Instructions for Use

Borrelia burgdorferi
Veterinary ELISA
Species B. burgdorferi sensu stricto

to determine IgG antibodies to Borrelia burgdorferi in dog or horse serum

Order No.: DC122.00
Color code: gold

The German Instructions for Use have been approved in accordance with §17c TierSG under the Registration Number FLI-B 397.

FOR IN VITRO DIAGNOSIS ONLY

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Druckdatum 03.02.2014
REV 2 / Borrelia burgdorferi Veterinär ELISA IgG GB
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1. **Intended Use**

The Virotech Borrelia burgdorferi (Species B. burgdorferi sensu stricto) Veterinary ELISA is an indirect ELISA for the semiquantitative and qualitative detection of specific IgG antibodies in dog or horse serum.

2. **Diagnostic Relevance**

**General**

The pathogen for borreliosis, the spirochaete Borrelia burgdorferi, was discovered in 1981 by Burgdorfer and Barbour and classified as a species of the genus Borrelia (1).

3 species of Borrelia burgdorferi sensu lato have now been identified: Borrelia burgdorferi sensu stricto, Borrelia garinii and Borrelia afzelii (2).

**Epidemiology:**

Borreliosis occurs endemically in all temperate zones (3). There are however differences in the relative frequency of the 3 pathogens in different regions. Thus, only B. burgdorferi sensu stricto has been observed in North America, but all three species have been found in Europe (2).

**Transmission:**

B. burgdorferi is predominantly transmitted by hard ticks of the genus Ixodes when they bite or suck blood. Ixodes scapularis is the vector known in the USA; Ixodes ricinus is the main vector in Europe (4).

**Clinical picture in the dog:**

It is generally accepted that the most characteristic symptoms of Lyme borreliosis in the dog are impaired well-being, with anorexia and fever, with fluctuating lameness and arthritis. Lameness and arthritis are really pathognomic symptoms. Aside from these symptoms, lymphadenopathies are observed in about 5% of cases and impairment of renal function in about 2% of cases. The latter may be severe (5).

**Clinical picture in the horse:**

Horses are often bitten by ticks when they are grazing on the meadow. In western and central Europe, these are almost always of the species Ixodes ricinus, which sucks the blood of horses in all three stages – the adult, the nymph and the larva (5).

In a German study (Hannover), the symptoms of the disease were determined in 50 horses. The high proportion of eye diseases - conjunctivitis, keratoconjunctivitis, retinitis - was striking; this is consistent with observations in the USA. On the other hand, the horses were most often taken to the vet because of general and less specific symptoms - such as loss of weight and energy (24%), inflamed joints (12%) and lameness (10%). Polyarthritis was often found and this can occur in almost any joint in the extremities (6).

3. **Test Principle**

The test antibodies in the animal serum form an immune complex with the antigen fixed on the microtitre plate. The antibody searched for in the human serum forms an immune complex with the antigen coated on the microtitre plate. Unbound immunoglobulins are removed by washing processes. The enzyme conjugate attaches to this complex. Unbound conjugate is again removed by washing processes. After adding the substrate solution (TMB), a blue dye is produced by the bound enzyme (peroxidase). The color changes to yellow when the stopping solution is added.

4. **Package Contents**

1. **1 Microtitre-Plate [MTP]** consisting of 96 with antigen coated, breakable single wells, lyophilised
2. **PBS-Dilution Buffer, (blue, ready to use), 2x50ml**, pH 7.2, with preservative and Tween 20
3. **PBS-Washing Solution, 50ml**, (20x concentrated), pH 7.2, with preservative and Tween 20
4. **IgG negative control dog [NEG IgG Dog], 1300µl**, dog serum with protein stabilizers and preservative, ready-to-use
5. **IgG cut-off control dog [CO IgG Dog], 1300µl**, dog serum with protein stabilizers and preservative, ready-to-use
6. **IgG positive control dog [POS IgG Dog], 1300µl**, dog serum with protein stabilizers and preservative, ready-to-use
7. **Anti-Dog-IgG conjugate, 11ml**, rabbit horseradish peroxidase conjugate, contains preservative, ready-to-use
8. **IgG negative control horse [NEG IgG Horse], 1300µl**, horse serum with protein stabilizers and preservative, ready-to-use
5. **Storage and Shelflife of the testkit and the ready to use reagents**

Store the testkit at 2-8°C. The shelf life of all components is shown on each respective label; for the kit shelf life please see Quality Control Certificate.

1. Microtiter strips/single wells are to be resealed in package after taking out single wells and stored with desiccant at 2-8°C. Reagents should immediately be returned to storage at 2-8°C after usage.
2. The ready to use conjugate and the TMB-substrate solution are sensitive to light and have to be stored in dark. Should there be a color reaction of the substrate dilution due to incidence of light, it is not useable anymore.
3. Take out only the amount of ready to use conjugate or TMB needed for the test insertion. Additional conjugate or TMB taken out may not be returned but must be dismissed.

<table>
<thead>
<tr>
<th>Material</th>
<th>Status</th>
<th>Storage</th>
<th>Shelflife</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Samples</td>
<td>Diluted</td>
<td>+2 to +8°C</td>
<td>max. 6h</td>
</tr>
<tr>
<td>Controls</td>
<td>Undiluted</td>
<td>+2 to +8°C</td>
<td>1 week</td>
</tr>
<tr>
<td>Microtiter plate</td>
<td>After Opening</td>
<td>+2 to +8°C (storage in the provided bag with desiccant bag)</td>
<td>3 months</td>
</tr>
<tr>
<td>Rheumatoid factor - Absorbent</td>
<td>Undiluted, After Opening</td>
<td>+2 to +8°C</td>
<td>3 months</td>
</tr>
<tr>
<td>Conjugate</td>
<td>Diluted</td>
<td>+2 to +8°C</td>
<td>1 week</td>
</tr>
<tr>
<td>Tetramethylbenzidine</td>
<td>After Opening</td>
<td>+2 to +8°C (protect from light)</td>
<td>3 months</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>After Opening</td>
<td>+2 to +8°C</td>
<td>3 months</td>
</tr>
<tr>
<td>Washing Solution</td>
<td>After Opening</td>
<td>+2 to +8°C</td>
<td>3 months</td>
</tr>
<tr>
<td>Final Dilution (ready-to-use)</td>
<td>After Opening</td>
<td>+2 to +25°C</td>
<td>4 weeks</td>
</tr>
</tbody>
</table>

6. **Precautions and Warnings**

1. Only sera which have been tested and found to be negative for HIV-1 antibodies, HIV-2 antibodies, HCV antibodies and Hepatitis-B surface-antigen are used as control sera. Nevertheless, samples, diluted samples controls, conjugate and microtiter strips should be treated as potentially infectious material. Please handle products in accordance with laboratory directions.
2. Those components that contain preservatives, the Citrate Stopping Solution and the TMB have an irritating effect to skin, eyes and mucous. If involved body parts are contacted, immediately wash under flowing water and possibly look up a doctor.
3. The disposal of the used materials is effected referring to the country-specific guidelines.

7. **Material required but not supplied**

1. Aqua dest./demin.
2. Eight-channel pipette 50µl, 100µl
3. Micropipettes: 10µl, 100µl, 1000µl
4. Test tubes
5. Paper towels or absorbent paper
6. Cover for ELISA-plates
7. Disposal box for infectious material
8. ELISA handwasher or automated EIA plate washing device
9. ELISA plate spectrophotometer, wavelength = 450nm, reference length = 620nm (Reference Wavelength 620-690nm)
8. Test Procedure

Working exactly referring to the Sekisui Virotech user manual is the prerequisite for obtaining correct results.

8.1 Examination Material
Sera are used as examination material.
Always prepare samples-dilution freshly.
For a longer storage the sera must be frozen. Repeated defrosting shall be avoided.

1. Only fresh non-inactivated sera should be used.
2. Hyperlipaemic, haemolytic, microbially contaminated and turbid sera should not to be used (false positive/negative results).

8.2 Preparation of Reagents
The ready-to-use controls (positive control, cut-off control, negative control) are parameter- and species-specific and are to be used exclusively with the plate batch in the kit.
The ready-to-use conjugates are also parameter- and species-specific, but may be used for different plate batches.

1. Set incubator to 37°C and check proper temperature setting before start of incubation.
2. Bring all reagents to room temperature before opening package of microtiter strips.
3. Shake all liquid components well before use.
4. Make up the washing solution concentrate to 1 L with distilled or demineralised water. If crystals have formed in the concentrate, please bring the concentrate to room temperature before use and shake well before use.

8.3 Virotech ELISA Test Procedure
1. For each test batch, pipette 100µl each of the ready-to-use dilution buffer (blank), the negative, positive and cut-off IgG controls, together with the diluted animal sera. We recommend that adjacent duplicates should be performed for the blank, controls and sera samples; it is absolutely essential that the cut-off control should be performed in duplicate.
   Working dilution of the animal sera: 1:400; e.g. predilution: Mix 10µl serum + 90µl dilution buffer (1:10); Second dilution: Take 10µl of this mixture and pipette 390µl dilution buffer into it (1:40). This corresponds to a dilution of 1:400.
2. After pipetting start incubation for 30 min. at 37°C (with cover).
3. End incubation period by washing microtiter strips 4 times with 350 µl washing solution per well. Do not leave any washing solution in the wells. Remove residues on a cellulose pad.
4. Pipette 100µl of the ready-to-use animal-specific conjugate into all wells.
5. Incubation of the conjugate: 30 min. at 37°C (with cover)
6. Stop conjugate incubation by washing 4 times (pls. refer to point 3 above).
7. Pipette 100µl of ready to use TMB into each well.
8. Incubation of substrate solution: 30 min. at 37°C (with cover, keep in dark).
9. Stopping the substrate reaction: Pipette 50µl citrate stop solution into each cavity. Carefully and thoroughly shake the plate until the fluids are well mixed and the colour in each well is homogenous.
10. Measure extinction (OD) at 450/620nm (Reference Wavelength 620-690nm). Set your photometer in such a way that the blank value is deducted from all other extinctions. Extinctions should be measured within 1 hour after adding the stopping solution!

Pls. refer to last page for Test Procedure Schema

8.4 Usage of ELISA processors
All Sekisui Virotech ELISAs can be used on ELISA processors. The user is bound to proceed a validation of the devices (processors) on a regular basis.
Sekisui Virotech recommends the following procedure:

1. Sekisui Virotech recommends to proceed the validation of device referring to the instructions of the device manufacturer during the implementation of the ELISA processor respectively after bigger reparations.
2. It is recommended to check the ELISA-processor with the Validation kit (EC250.00) afterwards. A regular check using the Validation kit shall be proceeded minimum once a quarter to test the accuracy of the processor.
3. The release criteria of the Quality Control Certificate of the product must be fulfilled for each test run. This procedure ensures the accuracy of your ELISA processor and serves additionally for the Quality Assurance of your laboratory.

9. Test Evaluation

The ready-to-use controls serve as quantification controls for the semiquantitative determination of specific IgG antibodies; the concentration can be given in Virotech Units = VE. The method can correct for fluctuations in the test procedure, leading to high reproducibility. The mean OD value is used to calculate the VE.

9.1 Test function control

a) OD-values

The OD of the blank should be < 0.15.

The OD-values of the negative controls should be lower than the OD-values mentioned in the Quality Control Certificate. The OD-values of the positive controls as well as of the cut-off controls should be above the OD-values mentioned in the Quality Control Certificate.

b) Virotech Units (VE)

The Virotech Units (VE) of the cut-off controls are defined as 10. The calculated VE of the positive controls should be within the ranges mentioned in the Quality Control Certificate. If those requirements (OD-values, VE) are not fulfilled, the test has to be repeated.

9.2 Calculation of the Virotech Units (VE)

The mean is calculated from the duplicate OD values. The extinction of the blank value (450/620nm) has to be subtracted from all other extinctions.

\[
VE_{(\text{positive control})} = \frac{OD_{(\text{positive control})}}{OD_{(\text{controls})}} \times 10
\]

\[
VE_{(\text{animal serum})} = \frac{OD_{(\text{animal serum})}}{OD_{(\text{controls})}} \times 10
\]

9.3 Interpretation Scheme IgG

<table>
<thead>
<tr>
<th>Result (VE)</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 8.0</td>
<td>negative</td>
</tr>
<tr>
<td>8.0 - 12.0</td>
<td>grey area</td>
</tr>
<tr>
<td>&gt; 12.0</td>
<td>positive</td>
</tr>
</tbody>
</table>

1. If the VE value measured for the sample is greater than 12 VE, the sample is considered to be positive.
2. If the VE value measured lies within the grey area, there is no significant increase in antibody concentration and the samples are regarded as threshold.

If an infection is to be reliably detected, the antibody concentration must be determined in two serum samples: a serum sample directly after the start of the infection and a second sample two weeks later. The antibody concentrations in the two samples must be measured in parallel, in a single test batch. A correct diagnosis cannot be made on the basis of a single serum sample.
3. If the measured values are under the defined grey area, there are no measurable antigen-specific IgG antibodies in the sample. The sample is classified as negative.

9.4 Limits of the Test
1. The interpretation of serological results shall always include the clinical picture, epidemiological data and all further available laboratory results.

10. Performance Data

10.1 Sensitivity and Specificity

IgG Detection in the Dog
In order to determine the performance of the Borrelia burgdorferi Veterinary ELISA, 351 sera were tested in the Sekisui Virotech ELISA and in the reference tests (ELISA and Western blot). The tests were performed in the laboratories of Sekisui Virotech GmbH, Rüsselsheim, and of Maastricht University.
35 sera were positive in both tests and 278 sera negative in both tests. For 6 sera, the results of the two tests did not agree. Threshold results (32) were excluded from the calculation.

The result is shown in the following table.

<table>
<thead>
<tr>
<th>Reference Test</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virotech</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>278</td>
</tr>
</tbody>
</table>

Specificity = 98%
Sensitivity = 100%

IgG Detection in the Horse
In order to determine the performance of the Borrelia burgdorferi Veterinary ELISAs, 119 sera were tested in the Sekisui Virotech ELISA and in the reference test (Western Blot). The tests were performed in the laboratories of Sekisui Virotech GmbH, Rüsselsheim.
3 sera were positive in both tests, 69 sera negative in both tests and 13 sera gave threshold values in both tests. For 3 sera, the results of the two tests did not agree. Threshold results (34) were excluded from the calculation.

The result is shown in the following table.

<table>
<thead>
<tr>
<th>Reference Test</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virotech</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>69</td>
</tr>
</tbody>
</table>

Specificity = 96%
Sensitivity* = 100%

*This is calculated as 100% sensitivity, however, this value is statistically unreliable, as only 3 positive Borrelia IgG sera were available for testing.

10.2 Cross-Reactivity
There has been much discussion about cross-reactions between B. burgdorferi and other spirochaetes, particularly Leptospira (5).
In a study of Leptospira-positive dog and horse sera, no cross-reactivity was found in the Borrelia burgdorferi Veterinary ELISA.

11. Literature
### 12. Test Procedure Scheme

#### Preparation of Dog- /Horse Serum and Washing Solution

- **Washing Solution:** Fill up concentrate to 1 liter with aqua dest./demin.

- **Samples Dilution**
  
  1:400
  
  *z.B.:*
  
  Mix 10 µl serum + 90µl dilution buffer (serum dilution buffer is ready-to-use)
  
  Mix 10µl of this mixture with 390µl dilution buffer.

---

### Testprocedure

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples Incubation</td>
<td>30 minutes at 37°C</td>
</tr>
<tr>
<td>Wash 4 times</td>
<td>100 µl Dog- /Horse Serum&lt;br&gt;blank value (Dilution Buffer) and controls</td>
</tr>
<tr>
<td>Conjugate Incubation</td>
<td>30 minutes at 37°C</td>
</tr>
<tr>
<td>Wash 4 times</td>
<td>350 µl Washing Solution&lt;br&gt;Remove Residues on a Cellulose Pad</td>
</tr>
<tr>
<td>Substrate Incubation</td>
<td>30 minutes at 37°C</td>
</tr>
<tr>
<td>Stopping</td>
<td>100 µl Conjugate IgG</td>
</tr>
<tr>
<td>Measure Extinctions</td>
<td>350 µl Washing Solution&lt;br&gt;Remove Residues on a Cellulose Pad</td>
</tr>
<tr>
<td></td>
<td>100 µl Substrate</td>
</tr>
<tr>
<td></td>
<td>50 µl Stopping Solution&lt;br&gt;shake carefully</td>
</tr>
<tr>
<td></td>
<td>Photometer at 450/620nm&lt;br&gt;(Reference Wavelength 620-690nm)</td>
</tr>
</tbody>
</table>