ECOLI

eSens

RUO

Intended for Research Use Only

eSens Bacillus anthracis QL PCR kit

REF ES3806B

Instructions for Use

1 INTENDED USE

eSens Bacillus anthracis QL PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of DNA of vegetative and cryptogamic forms of *Bacillus anthracis* in the biological material and environmental samples and for determination of *Bacillus anthracis* plasmid composition by identification of *pagA* (plasmid pXO1) and *capA* (plasmid pXO2) genes using real-time hybridization-fluorescence detection of amplified products. Intended for Research Use Only.

NOTE: The results of PCR analysis are NOT to be taken into account in complex diagnostics of disease.

2 PRINCIPLE OF PCR DETECTION

Bacillus anthracis DNA detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific *Bacillus anthracis* primers. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which 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.

eSens Bacillus anthracis QL PCR kit is a qualitative test that contains the Internal Control (Internal Control STI-704 (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.

eSens Bacillus anthracis QL PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by the separation of nucleotides and Taq-polymerase using a wax layer. Wax melts and reaction components mix only at 95 °C.

The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE	ROX
DNA-target	*Bacillus anthracis* pXO1 DNA	*Bacillus anthracis* pXO2 DNA	Internal Control STI-704 DNA
Target gene	Protective antigen precursor (pagA) gene	Encapsulation protein gene (capA)	Artificially synthesized sequence

3 CONTENT

eSens Bacillus anthracis QL PCR kit (ES3806B) includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FRT *Bacillus anthracis* ready-to-use single-dose test tubes (*under wax*)	colorless clear liquid	0.008	55 tubes
PCR-mix-2-FL	colorless clear liquid	0.77	1 tube
Positive Control DNA *Bacillus anthracis* pXO1 (C+*Bacillus anthracis* pXO1)	colorless clear liquid	0.1	1 tube
Positive Control DNA *Bacillus anthracis* pXO2 (C+*Bacillus anthracis* pXO2)	colorless clear liquid	0.1	1 tube
Positive Control STI-88 (CS+)	colorless clear liquid	0.1	1 tube
Negative Control (C-)*	colorless clear liquid	1.2	1 tube
Internal Control STI-704 (IC)**	colorless clear liquid	0.5	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube

***** 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.

eSens Bacillus anthracis QL PCR kit is intended for 55 reactions (including controls).

4 ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol filters (up to 200 µl).
- Tube racks.
- Vortex mixer.
- PCR box.
- Real-time instruments (for example, Rotor-Gene Q (QIAGEN, Germany), CFX 96 Touch, CFX 96 Opus (Bio-Rad, USA), QuantStudio 5 (Thermo Fisher Scientific), or equivalent).

- Refrigerator for 2-8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.

5 GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Sampling, transportation and storage of material for research and handling with it must be carried out according to the instructional guidance documents that regulate the research on *B.anthracis*.
- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. 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 regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid samples and reagents contact with the skin, eyes, and mucous membranes. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- Safety Data Sheets (SDS) are available on request.
- Use of this product should be limited to personnel trained in techniques DNA amplification.
- Workflow in the laboratory process must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents to the area where the previous step was performed.

⚠ Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6 SAMPLING AND HANDLING

eSens Bacillus anthracis QL PCR kit is intended for the analysis of the DNA extracted with DNA extraction kits from the biological material and environmental samples.

Sampling

6.1 *Water* (wastewater, well water and drinking water) – 10-20 ml.

6.2 *Soil*.

6.3 *Washing fluids from air filters*.

6.4 *Powdery substances* (cattle food, meal, etc).

Human material:

6.5 Whole blood (5 ml).

Blood is collected after overnight fasting into Vacuette® tubes with 6 % EDTA solution (50 µl of EDTA solution per 1 ml of blood). Close the tubes with blood and overturn them gently several times (to mix the content).

6.6 Exudate from lesion foci.

Exudate from lesion foci (in case of skin form) should be placed to 200 µl of 0.9 % sterile saline solution and is used without pretreatment.

6.7 *Sputum*.

Sputum should be treated with **Mucolysin** reagent (180-CE) according to the Mucolysin *Instruction manual*. If it is necessary to repeat the analysis, the rest of pretreated sputum should be frozen.

Animal material:

6.8 Whole blood.

Whole blood (5 ml). Blood is collected into a Vacuette® tubes with 6 % EDTA solution (50 µl of EDTA solution per 1 ml of blood). Close the tube with blood and overturn them gently several times (to mix the content).

6.9 Cattle milk.

Cattle milk (pretreatment is not required).

6.10 Parenchymatous organs and lymph nodes.

NOTE: Biological material should be delivered to the laboratory in a container with ice within one day.

The above-mentioned biological material can be stored at 2-8 °C for 1 day before the test or at the temperature below minus 16 °C for 6 months. Only one freeze-thawing cycle is acceptable.

Pretreatment

6.11 Water and washing fluids from air filters.

Centrifuge water (10-20 ml) at 8000 g (10 000 rpm in a rotor with a radius of 70 mm or at 3 000 rpm in a rotor with a radius of 150 mm) for 15 min. Discard carefully the supernatant leaving ~ 100 µl. Resuspend the pellet in this solution (100 µl) and transfer the suspension to 1.5-ml tubes.

6.12 Soil.

Transfer 0.4-1.0 g (~ 1.0 ml) of soil into 5-ml tubes with tightly closing caps using individual spatula. Add 3 ml of a sterile 0.9 % saline solution, mix carefully, and incubate for 5 min at room temperature. Then transfer 1 µl of the obtained solution to 1.5-ml tubes with tightly closing caps and centrifuge the coarse fraction for 2–3 min at 300 g (2000 rpm in a rotor with a radius of 70 mm). Use the clarified supernatant in work.

6.13 Powdery substances.

Dissolve powdery substances (~ 0.05 cm^3) in 150 µl of sterile 0.9 % saline solution. Use the obtained solution in work.
Water-insoluble substances should be treated as soil samples.

6.14 Parenchymatous organs

Homogenize the pieces (with size of not less than 1 cm^3) and whole lymph nodes by trituration using sterile porcelain mortar and mallet or homogenizer, add equal volume of sterile 0.9 % saline solution (about 100 µl) and mix carefully. Suspension should be settled at the room temperature for 2-3 min. Then transfer upper phase to 1.5-ml tubes and use it on disinfection stage.

Disinfection

Disinfection is carried out in compliance with local authorities' requirements.

1. Spore germination.
   Inoculate preliminary prepared material (0.1 ml) into the tubes with Hottinger's broth (0.9 ml) (pH 7.2±0.1). Incubate the tubes using shake-flask propagator with vigorous aeration at (37±1) °C for 2.5 h.
2. Treatment with penicillin.
   Add a freshly prepared penicillin solution to the tubes (to final concentration 1000 U/ml) and incubate for another 15 min more at (37±1) °C.
3. Transfer 1 ml of obtained suspension to 1.5-ml tubes with tightly closing caps using an automatic pipette with tips with aerosol filter. Centrifuge at 12000 rpm for 10 min. Discard the supernatant, resuspend in 100 µl of 0.9 % saline solution, and incubated in a constant-temperature cabinet at (110±5) °C for 10 min.
4. Lysis Solution from the **DNA-sorb-B** (K1-2-100-CE) (if it was stored at 2–8 °C) should be heated at the temperature 60-65 °C until complete crystal dissolution. Add 300 µl of Lysis Solution to each tube with test samples (100 µl) and incubate at 65 °C for 15 min.

Further analysis is performed according to the **DNA-sorb-B** (K1-2-100-CE) protocol.

7 WORKING CONDITIONS

eSens Bacillus anthracis QL PCR kit should be used at 18–25 °C.

8 PROTOCOL

8.1 DNA extraction

Any commercial nucleic acid extraction kit, if validated for the indicated specimen types, could be used.

Ecoli Dx, s.r.o. recommends:

- For the manual extraction

- **DNA-sorb-B** (K1-2-100-CE)

- For the automatic extraction

- **ePure Bacterial DNA Extraction Kit** (E2006)

NOTE: Extract the DNA according to the manufacturer's protocol.
The DNA extraction for each sample is carried out in the presence of **Internal Control STI-704 (IC).**

8.2 Preparing PCR

8.2.1 Preparing tubes for PCR

The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, DNA and control samples into tubes.

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

1. Prepare the required number of tubes with **PCR-mix-1-FRT *Bacillus anthracis*** and wax for amplification of DNA from biological 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 into each tube ensuring that it does not fall under the wax and mix with **PCR-mix-1-FRT *Bacillus anthracis.***
3. Using tips with aerosol filter add **10 µl** of **DNA samples** obtained at the DNA extraction stage.
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 (C+*Bacillus anthracis*)** 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 (C+*Bacillus anthracis*)** to the tube labeled **C+*Bacillus anthracis pXO2*** (Positive Control of Amplification).
CS+	–	Add **10 µl** of **Positive Control STI-88** to the tube labeled **CS+** (Positive Control of Amplification).
C–	–	Add **10 µl** of **the sample extracted from the Negative Control (C–) reagent** to the tube labeled C– (Negative control of Extraction).

8.2.2 Amplification

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

Table 2

eSens-1 RG program

	Rotor-type Instruments (e.g Rotor-Gene Q or equivalent)		**Plate-type Instruments** e.g CFX 96 Touch, CFX 96 Opus, QuantStudio 5 or equivalent.
Step	**Temperature, °C**	**Time**	**Cycles**
1	**95**	**5 min**	**1**
2	**95**	**10 s**	**10**
	60	**25 s**	
	72	**10 s**	
3	**95**	**10 s**	**35**
	56	**25 s Fluorescence acquiring**	
	72	**10 s**	

Fluorescent signal is detected in the channels for the **FAM, JOE** and **ROX** fluorophores.

2. Adjust the fluorescence channel sensitivity.
3. Insert tubes into the reaction module of the device.
4. Run the amplification program with fluorescence detection.
5. Analyze results after the amplification program is completed.

9 DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in three channels.

- The signal of the *Bacillus anthracis* pXO1 DNA amplification product is detected in the channel for the FAM fluorophore.
- The signal of the *Bacillus anthracis* pXO2 DNA amplification product is detected in the channel for the JOE fluorophore.
- The signal of the IC DNA amplification product is detected in the channel for the ROX fluorophore.

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the cDNA sample in the corresponding column of the results grid.

Principle of interpretation is the following:

- ***Bacillus anthracis* pXO1+ and pXO2+ DNA** is detected in a sample if the *Ct* value determined in the results grid in the channels for FAM and JOE fluorophores is less than the specified boundary *Ct* value, regardless of the *Ct* value determined in the channel for ROX fluorophore.

- ***Bacillus anthracis* pXO1+ DNA** is detected in a sample if the *Ct* value determined in the results grid in the channel for FAM fluorophore is less than the specified boundary *Ct* value, regardless of the *Ct* value determined in the channel for ROX fluorophore.
- ***Bacillus anthracis* pXO2+ DNA** is detected in a sample if the *Ct* value determined in the results grid in the channel for JOE fluorophore is less than the specified boundary *Ct* value, regardless of the *Ct* value determined in the channel for ROX fluorophore.
- The sample is considered to be **negative** if the *Ct* value is absent in the channels for the FAM and JOE fluorophores, whereas the *Ct* value determined in the channel for the ROX fluorophore is less than the specified boundary *Ct* value.

Table 3

Results interpretation

***Ct* value in the channel for the fluorophore**			**Result**
FAM	**JOE**	**ROX**	
absent	absent	≤ boundary value	*Bacillus anthracis* is not detected
< boundary value	absent	≤ boundary value or absent	*Bacillus anthracis* (pXO1+/pXO2-)
< boundary value	< boundary value	≤ boundary value or absent	*Bacillus anthracis* (pXO1+/pXO2+)
absent	< boundary value	≤ boundary value or absent	*Bacillus anthracis* (pXO1-/pXO2+)
absent	absent	absent or > boundary value	Repeat the sample analysis beginning with the DNA extraction stage

The result of the analysis is considered reliable only if the results obtained for the Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see Table 4 and Table 5).

Table 4

Results for controls

Control	**Stage for control**	***Ct* value in the channel for fluorophore**		
		FAM	**JOE**	**ROX**
C–	DNA extraction	Absent	Absent	<boundary value
NCA	PCR	Absent	Absent	Absent
C+*Bacillus anthracis* pXO1	PCR	<boundary value	Absent	Absent
C+*Bacillus anthracis* pXO2	PCR	Absent	<boundary value	Absent
CS+	PCR	Absent	Absent	<boundary value

Table 5

Boundary *Ct* values

Sample	Channel	*Ct* value
C–	ROX/Orange	31
C+ *Bacillus anthracis* pXO1	FAM/Green	33
C+ *Bacillus anthracis* pXO2	JOE/Yellow	33
CS+	ROX/Orange	31
Test samples	FAM/Green	33
	JOE/Yellow	33
	ROX/Orange	31

10 TROUBLESHOOTING

The results of analysis are not taken into account in the following cases:

1. If the *Ct* value is absent for the Positive Controls of Amplification (C+), this indicates incorrectly chosen amplification program or other errors of amplification stage. PCR should be repeated.
2. If the *Ct* value determined in the channel for the FAM fluorophore is greater than the specified boundary *Ct* value, whereas the *Ct* value determined in the channel for the ROX fluorophore is less than the boundary *Ct* value, PCR should be repeated. The result of analysis is **positive** if the same result has been obtained or if the *Ct* value determined in the channel for the FAM fluorophore is less than the specified boundary *Ct* value.
3. If the *Ct* value determined in the channel for the JOE fluorophore is greater than the specified boundary *Ct* value, whereas the *Ct* value determined in the channel for the ROX fluorophore is less than the boundary *Ct* value, PCR should be repeated. The result of analysis is **positive** if the same result has been obtained or if the *Ct* value determined in the channel for the JOE fluorophore is less than the specified boundary *Ct* value.
4. If the *Ct* value is absent in the channels for FAM and JOE fluorophores, whereas the *Ct* value in the channel for the ROX fluorophore is greater than the specified boundary *Ct* value or absent, the amplification and detection should be repeated. If the same result is obtained, analysis of the sample should be repeated beginning with the DNA extraction stage.
5. If any *Ct* value is determined for the Negative Control of Extraction (C–) in the channels for the FAM and/or JOE fluorophores and for the Negative Control of amplification (NCA) (DNA-buffer) in any channel, it indicates contamination of reagents or samples. In this case, the results of analysis for all samples are **invalid**. The analysis for all samples should be repeated and measures for detecting and elimination of contamination source must be taken.

11 TRANSPORTATION

eSens Bacillus anthracis QL PCR kit should be transported at 2–8 °C for no longer than 5 days.

12 STABILITY AND STORAGE

All components of the **eSens Bacillus anthracis QL PCR kit** are to be stored at 2–8 °C when not in use. All components of the **eSens Bacillus anthracis QL PCR kit** are stable until the expiry date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-mix-1-FRT *Bacillus anthracis* is to be kept away from the light.

13 SPECIFICATIONS

13.1 Sensitivity

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

NOTE: The claimed analytical features of **eSens Bacillus anthracis QL PCR kit** are guaranteed only when additional reagent kit **DNA-sorb-B** (K1-2-100-CE) (manufactured by FBIS CRIE) is used.

13.2 Specificity

The analytical specificity of **eSens Bacillus anthracis QL PCR kit** is ensured by selection of specific primers and probes and stringent reaction conditions. The primers and probes have been checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The testing specificity of **eSens Bacillus anthracis QL PCR kit** was confirmed in laboratory trials.

14 QUALITY CONTROL

The production process, including batch release, is carried out in accordance with highest possible quality standards.

15 KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code		Contains sufficient for <n> tests
RUO	Research Use Only		Use-by Date
VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer	**NCA**	Negative control of amplification
	Date of manufacture	**C-**	Negative control of extraction
IC	Internal control	**C+**$_{\text{Bacillus anthracis pXO1}}$, **C+**$_{\text{Bacillus anthracis pXO2}}$	Positive control of amplification

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
01_04/2022		
02_11/2025	Throughout the entire document	Change of the intended use from IVD to RUO.

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