

Please use only the valid version of the package insert provided with the kit.

INTRODUCTION

Leishmania are protozoa belonging to the family trypanosomatidae. The parasites exist in two forms: the promastigotes in the midgut of the vector insect, and the amastigotes within the phagolysosomes of macrophages in their mammalian hosts. In the macrophages they live as round, non-motile amastigotes (3-7 mm in diameter). The macrophages are ingested by the sandfly during blood-meal and the amastigotes are released into their stomach. Almost immediately the amastigotes transform in to the motile, elongated (10-20µm), flagellate promastigote form, which migrate to the alimentary tract of the fly and after multiplication move forward to the salivary glands of the insect. Leishmaniasis are a globally widespread group of parasitic diseases; the “type” is determined by the primary location of the macrophages that are infected. In humans four different forms of Leishmaniasis with a broad range of clinical manifestations are present; all can have devastating consequences. Leishmaniasis currently affects some 12 million people in 88 countries, all but 16 of which are in the developing world. It is estimated that 350 million people are exposed to the risk of infection by the different species of Leishmania parasite; the annual incidence of new cases is about 2 million (1-1.5 million cases of CL, 500,000 cases of VL). Visceral leishmaniasis (VL) is the most severe form of the disease, which, if untreated, has a mortality rate of almost 100%. Like many other tropical diseases, the leishmaniasis are related to economic development and man-made environmental changes, which increase exposure to the sandfly vector. The geographical distribution is limited by the distribution of the sandfly. AIDS and other immunosuppressive conditions increase the risk of Leishmania infected people developing visceral illness (VL). Leishmania/HIV co-infections are considered to be a real “emerging disease”, especially in south-western Europe, where 25-70% of adult VL cases are related to HIV infection, and 1.5-9.5% of AIDS cases suffer from newly acquired or reactivated VL. Intravenous drug users have been identified as the main population at risk.

Species	Disease	Symptoms	Mechanism of Infection
Leishmania infantum Leishmania donovani	Visceral leishmaniasis (VL) or kala-azar	Irregular bouts of fever, substantial weight loss, swelling of spleen and liver and anaemia	Transmission (along with the saliva) by the bite of infected female sandflies of the genus Phlebotomus in the Old World and Lutzomyia in the New World. The insects are 2 to 3 mm long and are found throughout the tropical and temperate parts of the world. Transmission primarily zoonotic, but anthroponotic forms exist.
Leishmania major	Cutaneous leishmaniasis (CL)	self-limiting skin lesions	
Leishmania brasiliensis	mucocutaneous leishmaniasis (MCL)	Skin ulcers, which can spread and cause hideous tissue destructions	
Leishmania mexicana	diffuse cutaneous leishmaniasis (DCL)	Disseminated and chronic lesions which resemble those of lepromatous leprosy	

Infection may be identified by

- Microscopy
- Serology: IFA, ELISA

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USA: **INTENDED USE**

The DRG Leishmania ELISA is intended for the qualitative determination of antibodies against *Leishmania infantum* in human serum or plasma (citrate).

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 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 alternative 450 nm is read using an ELISA microwell plate reader.

MATERIALS**Reagents supplied**

- **Leishmania infantum Coated Wells:** 12 breakapart 8-well snap-off strips coated with *Leishmania infantum* antigen; in resealable aluminium foil.
- **IgG 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 Conjugate**:** 1 bottle containing 20 ml of peroxidase Protein A; coloured 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.
- **Leishmania infantum Positive Control***:** 1 bottle containing 2 ml; coloured yellow; ready to use; red cap.
- **Leishmania infantum Cut-off Control***:** 1 bottle containing 3 ml; coloured yellow; ready to use; green cap.
- **Leishmania infantum Negative Control***:** 1 bottle containing 2 ml; coloured yellow; ready to use; blue cap.

* contains 0.1 % Bronidox L after dilution

** contains 0.2 % Bronidox L

*** contains 0.1 % Kathon

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USA: **Materials supplied**

- 1 Strip holder
- 1 Cover foil
- 1 Test protocol
- 1 distribution and identification plan

Materials and Equipment needed

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450/620nm
- 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

STABILITY AND STORAGE

The reagents are stable up to the expiry date stated on the label when stored at 2°C - 8°C.

REAGENT PREPARATION

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

Coated snap-off Strips

The ready to use breakapart snap-off strips are coated with Leishmania antigen. Store at 2°C - 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°C - 8°C; stability until expiry date.*

Leishmania Protein A Conjugate

The bottle contains 20 ml of a solution with Protein A, horseradish peroxidase, buffer, stabilizers, preservatives and an inert blue dye. Protein A is an immunoglobulin Fc-binding protein with a molecular weight of 42,000 Daltons. The solution is ready to use. Store at 2°C - 8°C. *After first opening stability until expiry date when stored at 2°C - 8°C.*

Controls

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

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USA: **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°C - 8°C. *After first opening stability until expiry date when stored at 2°C - 8°C.*

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*

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°C - 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°C - 8°C.*

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°C - 8°C. *After first opening stability until expiry date.*

SPECIMEN COLLECTION AND PREPARATION

Use human serum or plasma (citrate) samples with this assay.

If the assay is performed within 5 days after sample collection, the specimen should be kept at 2°C - 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. *Do not heat inactivate the serum and avoid repeated freezing and thawing.*

Heat inactivation of samples is not recommended.

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.

ASSAY PROCEDURE**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

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automatic systems we recommend to increase the washing steps from three to five and the volume of washing solution from 300µl to 350µl 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 300µl of Washing Solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be >5sec. 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 100µl Leishmania Protein A 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 100µl TMB Substrate Solution into all wells
- 9. Incubate for exactly 15 min at room temperature in the dark.**
10. Dispense 100µl 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 450/620nm within 30 min after addition of the Stop Solution or read results visually.

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.

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Dual wavelength reading using 620 nm as reference wavelength is recommended.
Where applicable calculate the **mean absorbance values** of all duplicates.

RESULTS**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.

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

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.

Results in DRG Units

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

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

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

SPECIFIC PERFORMANCE CHARACTERISTICS**Precision**

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

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 85 %.

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 91 %.

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.*

IMITATIONS 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.

In immunocompromised patients and newborns serological data only have restricted value.

A cross reaction with antibodies against Trypanosoma cruzi (Chagas) cannot be excluded.

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USA: **PRECAUTIONS AND WARNINGS**

- In compliance with article 1 paragraph 2b European directive 98/79/EC the use of the in vitro diagnostic medical devices is intended by the manufacturer to secure suitability, performances and safety of the product. Therefore the test procedure, the information, the precautions and warnings in the instructions for use have to be strictly followed. The use of the testkits 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 in-vitro use.
- 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. Nevertheless, 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 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!

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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USA: **BIBLIOGRAPHY**

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SCHEME OF THE ASSAY

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

	Substrate blank (e.g. A1)	Negative control	Positive control	Cut-off control	Sample (diluted 1+100)
Negative control	-	100 µl	-	-	-
Positive control	-	-	100 µl	-	-
Cut-off control	-	-	-	100 µl	-
Sample (diluted 1+100)	-	-	-	-	100 µl
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					
Conjugate	-	100 µl	100 µl	100 µl	100 µl
Cover wells with foil supplied in the kit Incubate for 30 min at room temperature Wash each well three times with 300 µl of washing solution					
TMB Substrate	100 µl	100 µl	100 µl	100 µl	100 µl
Incubate for exactly 15 min at room temperature in the dark					
Stop Solution	100 µl	100 µl	100 µl	100 µl	100 µl
Photometric measurement at 450 nm (reference wavelength: 620 nm)					

Symbols used with DRG Assays

Symbol	English	Deutsch	Français	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konformitätskennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeitsdatum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità
MTP	Microplate	Mikrotiterplatte	Microplaque	Microplaca	Micropiastra
CONJ	Conjugate	Konjugat	Conjugué	Conjugado	Coniugato
CONTROL -	Control serum, negative	Kontrollserum, negative	Sérum de contrôle négatif	Suero control negativo	siero di controllo, negativo
CONTROL +	Control serum, positive	Kontrollserum, positiv	Sérum de contrôle positif	Suero de control positivo	siero di controllo, positivo
CUT OFF	Cut off control serum	Cut off Kontrollserum	Sérum de contrôle du cut-off	Suero control Cut-off	siero di controllo, cut-off
DIL G	Sample diluent buffer IgG	IgG-Probenverdünnungspuffer	Tampon diluant pour échantillon IgG	soluzione solución tampón para muestras IgG	tampone per i campioni IgG
SOLN STOP	Stop solution	Stopplösung	Solution d'arrêt	Solución de parada	Soluzione bloccante
SUB TMB	TMB Substrate solution	TMB-Substratlösung	Substrat TMB	solción substrato TMB	soluzione substrato TMB
WASH BUF 20x	Washing solution 20x concentrated	Waschlösung 20x konzentriert	Solution de lavage concentré 20 x	solución de lavado concentrado x20	soluzione di lavaggio concentrazione x20