sources or manufacturers.
11. The TMB solution should be colourless, 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 colour prematurely. **Do not use** the TMB if it is noticeably blue in colour.
12. Never pipette via the mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.
13. Cross contamination of reagents and/or samples could cause erroneous results.
14. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
15. Do not expose reagents to strong light during storage or incubation.
16. Collect the wash solution in a disposal basin. Treat the waste solution with disinfectant. Avoid exposure of reagents to bleach fumes.
17. **Caution**: Neutralize any liquid waste of an acidic pH before adding a bleach solution.
18. Do not use an ELISA plate if the indicator strip on the desiccant pouch has turned from blue to pink.
19. Do not use reagents after the expiry date (6 months after the manufacture date).