ENGLISH

1. INTRODUCTION

Lyme disease (Borreliosis) is a tick-borne, bacterial disease of domestic animals and humans. It is caused by spirochetes of the Borrelia burgdorferi sensu lato group. In Europe, the most frequently isolated pathogenic genospecies of humans and dogs are Borrelia afzelii, Borrelia garinii and Borrelia burgdorferi sensu stricto. Borrelia are helically wound, flexible, highly motile bacteria. By rotation of their axial filaments (periplasmatic flagella) they are able to move efficiently in corkscrew fashion through viscous media (serum). Thereby they can disseminate throughout the body within days to weeks of infection. The pathogens are transmitted by various tick species of the genus Ixodes. In Europe, Ixodes ricinus is the most important vector. However, infestation rates with Borrelia vary depending on the region. In endemic areas of Germany, approximately 3-7 % of the larvae and 10-34 % of nymphs and adult ticks are infected by Borrelia burgdorferi sensu lato. Natural reservoirs are wild animals, including rodents as well as many other small mammals and birds. The ticks take their meals (blood) from these hosts. Since dogs frequent the areas ticks live, they are more affected than humans. Typical tick habitats are the edge of the woods, bushes, undergrowth and tall grass; but infected ticks can also be found in public parks. Symptoms of Lyme disease in dog comprise fever, apathy, loss of appetite and anorexia as well as recurrent and shifting lameness and polyarthritis. The characteristic rash or the circular area of redness around the bite (erythema chronicum migrans) which is seen in man may be absent or is overlooked due to hair coat or dark pigmentation.

2. INTENDED USE

The GenWay Lyme Borrelia Canine IgG-ELISA is intended for the qualitative determination of IgG-class antibodies against Borrelia burgdorferi in canine serum.

3. PRINCIPLE OF THE ASSAY

The qualitative immunoenzymatic determination of IgG-class antibodies against Borrelia is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique. Microwell strip wells are pre-coated with Lyme Borrelia antigens incl. VlsE to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled anti-dog IgG conjugate is added. This conjugate binds to the captured Borrelia-specific antibodies. The immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine-(TMB) substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of Borrelia-specific IgG antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm is read using an ELISA microwell plate reader.

4. MATERIALS

4.1. Reagents supplied

- Lyme Borrelia Microtiter Strips (IgG); 12 break apart 8-well snap-off strips coated with Borrelia burgdorferi antigens incl. VlsE; in resealable aluminum foil.
- IgG Sample Diluent**: 1 bottle containing 100 ml of buffer for sample dilution; pH 7.2 ± 0.2; colored yellow; ready to use; white cap.
- Stop Solution: 1 bottle containing 15 ml sulfuric acid, 0.2 mol/l; ready to use; red cap.
- Washing Solution (20x conc.)*: 1 bottle containing 50 ml of a 20-fold concentrated buffer (pH 7.2 ± 0.2) for washing
the wells; white cap.

**Lyme Borrelia anti-dog IgG Conjugate**: 1 bottle containing 20 ml of peroxidase labeled anti-dog IgG, colored blue, ready to use; black cap.

**TMB Substrate Solution**: 1 bottle containing 15 ml 3,3’,5,5’-tetramethylbenzidine (TMB); ready to use; yellow cap.

**Lyme Borrelia IgG Positive Control**: 1 bottle containing 2 ml; colored yellow; ready to use; red cap.

**Lyme Borrelia IgG Negative Control**: 1 bottle containing 2 ml; colored yellow; ready to use; blue cap.

**Lyme Borrelia IgG Cut-off Control**: 1 bottle containing 3 ml, colored yellow, ready to use, green cap.

* contains 0.1 % Bronidox L after dilution ** contains 0.2 % Bronidox L *** contains 0.1 % Kathon

4.2. Materials supplied
- 1 Strip holder
- 1 Cover foils
- 1 Test protocol
- 1 distribution and identification plan

4.3. Materials and Equipment needed
- ELISA microwell plate reader, equipped for the measurement of absorbance at 450/620 nm
- Incubator 37°C
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Deionised or (freshly) distilled water
- Disposable tubes
- Timer

5. STABILITY AND STORAGE
The reagents are stable up to the expiry date stated on the label when stored at +2...+8 °C.

6. REAGENT PREPARATION

It is very important to bring all reagents, samples and controls to room temperature (+20...+25 °C) before starting the test run!

6.1. Microtiter Strips
The ready to use break apart snap-off strips are coated with Borrelia burgdorferi antigens incl. VlsE. Store at +2...+8 °C. Immediately after removal of strips, the remaining strips should be resealed in the aluminum foil along with the desiccant supplied and stored at +2...+8 °C; stability until the expiry date.

6.2. Lyme Borrelia anti-dog IgG Conjugate
The bottle contains 20 ml of a solution with anti dog-IgG conjugated to horseradish peroxidase, buffer, stabilizers, preservatives and an inert blue dye. The solution is ready to use. Store at +2...+8 °C. After first opening stability until the expiry date when stored at +2...+8 °C.

6.3. Controls
The bottles contain a ready to use control solution. They have to be stored at +2...+8 °C. After first opening stability until the expiry date when stored at +2...+8 °C.

6.4. IgG Sample Diluent
The bottle contains 100 ml phosphate buffer, stabilizers, preservatives and an inert yellow dye. It is used for the dilution of the patient specimen. This ready to use solution has to be stored at +2...+8 °C. After first opening stability until the expiry date when stored at +2...+8 °C.

6.5. Washing Solution (20x conc.)
The bottle contains 50 ml of a concentrated buffer, detergents and preservatives. Dilute washing solution 1+19; e.g. 10 ml washing solution + 190 ml fresh and germ free redistilled water. The diluted buffer is stable for 5 days if stored at room temperature. Crystals in the solution disappear by warming up to +37 °C in a water bath. After first opening the concentrate is stable until the expiry date.

6.6. TMB Substrate Solution
The bottle contains 15 ml of a tetramethylbenzidine/hydrogen peroxide system. The reagent is ready to use and has to be stored at +2...+8 °C, away from the light. The solution should be colorless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away. After first opening stability until the expiry date when stored at +2...+8 °C.

6.7. Stop Solution
The bottle contains 15 ml 0.2 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at +2...+8 °C. After first opening stability until the expiry date.

7. SPECIMEN COLLECTION AND PREPARATION
Use dog serum samples with this assay. If the assay is performed within 5 days after sample collection, the specimens should be kept at +2...+8 °C; otherwise they should be aliquoted and stored deep-frozen (-20 to -70 °C). If samples are stored frozen, mix thawed samples well before testing. Avoid repeated freezing and thawing. Heat inactivation of samples is not recommended.

7.1. Sample Dilution
Before assaying, all samples should be diluted 1 + 100 with IgG Sample Diluent. Dispense 10 µl sample and 1 ml IgG Sample Diluent into tubes to obtain a 1 +100 dilution and thoroughly mix with a Vortex.

8. ASSAY PROCEDURE

8.1. Test Preparation
Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described. If performing the test on ELISA automatic systems we recommend to increase the washing steps from three to five and the volume of washing solution from 300 µl to 350 µl to avoid washing effects. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder.

Please allocate at least:1 well (e.g. A1) for the substrate blank,1 well (e.g. B1) for the Negative Control,2 wells (e.g. C1+D1) for the Cut-off Control and1 well (e.g. E1) for the Positive Control.

It is recommended to determine controls and patient samples in duplicate.

Perform all assay steps in the order given and without any appreciable delays between the steps. A clean, disposable tip should be used for dispensing each control and sample. Adjust the incubator to +37 ± 1 °C.

1. Dispense 100 µl controls and diluted samples into their respective wells. Leave well A1 for substrate blank.
2. Cover wells with the foil supplied in the kit.
3. Incubate for 60 min ± 5 min at +37 ± 1 °C.
4. When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300 µl of Washing Solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be > 5 sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step!

Note: Washing is critical! Insufficient washing results in poor precision and falsely elevated absorbance values.

1. Dispense 100 µl Lyme Borrelia anti-dog IgG Conjugate into all wells except for the blank well (e.g. A1). Cover with foil.
2. Incubate for 30 min at room temperature. Do not expose to direct sunlight.
3. Repeat step 4.
4. Dispense 100 µl TMB Substrate Solution into all wells
5. Incubate for exactly 15 min at room temperature in the dark.
6. Dispense 100 µl Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution.

Any blue color developed during the incubation turns into yellow.

11. Measure the absorbance of the specimen at 450/620 nm within 30 min after addition of the Stop Solution.

8.2. Measurement Adjust the ELISA Microwell Plate Reader to zero using the substrate blank in well A1.

If due to technical reasons the ELISA reader cannot be adjusted to zero using the substrate blank in well A1, subtract the absorbance value of well A1 from all other absorbance values measured in order to obtain reliable results!

Measure the absorbance of all wells at 450 nm and record the absorbance values for each control and patient sample in the distribution and identification plan.

Dual wavelength reading using 620 nm as reference wavelength is recommended.

Where applicable calculate the mean absorbance values of all duplicates.
9. RESULTS

9.1. Run Validation Criteria
In order for an assay to be considered valid, the following criteria must be met:

- **Substrate blank** in A1: Absorbance value \(< 0.100\).
- **Negative control** in B1: Absorbance value \(< 0.200\) and \(< \text{cut-off}\)
- **Cut-off control** in C1 and D1: Absorbance value \(0.150 – 1.30\).
- **Positive control** in E1: Absorbance value \(> \text{cut-off}\). If these criteria are not met, the test is not valid and must be repeated.

9.2. Calculation of Results
The Cut-off value is calculated as the mean value of the determinated Cut-off controls in C1 and D1.

*Example:* Absorbance value Cut-off Control 0.39 + absorbance value Cut-off Control 0.37 = 0.76 / 2 = 0.38 Cut-off = 0.38

9.3. Interpretation of Results
Samples are considered **POSITIVE** if the absorbance value is higher than 10% over the cut-off.

Samples with an absorbance value of 10% above or below the cut-off should not be considered as clearly positive or negative • grey zone

It is recommended to repeat the test again 2 -4 weeks later with a fresh sample. If results in the second test are again in the grey zone the sample has to be considered **NEGATIVE**.

Samples are considered **NEGATIVE** if the absorbance value is lower than 10% below the cut-off.

9.3.1. Results in GenWay Units

Patient (mean) absorbance value x 10 = [GenWay-Units = NTU]

*Example:* 1.786 x 10 = 42 NTU (GenWay Units)

Cut-off: 10 NTU Grey zone: 9-11
NTU Negative: <9 NTU Positive:
>11 NTU

10. SPECIFIC PERFORMANCE CHARACTERISTICS

10.1. Precision

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean (NTU)</th>
<th>Cv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos. Serum</td>
<td>12</td>
<td>59.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Intraassay</td>
<td>n</td>
<td>Mean (OD)</td>
<td>Cv (%)</td>
</tr>
</tbody>
</table>

Pos. Serum 23 1.95 2.0

10.2. Diagnostic Specificity

The diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of the specific analyte. It is \(>95\%\).

10.3 Diagnostic Sensitivity

The diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of the specific analyte. It is 93.3 %.

*Note:* The results refer to the groups of samples investigated; these are not guaranteed specifications.

11. LIMITATIONS OF THE PROCEDURE

Bacterial contamination or repeated freeze-thaw cycles of the specimen may affect the absorbance values. Diagnosis of an infectious disease should not be established on the basis of a single test result. A precise diagnosis should take into consideration clinical history, symptomatology as well as serological data. A negative result (IgG or IgM) cannot exclude an infection with B. burgdorferi. Especially in the early phase of infection there is the possibility that none or no detection quantities of the antibodies
do exist. In the case of infection or a grey zone result we recommend a further sample after 2-3 weeks. A positive result of IgG does not always mean that an acute infection exists because the antibodies of a previous infection can persist.

12. PRECAUTIONS AND WARNINGS

The test procedure, the information, the precautions and warnings in the instructions for use have to be strictly followed. The use of the test kits with analyzers and similar equipment has to be validated. Any change in design, composition and test procedure as well as for any use in combination with other products not approved by the manufacturer is not authorized; the user himself is responsible for such changes. The manufacturer is not liable for false results and incidents for these reasons. The manufacturer is not liable for any results by visual analysis of the patient samples.

Only for research use only.

All materials should still be regarded and handled as potentially infectious.

Do not interchange reagents or strips of different production lots.

No reagents of other manufacturers should be used along with reagents of this test kit.

Do not use reagents after the expiry date stated on the label.

Use only clean pipette tips, dispensers, and lab ware.

Do not interchange screw caps of reagent vials to avoid cross-contamination.

Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.

After first opening and subsequent storage check conjugate and control vials for microbial contamination prior to further use.

To avoid cross-contamination and falsely elevated results pipette patient samples and dispense conjugate without splashing accurately to the bottom of wells.

The GenWay ELISA is only designed for qualified personnel who are familiar with good laboratory practice.

WARNING: In the used concentration Bronidox L has hardly any toxicological risk upon contact with skin and mucous membranes!

WARNING: Sulphuric acid irritates eyes and skin. Keep out of the reach of children. Upon contact with the eyes, rinse thoroughly with water and consult a doctor!

12.1. Disposal Considerations

Residues of chemicals and preparations are generally considered as hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations. Contact your local authorities or waste management companies which will give advice on how to dispose hazardous waste.

13. ORDERING INFORMATION
**Lyme Borrelia Canine IgG-ELISA**

**Test Preparation**

Prepare reagents and samples as described. Establish the distribution and identification plan for all specimens and controls on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder.

**Assay Procedure**

<table>
<thead>
<tr>
<th>Substrate blank (e.g. A1)</th>
<th>Negative Control</th>
<th>Positive Control</th>
<th>Cut-off Control</th>
<th>Sample (diluted 1+100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>100µl ---</td>
<td>-100µl --</td>
<td>--100µl -</td>
<td>---100µl</td>
</tr>
<tr>
<td>Control Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cut-off Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Sample (diluted 1+100)</td>
<td></td>
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</tr>
</tbody>
</table>

Cover wells with foil supplied in the kit **Incubate for 1 h at 37°C** Wash each well three times with 300µl of washing solution

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>-</th>
<th>100µl</th>
<th>100µl</th>
<th>100µl</th>
<th>100µl</th>
</tr>
</thead>
</table>

Cover wells with foil supplied in the kit **Incubate for 30 min at room temperature** Wash each well three times with 300µl of washing solution

<table>
<thead>
<tr>
<th>TMB Substrate</th>
<th>100µl</th>
<th>100µl</th>
<th>100µl</th>
<th>100µl</th>
<th>100µl</th>
</tr>
</thead>
</table>

**Incubate for exactly 15 min at room temperature in the dark**

<table>
<thead>
<tr>
<th>Stop Solution</th>
<th>100µl</th>
<th>100µl</th>
<th>100µl</th>
<th>100µl</th>
<th>100µl</th>
</tr>
</thead>
</table>

Photometric measurement at 450 nm (reference wavelength: 620 nm)