IVD For *in Vitro* Diagnostic Use

CE

Brucella Real-TM

Handbook

Real Time PCR kit for qualitative detection of Brucella species

REF B10-50FRT

Σ 50

NAME

Brucella Real-TM

INTRODUCTION

Brucellosis is a worldwide zoonosis caused by infection with the bacterial *Brucella.* These organisms, which are small aerobic intracellular coccobacilli, localize in the reproductive organs of host animals, causing abortions and sterility. They are shed in large numbers in the animal's urine, milk, placental fluid, and other fluids. Exposure to infected animals and animal products causes brucellosis in humans. The global burden of human brucellosis remains enormous; it causes more than 500,000 infections per year worldwide. Among the 4 *Brucella* species known to cause disease in humans (*B. abortus, B. melitensis, B. canis, B. suis*), *B. melitensis* is thought to be most virulent and causes the most severe and acute of brucellosis.

INTENDED USE

The **Brucella Real-TM** is a "Real-Time PCR Amplification" test for the qualitative detection of *Brucella species* (B.melitensis, B.abortus, B.suis, B.ovis, B.canis) in the human and animal biological materials (whole blood, milk, tissue, etc).

PRINCIPLE OF ASSAY

Kit **Brucella Real-TM** is based on two major processes: isolation of DNA from specimens and Real Time amplification. *Brucella species* DNA is extracted from the specimens, amplified using Real-Time amplification and detected fluorescent reporter dye probes specific for *Brucella species* 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 *Brucella species.*

MATERIALS PROVIDED

Real Time PCR kit (B10-50FRT)

“Brucella Real-TM”: Real Time amplification kit

- **PCR-mix-1 *Brucella***, 55 ready-to-use single-dose test tubes;
- **PCR-mix-2-Flu**, 0,77 ml;
- ***Brucella* C+,** 0,1 ml;
- **Pos IC C+**, 0,1 ml ;
- **Negative Control C-,** 1,2 ml;*
- **IC (Internal Control),** 0,5 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*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit
- 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
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

Brucella Real-TM must be stored at 2-8°C. The kit can be shipped at 2-8°C and should be stored at 2-8°C immediately on receipt.

STABILITY

Brucella 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

Brucella 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;
- *sinovial liquid* collected in sterile disposable tube*;*
- *milk:* centrifuge 10 ml at 3000g/min for 10-15 min. If pellet is not visible add 10 ml more to same tube and repeat centrifuge. Discard the supernatant leaving about 200 µl of liquid above the pellet. Resuspend the pellet in this liquid and use 100 µl of the suspension 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 kit:

⇨ **DNA-Sorb-B** (Sacace, REF K-1-1/B);

⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006).

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.

PROTOCOL (25 µl reaction mix):

1. Prepare required quantity of **PCR-mix-1 Brucella** tubes for samples and controls.
2. Add **7 µl** of **PCR-mix-2 Flu** into each tube (without disturbing the wax layer).
3. Add **10 µl** of **extracted DNA** sample to appropriate tube.
4. Prepare for each panel 3 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of ***Brucella* C+** to the tube labeled C+Bru;
   - add **10 µl** of **Pos IC C+** to the tube labeled IC C+;
5. Gently spin the tubes (2-3 seconds) and insert them in the thermalcycler.

Amplification

Create a temperature profile on your instrument as follows:

	Rotor-type instruments[1]			**Plate- or modular type instruments[2]**		
Step	***Temperature, °C***	***Time***	**Cycles**	***Temperature, °C***	***Time***	**Cycles**
Hold	**95**	5 min	1	**95**	5 min	1
Cycling 1	**95**	10 s	10	**95**	10 s	10
	65	25 s		**65**	25 s	
	72	10 s		**72**	10 s	
Cycling 2	**95**	10 s	35	**95**	10 s	35
	56	25 s *fluorescent signal detection*		**56**	30 s *fluorescent signal detection*	
	72	10 s		**72**	10 s	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)
[2] For example, SaCycler-96™ (Sacace), CFX96/iQ5™ (Biorad), Mx3000P/3005P™ (Agilent)

Fluorescence detection on the channels Fam(Green) and JOE(Yellow)/HEX/Cy3 on the 2-nd cycling (56°C).

INSTRUMENT 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*	0.1	10 %	on
JOE/Yellow	*from 5 Fl to 10 Fl*	0.1	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 in the log-linear phase of the curve, at a level corresponding to 10-20% of fluorescence value reached by the positive control sample in the last amplification cycle.

RESULTS ANALYSIS:

The fluorescent signal intensity is detected in two channels

– The signal from the ***Brucella*** **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**.

Table. Results for controls

Control	Stage for control	Ct channel Fam (Green)	Ct channel Joe (Yellow)	Interpretation
NCE	DNA isolation	Pos (Ct < 31)	Neg	Valid result
NCA	Amplification	Neg	Neg	Valid result
Brucella C+	Amplification	Neg	Pos (Ct < 33)	Valid result
Pos IC C+	Amplification	Pos (Ct < 31)	Neg	Valid result

- *Brucella* DNA is **detected** in a sample if its Ct ≤ 33 value is defined in the results grid in the JOE(Yellow)/HEX/Cy3 channel.
- *Brucella* 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) and in the results table on the channel Fam (Green) the Ct value is lower than 31.
- The result of analysis is **invalid** if the Ct value is not defined (or > 31) 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.

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 *Brucella* primers and probes. The specificity of the kit **Brucella Real-TM** was 100%. The potential cross-reactivity of the kit **Brucella Real-TM** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **Brucella Real-TM** allows to detect *Brucella DNA* in 100% of the tests with a sensitivity of not less than 1000 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

Target region: WboA gene

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.

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.
        - ⇒ 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
    - 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 sample 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 sample 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.
5. If the Ct value in JOE/HEX/Yellow is greater than 33, and Ct value determined for the FAM is less than 31, PCR amplification should be repeated. The result of analysis is considered positive if the same result has been obtained or if the Ct value determined in the channel for the JOE/HEX/Yellow is less than 33.

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
* Rotor-Gene™ is a registered trademark of Qiagen
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* MX 3000P/3005P® is a registered trademark of Agilent Technologies

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