Leishmania spp. Real-TM

Handbook

Real Time PCR kit for qualitative detection of *Leishmania spp.*

REF N3-50FRT

REF TN3-50FRT

Σ 50

NAME

Leishmania spp. Real-TM

INTRODUCTION

Leishmaniasis is a parasitic disease that is found in parts of the tropics, subtropics, and southern Europe. It is classified as a Neglected Tropical Disease (NTD). Leishmaniasis is caused by infection with *Leishmania* parasites, which are spread by the bite of phlebotomine sand flies. There are several different forms of leishmaniasis in people. The most common forms are **cutaneous leishmaniasis**, which causes skin sores, and **visceral leishmaniasis**, which affects several internal organs (usually spleen, liver, and bone marrow).

Overall, infection in people is caused by more than 20 species (types) of *Leishmania* parasites, which are spread by about 30 species of phlebotomine sand flies.

The number of new cases per year is not known with certainty. For **cutaneous leishmaniasis**, estimates of the number of cases range from approximately 0.7 million to 1.2 million. For **visceral leishmaniasis**, estimates of the number of cases range from approximately 0.2 million to 0.4 million.

INTENDED USE

Kit **Leishmania spp. Real-TM** is a test for the qualitative detection of *Leishmania spp.* in the tissue specimens such as from skin sores (for cutaneous leishmaniasis) or from bone marrow (for visceral leishmaniasis).

PRINCIPLE OF ASSAY

Kit **Leishmania spp. Real-TM** is based on two major processes: isolation of DNA from specimens and Real Time amplification. *Leishmania spp.* DNA is extracted from the specimens, amplified using Real-Time amplification and detected by fluorescent reporter dye probes specific for L*eishmania spp.* DNA and Internal Control. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition. IC is detected in a channel other than the *Leishmania spp*.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (N3-50FRT)

Part N° 2 –" **Leishmania spp. Real-TM":** Real Time amplification

- **PCR-mix-1-FRT**, 0,6 ml;
- **PCR-Buffer-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **Pos Leishmania spp. C+**, 0,2 ml;
- **Negative Control C-**, 1,2 ml;*
- **Internal Control IC**, 1,0 ml;**
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TN3-50FRT)

Part N° 1 – **"DNA/RNA Prep":** Sample preparation

- **Lysis Sol**, 15 ml;
- **Prec Sol**, 20 ml;
- **Washing Sol 3**, 25,0 ml;
- **Washing Sol 4**, 10,0 ml
- **RE-buffer**, 4 x 1,2 ml;

Contains reagents for 50 extractions

Part N° 2 –" **Leishmania spp. Real-TM":** Real Time amplification

- **PCR-mix-1-FRT**, 0,6 ml;
- **PCR-Buffer-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **Pos Leishmania spp. C+**, 0,2 ml;
- **Negative Control C-**, 1,2 ml;*
- **Internal Control IC**, 1,0 ml;**
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

**must be used in the isolation procedure as Negative Control of Extraction.*
***add 10 µl of Internal Control during the DNA isolation directly to the sample/lysis mixture (see DNA/RNA Prep REF K-2-9 protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator, freezer

Zone 2: Real Time amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

Leishmania spp. Real-TM must be stored at -20°C. DNA/RNA Prep must be stored at+2-8°C. The kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt. Store **DNA/RNA Prep** kit at 2-8°C.

STABILITY

Leishmania spp. Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

-  Component Lysis Sol contains guanidine thiocyanate. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/38; S: 36/37/39). **Risk Phrases:**R 20/21/22 Harmful by inhalation, in contact with the skin and if swallowed, R 22 Harmful if swallowed, R 36/38 Irritating to eyes and skin. **Safety Phrases:** S 13 Keep away from food, drink and animal feedstuffs
-  Component Prec Sol contains 2-propanol: flammable. Irritant. (R10-36-67, S7-16-24/25-26). **Risk Phrases:** R10: Flammable, R36/37/38: Irritating to eyes, respiratory system and skin, R67: Vapors may cause drowsiness and dizziness. **Safety Phrases:** S7: Keep container tightly closed, S16: Keep away from sources of ignition - No smoking, S24/25: Avoid contact with skin and eyes, S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice;
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.

- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Leishmania spp. Real-TM can analyze DNA extracted from:

- *tissue specimens* from skin sores (for cutaneous leishmaniasis);
- *tissue specimens* from bone marrow (for visceral leishmaniasis).

Specimens can be stored at +2-8°C for no longer than 48 hours, or frozen at -20°C to -80°C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

The following kit is recommended:

⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);

⇨ **SaMag Tissue DNA Extraction kit** (Sacace, REF SM004).

Please carry out DNA extraction according to the manufacture's instruction. Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION

1. Prepare required number of 1.5 ml disposable polypropylene micro centrifuge tubes including one tube for Negative Control of Extraction (**Negative Control**, **C-**).
2. Add to each tube **10 µl** of **IC** (Internal Control) and **300 µl** of **Lysis Sol**
3. Add **100 µl** of samples to the appropriate tubes using pipette tips with aerosol barriers.
4. Prepare Controls as follows:
    - add **100 µl** of **Negative Control C–** to the tube labeled C*neg*
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 7-10 sec.
6. Add **400 µl** of **Prec Sol** and mix by vortex. Centrifuge all tubes at 13,000 r/min for 5 min and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
7. Add **500 µl of Wash Sol 3** into each tube. Vortex vigorously to ensure pellet washing. Centrifuge all tubes at 13,000 r/min for 60 sec and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
8. Add **200 µl of Wash Sol 4** into each tube. Vortex vigorously to ensure pellet washing. Centrifuge all tubes at 13,000 r/min for 60 sec and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
9. Incubate all tubes with open caps at **65 °C for 5 min**.
10. Resuspend the pellet in **50 µl of RE-buffer** (elution volume can be increased up to 90 µl). Incubate for 5 min at 65°C and vortex periodically.
11. Centrifuge the tubes at 13000g for 60 sec.

The supernatant contains RNA/DNA ready for amplification. If amplification is not performed the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at - 20°/-80°C.

PROTOCOL:

1. Prepare required quantity of reaction tubes for samples (N) and controls (N+2).
2. Prepare in the new sterile tube for each sample **10*(N+1) µl** of **PCR-mix-1-FRT**, **5,0*(N+1)** of **PCR-Buffer-FRT** and **0,5*(N+1)** of **TaqF DNA Polymerase.** Vortex and centrifuge for 2-3 sec.
3. Add to each tube **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. Mix by pipetting.
4. Prepare for each panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **Positive Leishmania spp. C+** to the tube labeled Amplification Positive Control;
5. Insert the tubes in the thermalcycler.

Amplification

Create a temperature profile on your instrument as follows:

Step	*Temperature, °C*	*Time*	Cycles
1	**95**	15 min	1
2	**95**	10 s	45
	60	25 s *fluorescent signal detection*	
	72	10 s	

For example SaCycler-96™ (Sacace,) Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green and JOE/Yellow/Hex/Cy3 fluorescence channels.

Leishmania spp. is detected on the JOE(Yellow)/HEX/Cy3 channel, *IC DNA* on the FAM (Green) channel

NSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 3000/6000, RotorGene Q)

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	0.03	10 %	on
JOE/Yellow	*from 4 Fl to 8 Fl*	0.05	10 %	on

Plate- or modular type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

DATA ANALYSIS

The fluorescent signal intensity is detected in two channels

- The signal from the Leishmania spp. DNA amplification product is detected in the JOE(Yellow)/HEX/Cy3 channel;
- The signal from the Internal Control amplification product is detected in the FAM (Green) channel.

Interpretation of results

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line. To set threshold put the line at such level where curves of fluorescence are linear. Results are accepted as relevant if positive and negative controls of amplification and extraction are passed.

Results for controls

Control	Stage for control	Ct FAM (Green)	Ct JOE(Yellow)/HEX/Cy3	Interpretation
NCE	DNA isolation	POS	NEG	Valid result
NCA	Amplification	NEG	NEG	Valid result
C+	Amplification	POS	POS	Valid result

- Leishmania spp. DNA is **detected** in a sample if its Ct value is defined in the results grid in the JOE(Yellow)/HEX/Cy3 channel.
- Leishmania spp. DNA is **not detected** in a sample if its Ct value is not defined in the results grid in the JOE(Yellow)/HEX/Cy3 (the fluorescence curve does not cross the threshold line) whereas the Ct value in the in the results grid is defined.
- The result of analysis is **invalid** if the Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) in the FAM/Green channel. In this case, PCR should be repeated starting from the DNA extraction.

Boundary value of the cycle threshold, Ct

Sample	Channel for fluorophore	Ct boundary value	
		Rotor-type instruments	Plate-type instruments
C+	FAM/Green	30	30
	JOE/Yellow/Hex/Cy3	30	30
Clinical samples, C-	FAM/Green	30	30

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

SPECIFICATIONS

Analytical sensitivity and reproducibility.

The analytical sensitivity of the **Leishmania spp. Real-TM** kit was determined using the Standard DNA of the Leishmania spp. This Standard was serially diluted in the DNA-buffer.

The analytical sensitivity of the kit **Leishmania spp. Real-TM** was not less than 1000 copies/ml.

TROUBLESHOOTING

1. Weak or no signal of the IC (FAM (Green) for the Negative Control of extraction.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. JOE(Yellow)/HEX/Cy3 signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
	Expiration Date	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
IC	Internal Control	**RUO**	For Research Use Only

* SaCycler™ is a registered trademark of Sacace Biotechnologies
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* Rotor-Gene™ is a registered trademark of Qiagen
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* SmartCycler® is a registered trademark of Cepheid

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