IVD For *in Vitro* Diagnostic Use

CE

Coxiella burnetii Real-TM

Handbook

Real Time PCR kit for the detection of Coxiella burnetii

REF B85-50FRT

50

NAME

Coxiella burnetii Real-TM

INTRODUCTION

Q fever is a zoonosis caused by *Coxiella burnetii*, an obligate gram-negative intracellular bacterium. Most commonly reported in southern France and Australia, Q fever occurs worldwide. *C burnetii* infects various hosts, including humans, ruminants (cattle, sheep, goats), and pets. In rare cases, C burnetii infection in reptiles, birds, and ticks has been reported. *C burnetii* is excreted in urine, milk, feces, and birth products. These products, especially the latter, contain large numbers of bacteria that become aerosolized after drying. *Coxiella burnetii* is a highly infectious agent that is rather resistant to heat and drying. It can become airborne and inhaled by humans. A single *C. burnetii* organism may cause disease in a susceptible person. This agent could be developed for use in biological warfare and is considered a potential terrorist threat.

Molecular detection by PCR is unaffected by changes in the infection cycle, and enables rapid, sensitive and specific detection of *C. burnetii* in a sample.

INTENDED USE

The **Coxiella burnetii Real-TM** is a “Real-Time Amplification” test for the qualitative detection of *C.burnetii* in the human and animal biological materials (whole blood, urine, feces, tissue, milk, etc) and in the environment (soil, water). *C.burnetii* DNA is extracted from samples, amplified using real time amplification with fluorescent reporter dye probes specific for *C.burnetii* and *Internal Control (* IC).

PRINCIPLE OF ASSAY

In **Coxiella burnetii Real-TM** kit there are 2 independent reactions running in parallel in each tube: the first detects specific fragment of *C.burnetii* and the second detects internal control (IC) DNA which allows excluding unreliable results. Positive result of reference reaction 2 (IC) together with negative results of reactions 1 (*C.burnetii)* confirm the absence of *C.burnetii* in a sample. A negative result of both reactions is a sign of PCR inhibition, and this provides a way to avoid false-negative results.

MATERIALS PROVIDED

- **PCR-mix-1 *Coxiella burnetii***, 0,6 ml;
- **PCR-mix-2-FRT,** 0,3 ml;
- **TaqF polymerase**, 0,03 ml;
- **Pos Coxiella/IC** (concentration of specific fragment of *C.burnetii* 100-200 copies/reaction), 0,2 ml ;
- **Negative Control C-,** 1,2 ml;*
- **Internal Control, IC,** 0,6 ml;**
- **DNA-buffer,** 0,5 ml.

Contains reagents for 55 tests.

* *must be used in the extraction procedure as Negative Control of Extraction.*

** *add 10 µl of Internal Control during the DNA extraction procedure directly to the sample/lysis mixture (see DNA/RNA-prep* ***REF*** *K-2-9).*

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Automatic adjustable pipettes.
- Disposable tips with aerosol barriers (100 or 200 µl) in tube racks.
- Tube racks.
- Vortex mixer/desktop centrifuge.
- PCR box.
- Real Time PCR instrument.
- Disposable polypropylene microtubes for PCR or PCR-plate
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

STORAGE INSTRUCTIONS

All components of the **Coxiella burnetii Real-TM** PCR kit (except for PCR-mix-1, PCR-mix-2-FRT, and TaqF Polymerase) are to be stored at 2–8 ºC when not in use. All components of the **Coxiella burnetii Real-TM** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

PCR-mix-1, PCR-mix-2-FRT and TaqF Polymerase are to be stored at ≤ –16ºC.

PCR-mix-1 is to be kept away from light.

STABILITY

Coxiella burnetii 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. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

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. 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

Coxiella burnetii Real-TM can analyze DNA extracted from:

- *whole blood* collected in either ACD or EDTA tubes;
- *tissue* (≈1,0 gr) homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile. Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;
- *serous exudation from skin lesions:* insert the swab into the nuclease-free 1,5 ml tube and add 0,2 mL of Transport medium or sterile Saline solution. Vigorously agitate swabs in medium for 15-20 sec.
- *milk;*
- *water:* centrifuge 10-20 ml of water for 15 min at 8000g, discard the supernatant and leave about 100 µl of solution for DNA extraction
- *Ticks (Rhipicephalus, Haemaphysalis, Dermacentor, Ixodes)*
  Before ticks pretreatment, pools of ticks should be formed. Each pool can contain 5-7 non-sated ticks or 2-3 ticks of semi-sated ones. Fully sated ticks should be analyzed individually. Use sterile porcelain mortars and sterile pestles for ticks suspension preparation. If automatic homogenizer TissueLyser LT is used the following parameters are set: beads diameter – 7 mm; frequency – 50 Hz/sec; time of homogenization – 12-15 min; buffer volume – 700 µl (non-sated tick), 1000-1500 µl (sated tick and pools). In case of sated ticks, they should be punctured with sterile disposable needle prior to homogenization. Ticks can be washed in 70% ethanol if needed. Use sterile porcelain mortars and sterile pestles for ticks suspension preparation. Grind the ticks in 100 µl (if sample consist of 1 non-sated tick), in 1-1.5 ml (for pool or sated tick) of 0.15 M sodium chloride solution or PBS buffer. Mix solution with ticks by two portions. Centrifuge obtained suspension 1 min at 10000 g. It is acceptable to store material before analysis for 1 day at the temperature 2-8 C or 1 week at the temperature not more than minus 16 °C. Subsequent storage should be at the temperature not more than minus 68 °C.
- *soil:*
  1. Prepare required quantity of 5 ml tubes with 1,0 ml of 70% ethanol, add to each tube 1,0 g of soil. Incubate 10 min at room temperature with gentle shaking.
  2. Add to each tube 3 ml of sterile Saline solution, vortex vigorously and incubate 5 min at room temperature with gently shaking.
  3. Transfer 1 ml of soil solution into the new 1,5 ml tube and centrifuge for 3 min at 300g.
  4. Use supernatant for DNA extraction.

Specimens can be stored at +2-8°C for no longer than 12 hours, or freeze at -20°C to -80°C.

Transportation of clinical specimens and materials that contain or are suspected of containing infectious agents 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 kits:

- ⇒ **DNA/RNA Prep** (Sacace, REF K-2-9) – for blood plasma or tick suspension (non-sated or semi-sated ones);
- ⇒ **RIBO-Zol** (Sacace REF K-2-3) – for sated ticks;
- ⇒ **SaMag Bacterial DNA Extraction kit (**Sacace, REF K-2-9)

- DNA is extracted from each clinical sample in the presence of **Internal Control** (**10 µl** of **IC** is added to each sample).
- Transfer **100 µl** of **Negative Control** to the tube labeled NCE.

- Extract DNA according to the manual provided by the manufacturer.

PROTOCOL (Reaction volume 25 µl):

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 µl** of **PCR-mix-1**, **5,0*N** of **PCR-mix-2-FRT** and **0,5*N** of **TaqF Polymerase.** Vortex and centrifuge for 2-3 sec.
3. Add **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 (NCA);
   - add **10 µl** of ***Pos* Coxiella /IC** to the tube labeled. *C+*;

Coxiella burnetii on the JOE (Yellow)/HEX/Cy3 channel, IC is detected on the FAM (Green) channel.

Amplification

1. Create a temperature profile on your instrument as follows:

Amplification program for rotor-type instruments[1]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling2	95	5 s	–	40
	56	20 s	FAM/Green, JOE/Yellow	
	72	15 s		

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*

Amplification program for plate-type instruments[2]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
1	95 °C	15 min	–	1
2	95 °C	5 s	–	5
	60 °C	25 s	–	
	72 °C	15 s	–	
3	95 °C	5 s	–	40
	56 °C	25 s	FAM, HEX/Cy3/JOE	
	72 °C	15 s		

[2] *For example, SaCycler 96 (Sacace), CFX96/ iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

INSTRUMENT SETTINGS

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

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

Plate-type instruments (SaCycler, iQ5, Mx300P, ABI 7500/7300/StepOne)

Channel	Threshold
FAM	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 line at a level corresponding to 10–20% of the maximum fluorescence signal obtained for Pos C+ sample during the last amplification cycle

DATA ANALYSIS

Coxiella burnetii **DNA** amplification product is detected in the **JOE/Yellow/HEX** channel, Internal Control amplification product is detected in 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 comply with the following:

Results for controls

Control	Stage for control	Ct in channel		Interpretation
		FAM/Green	JOE/Yellow/HEX	
NCE	DNA extraction	≤ 28	Neg	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	≤ 31	≤31	OK

1. The sample is considered **positive** if Ct values detected in the FAM/Green and JOE/Yellow/HEX channel are less than the boundary Ct values (≤ 38) for these channels. The fluorescence curve should have a typical sigmoid shape and cross the threshold line in the region of significant fluorescence increase only once.
2. The sample is considered **negative** if its fluorescence curve does not cross the threshold line (Ct value is absent) and does not have the typical shape.

The results of analysis are considered reliable only if the results obtained for Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct.

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

Sensitivity

The analytical sensitivity of **Coxiella burnetii Real-TM** PCR kit is 10^3 *Coxiella burnetii* DNA copies/ml.

*The claimed analytical features of **Coxiella burnetii Real-TM** PCR kit are guaranteed only when additional reagent kit (DNA/RNA-prep) is used.*

Specificity

The analytical specificity of **Coxiella burnetii Real-TM** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. The clinical specificity of **Coxiella burnetii Real-TM** PCR kit was confirmed in laboratory clinical tests.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam/Green): retesting of the sample is required.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting 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 for the IC detection select the fluorescence channel reported in the protocol.
    - Improper DNA extraction.
        - ⇒ Repeat analysis starting from the DNA extraction stage
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak (Ct >39) signal on the Joe (Yellow)/Cy3/HEX channel: the result is considered equivocal. It is necessary to repeat the analysis twice. If a positive Ct value is detected twice, the sample is considered as positive.
3. Joe (Yellow)/Cy3/HEX 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 among tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
    - Contamination during PCR 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

Sacace Biotechnologies Srl
via Scalabrini, 44 – 22100 – Como – Italy Tel +390314892927 Fax +390314892926
mail: ***info@sacace.com*** *web:* ***www.sacace.com***