IVD For *in Vitro* Diagnostic Use

CE

Bacillus anthracis Real-TM

Handbook

Real Time PCR kit for the detection of Bacillus anthracis

REF B101-50FRT

REF TB101-50FRT

Σ **50**

NAME

Bacillus anthracis Real-TM

INTRODUCTION

Bacillus anthracis is a rod-shaped, Gram-positive, aerobic bacterium that is about 1 by 9 micrometers in length. It was shown to cause disease by Robert Koch in 1876. The bacterium normally rests in endospore form in the soil, and can survive for decades in this state. Herbivores are often infected whilst grazing or browsing, especially when eating rough, irritant or spiky vegetation: the vegetation has been hypothesized to cause wounds within the gastrointestinal tract permitting entry of the bacterial endo-spores into the tissues, though this has not been proven. Once ingested or placed in an open cut, the bacterium begins multiplying inside the animal or human and typically kills the host within a few days or weeks. The endo-spores germinate at the site of entry into the tissues and then spread via the circulation to the lymphatics, where the bacteria multiply.

INTENDED USE

The Bacillus anthracis Real-TM PCR kit is an in vitro nucleic acid amplification test for qualitative detection of DNA of vegetative and cryptogamic forms of Bacillus anthracis in biological material and environmental samples and for determination of Bacillus anthracis plasmid composition by identification of pagA (plasmid pXO1) and capA (plasmid pXO2) genes by using real-time hybridization-fluorescence detection..

PRINCIPLE OF ASSAY

Bacillus anthracis Real-TM kit is based on the amplification of pathogen genome specific region using special Bacillus anthracis primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run. **Bacillus anthracis Real-TM** kit is a qualitative test that contains the Internal Control (IC). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. **Bacillus anthracis Real-TM** kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions.

MATERIALS PROVIDED

Module No.1: Real Time PCR test (B101-50FRT)

Part N° 2– **"Bacillus anthracis Real-TM":** Real Time amplification kit		
PCR-mix-1-FRT *Bacillus anthracis* ready-to-use single-dose test tubes (*under wax)*	0.008ml	55 tubes
PCR-mix-2-FL	0.77ml	1 tube
Positive Control DNA *Bacillus anthracis* pXO1 (C+$_{Bacillus\ anthracis\ pXO1}$)	0.1ml	1 tube
Positive Control DNA *Bacillus anthracis* pXO2 (C+$_{Bacillus\ anthracis\ pXO2}$)	0.1ml	1 tube
Positive PCR Internal Control (CS+)	0.1ml	1 tube
Negative Control (C-)*	1.2ml	1 tube
Internal Control (IC)**	0.5ml	1 tube
DNA-buffer	0.5ml	1 tube

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TB101-50FRT)

Part N° 1 – **"DNA-Sorb-B":** Sample preparation kit		
Lysis Solution	15ml	1 tube
Washing Solution 1	15ml	1 tube
Washing Solution 2	50ml	1 tube
Sorbent	1,25ml	1 tube
DNA-eluent	5,0ml	1 tube

Contains reagents for 50 extractions

Part N° 2– **"Bacillus anthracis Real-TM":** Real Time amplification kit		
PCR-mix-1-FRT *Bacillus anthracis* ready-to-use single-dose test tubes (*under wax)*	0.008ml	55 tubes
PCR-mix-2-FL	0.77ml	1 tube
Positive Control DNA *Bacillus anthracis* pXO1 (C+$_{Bacillus\ anthracis\ pXO1}$)	0.1ml	1 tube
Positive Control DNA *Bacillus anthracis* pXO2 (C+$_{Bacillus\ anthracis\ pXO2}$)	0.1ml	1 tube
Positive PCR Internal Control (CS+)	0.1ml	1 tube
Negative Control (C-)*	1.2ml	1 tube
Internal Control (IC)**	0.5ml	1 tube
DNA-buffer	0.5ml	1 tube

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.*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- 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

Bacillus anthracis Real-TM must be stored at 2-8°C. **DNA-sorb-B** must be stored at 2-8°C. The kits can be shipped at 2-8°C but should be stored at 2-8°C immediately on receipt.

STABILITY

Bacillus anthracis 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:

- ⚠ 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***

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Bacillus anthracis 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
- *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 kit:

- ⇨ **DNA-Sorb-B** (Sacace, REF K1-1/B);
- ⇨ **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. Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the module no.2)

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 56°C until disappearance of ice crystals.
2. Prepare required quantity of 1.5 ml polypropylene tubes.
3. Add to each tube **10 µl** of **Internal Control** and **300 µl** of **Lysis Solution**.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control**) to labeled C*neg*.
6. Vortex the tubes, incubate for 5 min at 65°C and centrifuge for 3-5 sec.
7. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
8. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature.
9. 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.
10. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and 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. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and 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.
12. Repeat step 11.
13. Incubate all tubes with open cap for 5 min at 65°C.
16. Resuspend the pellet in **50 µl** of **DNA-eluent.** Incubate for 10 min at 65°C and vortex periodically.
17. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. The amplification can be performed on the same day of extraction.

PCR PROTOCOL

Preparing tubes for PCR

1. Prepare the required number of the tubes with **PCR-mix-1-FRT *Bacillus anthracis*** and wax for amplification of DNA from clinical and control samples (1 negative and 3 positive control samples).
2. Add **7 µl** of **PCR-mix-2-FL** to the surface of the wax layer of each tube ensuring that it does not fall under the wax and mix with **PCR-mix-1-FRT *Bacillus anthracis.***
3. Add **10 µl** of **DNA** obtained from clinical or control samples at the DNA extraction stage to the prepared tubes using tips with aerosol barrier.
4. Carry out the control amplification reactions:

NCA Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

C+*Bacillus anthracis* pXO1 Add **10 µl** of **Positive Control DNA *Bacillus anthracis* pXO1** to the tube labeled C+*Bacillus anthracis* pXO1 (Positive Control of Amplification).

C+*Bacillus anthracis* pXO2 Add **10 µl** of **Positive Control DNA *Bacillus anthracis* pXO2** to the tube labeled C+*Bacillus anthracis* pXO2 (Positive Control of Amplification).

CS+ Add **10 µl** of **Positive PCR Internal Control** to the tube labeled CS+ (Positive Control of Amplification).

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	95	10 s	10	95	10 s	10
	60	25 s		60	25 s	
	72	10 s		72	10 s	
Cycling 2	95	10 s	35	95	10 s	35
	56	25 s FAM/Green, JOE/Yellow, ROX/Orange		56	30 s FAM/Green, JOE/Yellow, ROX/Orange	
	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 is detected at the stage 2 of Cycling in FAM/Green, JOE/Yellow and ROX/Orange channels.

INSTRUMENT SETTINGS

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

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 20 Fl to 30 Fl*	0.025	10 %	Off
JOE/Yellow	*from 10 Fl to 15 Fl*	0.1	10 %	Off
ROX/Orange	*from 5 Fl to 10 Fl*	0.1	10 %	Off

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 at a level where fluorescence curves are linear and do not cross curves of the negative samples.

RESULTS ANALYSIS

The results are interpreted by the software analyzing the fluorescence curves that cross (or not) the threshold line.

Results are accepted as relevant if both positive and negative controls of amplification along with negative control of extraction are passed.

Results for controls

Control	Controlled stage	Ct FAM/Green	Ct JOE/Yellow	Ct ROX/Orange	Interpretation
C–	DNA extraction	Neg	Neg	Pos	OK
NCA	Amplification	Neg	Neg	Neg	OK
C+*Bacillus anthracis* pXO1	Amplification	Pos	Neg	Neg	OK
C+*Bacillus anthracis* pXO2	Amplification	Neg	Pos	Neg	OK
CS+	Amplification	Neg	Neg	Pos	OK

1. The sample is considered to be **positive** for DNA *Bacillus anthracis* pXO1+ and pXO2+ if the Ct value in FAM/Green and JOE/Yellow channels is less than 33, respectively, regardless of the Ct value in the ROX/Orange channel.
2. The sample is considered to be **positive** for DNA *Bacillus anthracis* pXO1+ if the Ct value in the FAM/Green channel is less than 33, regardless of the Ct value in the ROX/Orange channel.
3. The sample is considered to be **positive** for DNA *Bacillus anthracis* pXO2+ if the Ct value in the JOE/Yellow channel is less than 33, regardless of the Ct value in the ROX/Orange channel.
4. The sample is considered to be **negative** if the Ct value in FAM/Green and JOE/Yellow channels is absent and the Ct value in the ROX/Orange channel does not exceed 31.

SPECIFICATIONS

Sensitivity

The analytical sensitivity of **Bacillus anthracis Real-TM** PCR kit is not less than $1x10^3$ spores of *Bacillus anthracis* pXO1+ and pXO2+ per 1 ml.

Specificity

The analytical specificity of **Bacillus anthracis Real-TM** PCR kit is ensured by selection of specific primers and probes and 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 **Bacillus anthracis Real-TM** PCR kit was confirmed in laboratory clinical trials.

TROUBLESHOOTING

1. Weak or no signal of the IC (ROX/Orange 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. FAM/Green, 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. Any 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	**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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