# Product information

Useŕs Manual

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# Leishmania ELISA

Enzyme immunoassay for the qualitative determination of antibodies against Leishmania in veterinary mammalian serum

VET

REF **DEM-DE0310**

Σ **96 wells**

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JS
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## 1. INTRODUCTION

Leishmaniasis is an infectious disease transmitted by sand flies and caused by various species of Leishmania. The parasites can infect both humans and canines, and the resulting condition is known as visceral leishmaniasis. The disease is particularly common in Mediterranean basin (e.g., Italy, Spain and Portugal), the Balkans, central and southwest Asia, north and northwest China, north and sub-Saharan Africa, and parts of Central and South America. The domestic dog seems to be the main reservoir for human visceral leishmaniasis, rendering disease control that much more vital.
In dogs clinical manifestations include chronic wasting, epistaxis, diarrhea, conjunctivitis, ocular signs (anterior uveitis, retinitis), severe muscle atrophy, swollen limbs and joints, lameness, lymphadenopathy, polyarthritis, and protein-losing nephropathy, which may lead to renal failure. Assessment of renal function in all infected dogs is critically important.
Infection may be identified by

- Microscopy
- Serology: IFA, ELISA

## 2. INTENDED USE

The Leishmania ELISA is intended for the qualitative determination of antibodies against Leishmania in veterinary mammalian serum.

## 3. PRINCIPLE OF THE ASSAY

The qualitative immunoenzymatic determination of antibodies against Leishmania is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique. Microtiter strip wells are precoated with Leishmania antigens to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled Protein A/G conjugate is added. This conjugate binds to the captured Leishmania specific antibodies. The immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine (TMB) Substrate Solution which gives a blue reaction product. The intensity of this product is proportional to the amount of Leishmania specific 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

- **Leishmania Coated Wells:** 12 breakapart 8-well snap-off strips coated with Leishmania antigen; in resealable aluminium foil.
- **Sample Diluent**:** 1 bottle containing 100 ml of buffer for sample dilution; pH 7.2 ± 0.2; coloured yellow; ready to use; white cap.
- **Stop Solution**: 1 bottle containing 15 ml sulphuric 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.
- **Leishmania Protein A/G Conjugate**:** 1 bottle containing 20 ml of peroxidase Protein A/G; coloured yellow, ready to use; white cap.
- **TMB Substrate Solution:** 1 bottle containing 15 ml 3,3',5,5'-tetramethylbenzidine (TMB); ready to use; yellow cap.
- **Leishmania Positive Control**:** 1 bottle containing 2 ml; coloured yellow; ready to use; red cap.
- **Leishmania Cut-off Control**:** 1 bottle containing 3 ml; coloured yellow; ready to use; green cap.
- **Leishmania Negative Control**:** 1 bottle containing 2 ml; coloured yellow; ready to use; blue cap.

* contains 0.1 % Bronidox L after dilution

** contains 0.1 % Kathon

### 4.2. Materials supplied

- 1 Strip holder
- 1 Cover foil
- 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. Coated snap-off Strips
The ready to use breakapart snap-off strips are coated with Leishmania antigen. Store at 2...8 °C. Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2...8 °C; stability until expiry date.

### 6.2. Leishmania Protein A/G Conjugate
The bottle contains 20 ml of a solution with Protein A/G, horseradish peroxidase, buffer, stabilizers, preservatives and an inert yellow dye. The solution is ready to use. Store at 2...8 °C. After first opening stability until expiry date when stored at 2…8 °C.

### 6.3. Controls
The bottles labelled with Positive, Cut-off and Negative Control contain 2 resp. 3 ml of a ready to use control solution. It contains 0.1 % Kathon and has to be stored at 2...8 °C. After first opening stability until expiry date when stored at 2…8 °C.

### 6.4. 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 expiry date when stored at 2…8 °C.

### 6.5. Washing Solution (20xconc.)
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 will keep 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 colourless 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 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 expiry date.

## 7. SPECIMEN COLLECTION AND PREPARATION

Use veterinary mammalian serum samples with this assay. 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 Sample Diluent. Dispense 10 µl sample and 1 ml 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. The following test procedure is only validated for manual procedure. 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 and |
| 1 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 100µl 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 1 hour ± 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.
5. Dispense 100 µl Leishmania Protein A/G Conjugate into all wells except for the blank well (e.g. A1). Cover with foil.
6. **Incubate for 30 min at room temperature.** Do not expose to direct sunlight.
7. Repeat step 4.
8. Dispense 100 µl TMB Substrate Solution into all wells
9. **Incubate for exactly 15 min at room temperature in the dark.**
10. 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 colour developed during the incubation turns into yellow.
    Note: Highly positive patient samples can cause dark precipitates of the chromogen! These precipitates have an influence when reading the optical density. Predilution of the sample with physiological sodium chloride solution, for example 1+1, is recommended. Then dilute the sample 1+100 with dilution buffer and multiply the results in OD by 2.
11. Measure the absorbance of the specimen at 450/620 nm within 30 min after addition of the Stop Solution or read results visually.

### 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 < cut-off**
- **Cut-off control** in C1 and D1: Absorbance value **0.150 – 1.30.**
- **Positive control** in E1: Absorbance value **> 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 is the mean absorbance value of the Cut-off control determinations.
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 Units

$$\frac{\text{Patient (mean) absorbance value} \times 10}{\text{Cut-off}} = [\text{Units} = \text{U}]$$

Example: $\frac{1.216 \times 10}{0.38} = 32$ U (Units)

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

## 10. SPECIFIC PERFORMANCE CHARACTERISTICS

The performance data have been established with samples of selected veterinary mammalian species. Due to the nature of the Protein A/G conjugate this ELISA should react with other veterinary species also. More detailed information is available on request.

### 10.1. Precision

| **Interassay** | **n** | **Mean** | **Cv (%)** |
|---|---|---|---|
| Pos. Serum | 4 | 0.735 | 5.9 |
| **Intraassay** | **n** | **Mean** | **Cv (%)** |
| Pos. Serum | 7 | 1.609 | 7.4 |

### 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 > 98 %.

### 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 > 98 %.

### 10.4. Interferences

Interferences with hemolytic, lipemic or icteric sera are not observed up to a concentration of 10 mg/ml hemoglobin, 5 mg/ml triglycerides and 0.2 mg/ml bilirubin.
**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 cross reaction with antibodies against Trypanosoma cruzi (Chagas) cannot be excluded.

## 12. PRECAUTIONS AND WARNINGS

- All materials should be regarded and handled as potentially infectious.
- All components of human origin used for the production of these reagents have been tested for anti-HIV antibodies, anti-HCV antibodies and HBsAg and have been found to be non-reactive. 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 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 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.

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