IVD For *in Vitro* Diagnostic Use

CE

Rickettsia conorii Real-TM

Handbook

Real Time PCR Kit for qualitative detection of *Rickettsia conorii* in biological materials

REF H2741-50FRT

Σ **50**

NAME

Rickettsia conorii Real-TM

INTRODUCTION

Rickettsia conorii is a Gram-negative, obligate intracellular bacterium of the genus *Rickettsia* that causes human disease called Boutonneuse fever, Mediterranean spotted fever, or other names. It is a member of the spotted fever group, localized in most of the regions bordering on the Mediterranean Sea and Black Sea, Israel, Kenya, and other parts of North, Central, and South Africa, as well as India. The prevailing vector is the brown dog tick, *Rhipicephalus sanguineus.*

INTENDED USE

Kit **Rickettsia conorii Real-TM** is an *in vitro* nucleic acid amplification test for qualitative detection and identification of *Rickettsia conorii* in the biological materials (blood, eschar swabs, cerebrospinal fluid, ticks, tissue: autopsy, biopsy material).

PRINCIPLE OF ASSAY

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

MATERIALS PROVIDED

Module No.1: Real Time PCR test (H2741-50FRT)

Part N° 1 - "**Rickettsia conorii Real-TM**": Real Time amplification kit

- **PCR-mix-1-*Rickettsia conorii***, 0,6 ml;
- **PCR-Buffer-FRT**, 0,3 ml;
- **Rickettsia conorii C+***, 0,2 ml;
- **Negative Control C-****, 1,2 ml;
- **Internal Control IC*****, 0,5 ml
- **DNA-buffer******, 0,2 ml;

Contains reagents for 55 tests.

* *Rickettsia conorii C+ plasmid DNA must be used as Positive Amplification Control (see PCR PROTOCOL);*

** *must be used in the isolation procedure as Negative Control of Extraction (see SPECIMEN AND REAGENT PREPARATION and PCR PROTOCOL);*

*** *add 10 µl of Internal Control during the DNA isolation directly to the sample/lysis mixture (see SPECIMEN AND REAGENT PREPARATION);*

**** *DNA Buffer negative reagent must be used as Negative Amplification Control (see PCR PROTOCOL).*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Vortex
- 65°C ± 2°C dry heat block
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g)
- Tube racks
- Microcentrifuge tubes, 1,5 - 2,0 ml
- Pipettes with sterile, RNase-free filters tips
- Biohazard waste container
- Disposable gloves, powderless
- Refrigerator, Freezer

Zone 2: Real Time amplification:

- Real Time Thermalcycler
- Tubes or PCR plate
- Workstation
- Pipettes with sterile, RNase-free filters tips
- Tube racks

STORAGE INSTRUCTIONS

Rickettsia conorii Real-TM must be stored at **2-8°C** except the reagents **PCR-mix-1-*Rickettsia conorii*** and **PCR-Buffer-FRT** that must be stored at **-20°C**. The kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

Rickettsia conorii 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

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- 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.

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

Rickettsia conorii Real-TM can analyze extracted DNA from:

- *Liquor (CSF)* stored in "Eppendorf" tube*;*
- *Blood*;
- *Tissue (autopsy, biopsy) material;*
- *Ticks;*
- *Eschar swabs.*

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.

DNA 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 kit:

- ⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006) for cell free body fluids.

Please carry out DNA extraction according to the manufacture's instruction.

DNA is extracted from each clinical sample in the presence of Internal Control (10 µl of IC is added to each sample).

SPECIMEN AND REAGENT PREPARATION

DNA/RNA Prep (Sacace, REF K-2-9) extraction protocol (reagent provided separately):

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 **300 µl** of **Lysis Sol** and **10 µl** of **Internal Control.**
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.

PCR PROTOCOL (Reaction volume 25 µl):

Total reaction volume is **25 µl**, the volume of DNA sample is **10 µl.**

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

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

Rickettsia conorii is detected on the JOE (Yellow) channel, *IC DNA* on the FAM (Green) channel

AMPLIFICATION

1. Create a temperature profile on your rotor-type[1] or plate-type[2] instrument as follows:

Step	Temperature, °C	Time	Fluorescent signal detection	Cycles
Hold	50	15 min	–	1
Hold	95	15 min	–	1
Cycling	95	10 s	–	45
	60	30 s	**FAM, JOE**	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)
[2] For example, SaCycler-96™ (Sacace), iQ5™, CFX™ (BioRad); Mx3000P™/3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied), SmartCycler® (Cepheid)

INSTRUMENT SETTINGS

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

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Eliminate cycles before	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.03*	*5 %*	*5*	*On*
JOE/Yellow	*from 5 Fl to 10 Fl*	*0.03*	*5 %*	*5*	*On*

Plate-type instruments

Channel	Threshold
FAM	Set the threshold line at the level corresponding to 10 % of maximum fluorescence level obtained for C+ sample at the last amplification cycle
JOE	Set the threshold line at the level corresponding to 5 % of maximum fluorescence level obtained for C+ sample at the last amplification cycle

DATA ANALYSIS

***Rickettsia conorii* DNA** amplification product is detected in the **JOE/Yellow** channel. **IC DNA** amplification product is detected on the **FAM/Green** channel.

The results are interpreted by the software of the PCR instrument by the crossing (or not crossing) of the fluorescence curve with the threshold line.

The analysis results are considered valid, only if the control samples results are correct:

Results for controls:

Control	Control stage	Ct channel Fam (Green)	Ct channel ROX(Orange)	Interpretation
NCE	DNA isolation	POS	NEG	Valid result
DNA-buffer	Amplification	NEG	NEG	Valid result
C+	Amplification	POS	POS	Valid result

The result interpretation is done on the basis of the information reported in the following table:

Result interpretation:

***Ct* value in the fluorophore channel**		**Result**
FAM	**JOE**	
< boundary value	absent	*Rickettsia conorii* DNA **is not detected**
determined or absent	< boundary value	*Rickettsia conorii* DNA **is detected**
absent or > boundary value	absent or > boundary value	**Invalid** result*
< boundary value	> boundary value	**Equivocal** result**

* In case of invalid result, the PCR analysis should be repeated for the corresponding clinical sample starting from the DNA extraction stage.

** In case of equivocal result, the PCR analysis should be repeated for the corresponding clinical sample starting from the DNA extraction stage. If it is obtained the same result, the sample is considered positive. If it is obtained negative result in the second run then the sample is considered equivocal and re-sampling of the material for analysis is recommended.

The analysis results are considered valid, only if the control samples and clinical samples results fall within the following boundary values:

Boundary values for clinical samples and controls:

Sample	Control stage	Plate-type instrument		Rotor-type instrument	
		Channel for fluorophore			
		FAM	JOE	FAM	JOE
C+	Amplification	< 37	< 37	< 34	< 34
C–	DNA isolation	< 33	absent	< 30	absent
NCA	Amplification	absent		absent	
Clinical samples	DNA isolation	< 36	< 43	< 32	< 42

QUALITY CONTROL PROCEDURE

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 results (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *Rickettsia conorii* primers and probes. The potential cross-reactivity of the kit **Rickettsia conorii Real-TM** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical specificity was studied on the following samples of microorganisms:

Organisms	FAM channel (Internal control)	JOE channel (*Rickettsia conorii*)
West Nile virus	Valid	Negative
Japanese encephalitis virus	Valid	Negative
Omsk hemorrhagic fever virus	Valid	Negative
Tick-borne encephalitis virus	Valid	Negative
Anaplasma phagocytophillum	Valid	Negative
Bartonella henselae	Valid	Negative
Babesia microti	Valid	Negative
Leptospira kirschneri	Valid	Negative
L. borgpetersenii	Valid	Negative
Shigella sonne	Valid	Negative
S. flexneri	Valid	Negative
Salmonella typhi	Valid	Negative
S. enteritidis	Valid	Negative

Organisms	FAM channel (Internal control)	JOE channel (*Rickettsia conorii*)
Klebsiella pneumonia	Valid	Negative
Esherichia coli NCTC 9001	Valid	Negative
Enterococcus faecalis	Valid	Negative
Staphylococcus aureus	Valid	Negative
S. saprophyticus	Valid	Negative
Pseudomonas aeruginosa	Valid	Negative
Proteus mirabilis	Valid	Negative
Enterobacter cloacae	Valid	Negative
Yersinia pestis	Valid	Negative
25 samples of eschars swabs	Valid	Negative
25 blood samples from the patients with another causation of disease	Valid	Negative
25 biopsy material samples from the patients with another causation of disease	Valid	Negative
25 cerebrospinal fluid samples from the patients with another causation of disease	Valid	Negative
25 samples of *Dermacentor reticulatus* ticks	Valid	Negative

All tested samples did not give any false positive result. The specificity of the kit **Rickettsia conorii Real-TM** was 100%.

Analytical sensitivity

The kit **Rickettsia conorii Real-TM** allows to detect *Rickettsia conorii* DNA in 100% of the tests with a sensitivity of not less than 1000 copies/ml as specified in the table below:

Biological material	Volume of starting sample, µl	Nucleic acid extraction kit	Analytical sensitivity (limit of detection), copies/ml
Blood	Pellet + 100	DNA/RNA Prep	10^3
Tissue (autopsy, biopsy) material	100	DNA/RNA Prep	10^3
Cerebrospinal fluid	Pellet + 100	DNA/RNA Prep	10^3
Eschar swabs	100	DNA/RNA Prep	10^3
Ticks	100	DNA/RNA Prep	10^3

Target region:

ompA gene

TROUBLESHOOTING

1. Weak or no signal of the IC (FAM/Green channel) 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.
    - The reagents storage conditions didn't comply with the instructions.
        ⇒ Check the storage conditions
    - Improper DNA extraction.
        ⇒ Repeat analysis starting from the DNA extraction stage
    - 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 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. JOE/Yellow 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
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**C–**	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
* iQ5™ and CFX™ is a registered trademark of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Qiagen
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