Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

Leptospira 16s RNA Real-TM

Handbook

Real Time Kit for the qualitative detection of Leptospira 16s RNA

REF B49-50FRT

REF TB49-50FRT

50

NAME

Leptospira 16s RNA Real-TM

INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by pathogenic species of the genus ***Leptospira.*** In 90% of cases, leptospirosis manifests as an acute febrile illness with a biphasic course and an excellent prognosis. Nonspecific signs and symptoms of leptospirosis (eg, fever, headache, nausea, vomiting) are often confused with viral illness. In 10% of cases, the presentation is more dramatic, and the infection has a mortality rate of 10%. Known as Weil disease or icteric leptospirosis, the classic definition of this forms of leptospirosis includes fever, jaundice, renal failure, and hemorrhage. Other organ systems (ie, pulmonary system, cardiac system, CNS) are also frequently involved.

INTENDED USE

The **Leptospira 16s RNA Real-TM** is a Real-Time test for the qualitative detection of *Leptospira 16s RNA* in the biological materials. Test contains an IC which serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition.

PRINCIPLE OF ASSAY

Leptospira 16s RNA Real-TM Test is based on three major processes: isolation of RNA from specimens, one-step reverse transcription of the RNA and Real Time amplification of the cDNA. *Leptospira* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific primers and detection via fluorescent dyes. These dyes are linked with probes of oligonucleotides which bind specifically to the amplified product. The real-time PCR monitoring of fluorescence intensities allows the accumulating product detection without reopening of reaction tubes after the PCR run. **Leptospira 16s RNA Real-TM** PCR kit is a qualitative test which contains the Internal Control (IC). It must be used in the isolation procedure in order to control the process of each individual sample extraction and serves also to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B49-50FRT)

Part N° 2 – **"Controls":** Controls kit

- **Internal Control IC***, 5 x 0,12 ml;
- ***Leptospira 16s RNA* C+ rec****, 5 x 0,03 ml;
- **Negative Control C-*****, 1,2 ml;

Part N° 3 – **"Leptospira 16s RNA Real-TM":** Real Time amplification kit

- **RT-G-mix-2**, 2 x 0,01 ml;
- **RT-PCR-mix-1-TM Leptospira**, 0,6 ml;
- **RT-PCR-mix-2-TM**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **M-MLV Revertase**, 0,015 ml;
- ***Leptospira* cDNA C+**, 0,1 ml;
- **RT-eluent**, 2 x 0,07 ml

Contains reagents for 50 tests.

* *add 10 µl of Internal Control RNA during the RNA purification procedure directly to the sample/lysis mixture of all samples,Positive and Negative extraction controls.*
** *must be used in the isolation procedure as Positive Control of Extraction.*
*** *must be used in the isolation procedure as Negative Control of Extraction.*

Module No.2: Complete Real Time PCR test with RNA purification kit (TB49-50FRT)

Part N°1 – **Ribo-Sorb:** Sample preparation kit

- **Lysis Solution**, 22,5 ml;
- **Washing Solution**, 20,0 ml;
- **Sorbent**, 1,25 ml;
- **RNA-eluent**, 5 x 0,5 ml;
- Contains reagents for 50 tests.

Part N° 2 – **"Controls":** Controls kit

- **Internal Control IC***, 5 x 0,12 ml;
- ***Leptospira 16s RNA* C+ rec****, 5 x 0,03 ml;
- **Negative Control C-*****, 1,2 ml;

Part N° 3 – **"Leptospira 16s RNA Real-TM":** Real Time amplification kit

- **RT-G-mix-2**, 2 x 0,01 ml;
- **RT-PCR-mix-1-TM Leptospira**, 0,6 ml;
- **RT-PCR-mix-2-TM**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **M-MLV Revertase**, 0,015 ml;
- ***Leptospira* cDNA C+**, 0,1 ml;
- **RT-eluent**, 2 x 0,07 ml

Contains reagents for 50 tests.

* *add 10 µl of Internal Control RNA during the RNA purification procedure directly to the sample/lysis mixture of all samples,Positive and Negative extraction controls.*
** *must be used in the isolation procedure as Positive Control of Extraction.*
*** *must be used in the isolation procedure as Negative Control of Extraction.*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- RNA extraction kit (Module No. 1)
- Biosafety cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- 60°C ± 2°C dry heat block
- Vortex mixer
- Pipettors (capacity 5-40 µl; 40-200 µl; 200-1000 µl) with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Disposable gloves, powderless
- Tube racks
- 70% Ethanol (freshly prepared mixture of reagent grade 96% ethanol and distilled water)
- Acetone
- Refrigerator
- Freezer

Zone 2: RT and amplification:

- Real Time Thermalcycler
- Workstation
- Pipettors (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks

STORAGE INSTRUCTIONS

Ribo-Sorb **and** Controls **must be stored at 2-8°C. Leptospira 16s RNA Real-TM** must be stored at– 20°C. The kits can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

Leptospira 16s RNA Real-TM Test 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

IVD **_In Vitro_ Diagnostic Medical Device**

For _In Vitro_ Diagnostic Use Only

The user should always pay attention to the following:

- ⚠ Lysis Solution 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: 20/21/22-36/37/38; S: 36/37/39).
- 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

Leptospira 16s RNA Real-TM can analyze RNA extracted from:

- *whole blood* collected in either ACD or EDTA tubes;
- *liquor;*
- *urine (sediment)*;
- *tissue* homogenized with mechanical homogenizer and dissolved in PBS sterile;

It is recommended to process samples immediately after collection. Store samples at 2–8 °C for no longer than 24 hours, or freeze at –20/80°C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

⇒ **Ribo-Sorb-50** (Sacace, REF K-2-1): sample volume 100 µl

⇒ **Ribo Virus 50**– spin column extraction kit (Sacace, REF K-2-C): sample volume 150 µl

Please carry out the RNA extraction according to the manufacturer’s instructions. Add 10 µl of Internal Control during the RNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction** and one tube for **Positive Control of Extraction**.
2. Add to each tube **450 µl Lysis Solution** and **10 µl IC**.
3. Add **100 µl** of samples to the appropriate tube containing Lysis Solution and IC. Vortex vigorously and incubate 5 min at room temperature.
4. Prepare Controls as follows:
   - add **100 µl** of **Negative Control C–** to the tube labeled *Cneg* and *Cpos*.
   - add **10 µl** of ***Leptospira 16s RNA* C+ rec** to the tube labeled *Cpos*
5. Vortex the tubes and centrifuge for 5 sec at 5000g. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 1 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for RNA/DNA extraction
6. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
8. Centrifuge all tubes for 1 min at 10000g 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 tubes.
9. Add **400 µl** of **Washing Solution** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g. 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 tubes.
10. Add **500 µl** of **Etanolo al 70%** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g. 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 tubes.
11. Repeat step 10.
12. Add **400 µl** of **Acetone** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g 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 tubes.
13. Incubate all tubes with open cap for 10 min at 60°C.
14. Resuspend the pellet in **40 µl** of **RNA-eluent.** Incubate for 10 min at 60°C and vortex periodically. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains RNA ready for use.

RT AND AMPLIFICATION

1. Prepare Reaction mix by adding for each sample **10 µl** of **RT-PCR-mix-1-TM**, **5 µl** of **RT-PCR-mix-2-TM**, **0,5 µl** of **TaqF Polymerase 0,25 µl** of **M-MLV Revertase** and **0,25 µl** of **RT-G-mix-2**. Prepare required quantity of reaction tubes for samples and controls and add for each tube **15,0 µl** of **Prepared Mix**.
2. Add **10 µl** of **extracted RNA** sample to appropriate tube.

(**Re-centrifuge all the tubes with extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction!).*

3. Prepare for each panel 2 controls:
   - add **10 µl** of **RT-eluent** to the tube labeled Amplification Negative Control;
   - add **10 µl** of ***Leptospira* cDNA C+** to the tube labeled *C+*;

Create a temperature profile on your Real-time instrument as follows:

	***Rotor type instruments*[1]**				***Plate type or modular instruments*[2]**			
Stage	***Temp, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***	***Temp, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***
Hold	50	30 min	–	1	50	30 min	–	1
Hold	95	15 min	–	1	95	15 min	–	1
Cycling	95	20 s	–	10	95	20 s	–	10
	65	50 s	–		65	50 s	–	
	72	20 s	–		72	20 s	–	
Cycling 2	95	20 s	–	38	95	20 s	–	40
	61	50 s	FAM(Green), JOE(Yellow)		61	60 s	FAM, JOE/HEX/Cy3	
	72	20 s	–		72	20 s	–	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)
[2] For example, *SaCycler-96™ (Sacace),* iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7300/7500/StepOne Real Time PCR (Applera), SmartCycler® (Cepheid).

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

Leptospira 16s RNA is detected on the JOE(Yellow)/HEX/Cy3, *IC DNA* on the channel FAM (Green) channel

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 3 Fl to 7 Fl*	*0.03*	*10 %*	*On*
JOE/Yellow	*from 10 Fl to 20 Fl*	*0.03*	*10 %*	*On*

Plate-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.

RESULTS ANALYSIS

The result of analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Positive and Negative Control of extraction are correct (Table 1).

Table 1. Results for controls

Control	Stage for control	Ct channel Fam	Ct channel Joe	Interpretation
PCE	RNA isolation	POS	POS	Valid result
NCE	RNA isolation	POS	NEG	Valid result
NCA	Amplification	NEG	NEG	Valid result
C+	Amplification	NEG	POS	Valid result

Boundary Ct values for control and test samples for Rotor-type instruments

Control/Sample	Boundary *Ct* value
PCE	26
C+	25
All other test samples	32
Blood sediment and cerebrospinal fluid	24.5
Tissues homogenates	26.5
Urine sediment	27

Boundary Ct values for control and test samples for Plate-type instruments

Control/Sample	Boundary *Ct* value
PCE	27
C+	26
All other test samples	32
Blood sediment and cerebrospinal fluid	27
Tissues homogenates	27
Urine sediment	29

PERFORMANCE CHARACTERISTICS

The kit **Leptospira 16s RNA Real-TM** allows to detect Leptospira 16s RNA in 100% of the tests with a sensitivity of not less than 5000 copies/ml.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam (Green) channel): retesting of the sample is required.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended RNA extraction method and follow the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting the extracted RNA 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 for the IC detection select the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the RNA extraction procedure.
2. Weak signal on the Joe (Yellow)/Cy3/HEX channel: retesting of the sample is required.
3. Joe (Yellow)/Cy3/HEX signal with Negative Control of extraction.
    - Contamination during RNA 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 among tubes.
        - ⇒ Repeat the RNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
    - 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 controls at the end.
        - ⇒ 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
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
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* Rotor-Gene™ Technology is a registered trademark of Qiagen
* MX3000P® is a registered trademark of Agilent Technologies
*ABI® is a registered trademark of Applied Biosystems
* SmartCycler® is a registered trademark of Cepheid

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