IVD For *in Vitro* Diagnostic Use

CE

Borrelia burgdorferi Real-TM

Handbook

Real Time PCR Kit for qualitative detection of *Borrelia burgdorferi* in biological materials

REF B37-50FRT

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Sacace Biotechnologies S.r.l., via Scalabrini 44 – 22100 Como - ITALY

NAME

Borrelia burgdorferi Real-TM

INTRODUCTION

Lyme disease (LD) is a vector-borne, multisystem inflammatory disease caused by the spirochete bacterium *Borrelia burgdorferi sensu lato*. It is transmitted to humans by infected tiks of the *Ixodes* genus. After entering the circulation, the bacterium invades the cutaneous, synovial, cardiac, and nervous system. Spirochetes bacteria have also been demonstrated histologically in bone marrow, the spleen, lymph nodes, the liver, testes, and the placenta during early hematogenous dissemination.

INTENDED USE

Kit **Borrelia burgdorferi Real-TM** is an *in vitro* nucleic acid amplification test for qualitative detection and identification of *Borrelia burgdorferi* in the biological materials. The strains detected by the kit **Borrelia burgdorferi Real-TM** are the followings: *B.burgdorferi, B.afzelii, B.garinii, B.valaisiana, B.tanukii, B. bissetii, B.americana, B.spielmanii, B.sinica, B.lusitaniae, B.andersonii, B.turdi, B.japonica.*

PRINCIPLE OF ASSAY

Kit **Borrelia burgdorferi Real-TM** is based on two major processes: isolation of DNA from specimens and Real Time amplification. *Borrelia burgdorferi* 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 *Borrelia burgdorferi* DNA and IC. 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 *Borrelia burgdorferi*.

MATERIALS PROVIDED

"**Borrelia burgdorferi Real-TM**": Real Time amplification kit

- **PCR-mix-1-FRT**, 0,6 ml;
- **PCR-Buffer-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **Borrelia burgdorferi C+***, 0,1 ml;
- **Negative Control C-****, 1,6 ml;
- **Internal Control IC*****, 1,0 ml
- **DNA-buffer******, 0,5 ml;

Contains reagents for 55 tests.

* *Borrelia burgdoferi C+ plasmid DNA must be used as Positive Amplification Control (see PROTOCOL);*

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

Borrelia burgdorferi Real-TM must be stored at -20°C, **DNA/RNA Prep** must be stored at +2-8°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

Borrelia burgdorferi 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:

- ⚠ 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).
- ⚠ Component Prec Sol contains 2-propanol: flammable. Irritant. (R10-36-67, S7-16-24/25-26). 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

Borrelia burgdorferi Real-TM can analyze DNA extracted from:

- *liquor (CSF)* stored in “Eppendorf” tube*;*
- *sinovial liquid* stored in “Eppendorf” tube;
- *skin punch biopsy;*
- *urine (sediment)*;
- *plasma* (only during primary infection) collected blood in ACD or EDTA tubes;

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)

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).

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+1) µl** of **PCR-mix-1-FRT**, **5,0*(N+1)** of **PCR-Buffer-FRT** and **0,5*(N+1)** of **TaqF 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 3 controls:
   - add **10 µl** of extracted **Negative Control C-** to the tube labeled Negative Control of Extraction;
   - add **10 µl** of **DNA-buffer** to the tube labeled Negative Amplification Control;
   - add **10 µl** of **Borrelia burgdorferi C+** to the tube labeled Positive Amplification Control.
5. Insert the tubes in the thermalcycler.

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

Borrelia burgdorferi is detected on the FAM (Green) channel, *IC DNA* on the JOE(Yellow)/HEX/Cy3 channel

AMPLIFICATION

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

	Rotor-type Instruments[1]			Plate- or modular type Instruments[2]		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
1	**95**	15 min	1	**95**	15 min	1
2	**95**	15 s	10	**95**	15 s	10
	63	50 s		**63**	50 s	
	72	20 s		**72**	20 s	
3	**95**	15 s	40	**95**	15 s	40
	58	50 s *fluorescent signal detection*		**58**	55 s *fluorescent signal detection*	
	72	20 s		**72**	20 s	

[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), LineGeneK® (Bioer)

INSTRUMENT SETTINGS

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

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

DATA ANALYSIS

Borrelia burgdorferi DNA amplification product is detected in the **FAM/Green** channel. **IC DNA** amplification product is detected on the JOE(Yellow)/HEX/Cy3 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	Step control	Ct channel Fam (Green)	Ct channel JOE(Yellow)	Interpretation
NCE	DNA isolation	NEG	POS (Ct < 25)	Valid result
DNA-buffer	Amplification	NEG	NEG	Valid result
C+	Amplification	POS (Ct < 24)	NEG	Valid result

1. The sample is considered **positive** for *Borrelia burgdorferi* if Ct value is detected in the FAM/Green channel and the Ct value for the IC is detected in JOE(Yellow) channel, with Ct values less than the boundary values (see below table). 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** for *Borrelia burgdorferi* if its fluorescence curve does not cross the threshold line (Ct value is absent) in the FAM/Green channel and the Ct value for the IC is detected in JOE(Yellow) channel, with a Ct less than the boundary value (see table below).

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.

Boundary values:

Sample	Ct channel Fam (Green)	Ct channel JOE(Yellow)
NCE	Ct is absent	Ct < 25
DNA-buffer	Ct is absent	Ct is absent
C+	Ct < 24	Ct is absent
Clinical samples	Ct < 37	Ct < 25

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 *Borrelia burgdorferi* primers and probes. The potential cross-reactivity of the kit **Borrelia burgdorferi 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:

Genus of the microorganism	Kind of microorganism	Name of the strain	Source of isolation	Result
Flavivirus	West nile virus	Hp- 94, Egypt-101, Uganda		-
	Langat virus	TR-21		-
	Powassan virus	Baers		-
	Japanese encephalitis virus	Nakayama , Beijing - I , Jagar-01		-
	Omskhemorrhagic fever virus	The Veselovka - 752.Goloshubin, M1, Kr-4		-
Borrelia	Borrelia miyamotoi		blood patients	
Leptospira	L.interrogans	Strains serogroups pomona, icterohaemorragiae, canicola, szwajizak, autumnalis, australis, pryogenes, wolffi		-
	L. kirschneri	Strains serogroups Grippotyphosa, kabura, dja tzi		-
	L.borgpetersenii	Strains serogroups tarassovi polonica ballum		-
Treponema	Treponema pallidum			-
Rickettsia	Rickettsia conorii subsp. caspia		Blood of patients	-

	R.heilongiangensis		Blood of patients	
Coxiella	Coxiella burnetii		Blood of patients	-
Bartonella	Bartonella henselae		Blood of patients	
	Bartonella quantana		Blood of rodents	-

All tested samples did not reveal any false positive result.

The specificity of the kit **Borrelia burgdorferi Real-TM** was 100%.

Analytical sensitivity

The kit **Borrelia burgdorferi Real-TM** allows to detect *Borrelia burgdorferi* DNA in 100% of the tests with a sensitivity of not less than 500 copies/ml.

Kind of biological material	**Sample preparation of material (if necessary)**	**Kit for RNA / DNA extraction**	**Extraction volume, µL**	**Analytical Sensitivity (limit of detection)[1]**
Bacterial Blood Precipitate	Concentration of bacteria by successive centrifugation cycles	DNA/RNA Prep	Cell pellet and 100 µl of supernatant plasma	$5x10^2$ copies/ml
Autopsy and biopsy material	Preparation of 10% suspension	DNA/RNA Prep	100 µl	$5x10^2$ copies/ml
Liquor (CSF)	Concentration of bacteria by centrifugation	DNA/RNA Prep	Cell pellet and 100 µl of supernatant cerebrospinal fluid	$5x10^2$ copies/ml

Target region

The target region detected by the kit is **Borrelia burgdorferi Real-TM** the 16S rRNA gene of the following strains: *B.burgdorferi, B.afzelii, B.garinii, B.valaisiana, B.tanukii, B. bissetii, B.americana, B.spielmanii, B.sinica, B.lusitaniae, B.andersonii, B.turdi, B.japonica.*

[1] *Lowest concentration detected.*

TROUBLESHOOTING

1. Weak or no signal of the IC (JOE/Yellow 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. Fam 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. Fam 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

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