CE

IVD

For Professional Use Only

AmpliSens® *Bacillus anthracis*-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® *Bacillus anthracis*-FRT 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.

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

3. CONTENT

AmpliSens® *Bacillus anthracis*-FRT PCR kit is produced in 1 form:

AmpliSens® *Bacillus anthracis* -FRT PCR kit variant FRT (for use with RG)

REF R-B41(RG)-CE.

AmpliSens® *Bacillus anthracis*-FRT PCR kit variant FRT 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 (DNA-sorb-B, **REF** K1-2-50-CE).

AmpliSens® *Bacillus anthracis* -FRT PCR kit is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Tube racks.
- Vortex mixer.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia) or equivalent).
- Refrigerator at 2-8 °C.
- Deep-freezer at ≤ –16 °C.
- Waste bin for used tips.
- Agents kit for work space treatment.

5. GENERAL PRECAUTIONS

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 detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, protect eyes while samples and reagents handling. Thoroughly wash hands afterward.
- 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 samples and unused reagents in compliance with local authorities requirements.
- Samples 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 contact with the skin, eyes and mucosa. If skin, eyes and mucosa contact immediately flush with water, seek medical attention.
- 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 move to the Amplification and Detection Area. 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.

6. SAMPLING AND HANDLING

Obtaining samples of biological materials for PCR-analysis, transportation and storage are described in manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® *Bacillus anthracis* -FRT PCR kit is intended for the analysis of DNA extracted

by using DNA extraction kit from biological material and environmental samples.

The following material is used for analysis:

– Water (from water bodies, wastewater, and drinking water) – 10-20 ml;
– Soil;
– Washing fluids from air filters;
– Powdery substances (cattle food, meal, etc).

Human material:

– Whole blood (5 ml). Blood is taken fasting to a Vacuette® tube with 6 % EDTA (50 µl of EDTA per 1 ml of blood). Close the tube with blood and mixed the contents carefully by inverting several times.
– Exudate from lesion foci (in case of skin form) is placed to 200 µl of 0.9 % sterile NaCl solution (it can be used without pretreatment).
– Sputum is to be treated with Mucolysin reagent **REF** 180-CE according to Mucolysin instruction manual. If the analysis should be repeated, the remained sputum should be frozen.

Animal material:

– Whole blood (5 ml). Blood is taken to a Vacuette® tube with 6 % EDTA (50 µl of EDTA per 1 ml of blood). Close the tube with blood and mix by inverting several times.
– Cattle milk (without pretreatment).
– Parenchymal organs and lymph nodes.

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

Material pretreatment:

Water and washing fluids from air filters

10-20 ml of water is centrifuged 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. Carefully discard the supernatant leaving ~ 100 µl. Resuspended the pellet in this solution (100 µl) and transfer the suspension to 1.5-ml tubes.

Soil

Transfer 0.4-1.0 g (~ 1.0 ml) of soil to 5-ml tubes with tightly closed caps using individual spatula. Add 3 ml of a sterile 0.9 % NaCl 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 pellet the coarse fraction by centrifuging at 300 g (2000 rpm in a

rotor with a radius of 70 mm) for 2–3 min. Use the clarified supernatant in work.

Powdery substances

Powdery substances (~ 0.05 cm^3) are to be dissolved in 150 µl of a sterile 0.9 % NaCl solution. The obtained solution is used in work.

If the substances are not dissolvable in water, they should be treated as soil samples.

Parenchymal organs

Triturate the pieces with size of not less than 1 cm^3 and lymph nodes (as a whole) in sterile porcelain mortar, add near 100 µl of sterile 0.9 % NaCl solution and mix carefully. Suspension is settled at the room temperature during 2-3 min then upper phase is transferred to 1.5-ml tubes. Use on disinfection stage.

Disinfection:

Disinfection is carried out in compliance with local authorities' requirements

1. Spores germination.

   Seed preliminary prepared material *in quantity of* 0.1 ml to 0.9 ml of Hottinger broth (pH 7.2±0.1). Incubate at (37±1) °C for 2.5 h.
2. Treatment with penicillin.

   Add a freshly prepared solution to the tubes (final concentration, 1000 U/ml) and incubate for another 15 min more at (37±1) °C.
3. Transfer 1 ml of thus obtained suspension to 1.5-ml tubes with tightly closing caps using an automatic pipette with tips with aerosol barrier. Centrifuge at 12000 rpm for 10 min. Discard the supernatant, resuspend in 100 µl of 0.9 % NaCl, and incubated in a constant-temperature cabinet at (110±5) °C for 10 min.
4. Lysis Solution from the DNA-sorb-B kit (**REF** K1-2-50-CE), if stored at 2–8 °C, should be heated to 65 °C until the ice crystals disappear. Add 300 µl of Lysis Solution to each tube with 100 µl of decontaminated material and incubate at 65 °C for 15 min. Further analysis is performed according to the DNA-sorb-B **REF** K1-2-50-CE protocol.

7. WORKING CONDITIONS

AmpliSens® *Bacillus anthracis*-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. DNA extraction

It is recommended to use the following nucleic acid extraction kits:

- DNA-sorb-B, **REF** K1-2-50-CE.

Carry out the DNA extraction according to the manufacturer's instructions.

8.2. Preparing PCR

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

8.2.1 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 Control STI-88** to the tube labeled CS+ (Positive Control of Amplification).

8.2. 2. Amplification

1. Program the Rotor-Gene according to manufacturer's manual and Guidelines.
2. Create a temperature profile on your Rotor-Gene instrument as follows:

AmpliSens-1 RG program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycles*
Hold	95	5 min	–	1
Cycling	95	10 s	–	10
	60	25 s	–	
	72	10 s	–	
Cycling 2	95	10 s	–	35
	56	25 s	FAM/Green, JOE/Yellow, ROX/Orange	
	72	10 s	–	

3. Fluorescence is detected at the stage 2 of Cycling **(60 °C)** in FAM/Green, JOE/Yellow

and ROX/Orange fluorometer channels.

4. Make the adjustment of the fluorescence channel sensitivity according to Guidelines.

9. DATA ANALYSIS

See **Guidelines** for data analysis settings for Rotor-Gene 3000 or Rotor-Gene 6000.

Results interpretation

The results are interpreted by the software of Rotor-Gene 3000 or Rotor-Gene 6000 Instrument by the crossing (or not) of the fluorescence curve with the threshold line.

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

Table 1

Results for controls

Control	Controlled stage	Ct on channel FAM/Green	Ct on channel JOE/Yellow	Ct on channel ROX/Orange	Interpretation
C–	DNA extraction	Neg	Neg	Pos (< Z*)	OK
NCA	Amplification	Neg	Neg	Neg	OK
C+*Bacillus anthracis* pXO1	Amplification	Pos (< Y*)	Neg	Neg	OK
C+*Bacillus anthracis* pXO2	Amplification	Neg	Pos (< X*)	Neg	OK
CS+	Amplification	Neg	Neg	Pos (< N*)	OK

*For X, Y, and Z values, see Guidelines [2].

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 not less than Y and X, 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 Y, 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 X, 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 Z.

10. TROUBLESHOOTING

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

1. If no signal is detected for positive controls of amplification, this may indicate incorrect programming of the temperature profile of the PCR instrument used or other errors during PCR. PCR should be repeated.

2. If the Ct value in the FAM/Green channel exceeds Y and the Ct value in the ROX/Orange channel does not exceed Z, PCR should be repeated. The result of analysis is considered to be **positive** if it is the same or if the Ct value in the FAM/Green channel is less than Y.
3. If the Ct value in the JOE/Yellow channel exceeds X and the Ct value in the ROX/Orange channel does not exceed Z, PCR should be repeated. The result of analysis is considered to be **positive** if it is the same or if the Ct value in the JOE/Yellow channel is less than X.
4. If the Ct value is absent in FAM/Green and JOE/Yellow channels and the Ct value in the ROX/Orange channel exceeds Z or is absent, PCR with detection should be repeated. If the same result is obtained, analysis of the sample should be repeated from the DNA extraction stage.
5. If a Ct value is detected for C– in the JOE/Yellow and/or FAM/Green channel and for NCA (DNA-buffer) in any channel, this indicates contamination of reagents or samples. In this case, the results of analysis are considered to be **invalid**. Analysis should be repeated and measures to detect and eliminate the source of contamination should be taken.

If you have any further questions or if you encounter problems, please contact our Authorized representative in the European Community.

11. TRANSPORTATION

AmpliSens® ***Bacillus anthracis*-FRT** PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens®** ***Bacillus anthracis*-FRT** PCR kit are to be stored at 2–8 °C when not in use. All components of the **AmpliSens®** ***Bacillus anthracis*-FRT** PCR kit are stable until the expiration date on the label. The shelf life of opened reagents is the same as that of unopened reagents, unless otherwise stated.

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

13. SPECIFICATIONS

13.1. Sensitivity

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

The claimed analytical features of **AmpliSens® *Bacillus anthracis*-FRT** PCR kit are guaranteed only when additional reagent kit DNA-sorb-B (manufactured by Federal Budget Institute of Science "Central Research Institute for Epidemiology") is used.

13.2. Specificity

The analytical specificity of **AmpliSens® *Bacillus anthracis*-FRT** 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 **AmpliSens® *Bacillus anthracis*-FRT** PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook "Sampling, Transportation, and Storage of Clinical Material for PCR Diagnostics", developed by Federal Budget Institute of Science "Central Research Institute for Epidemiology" of Federal Service for Surveillance on Consumers' Rights Protection and Human Well-Being, Moscow, 2008.
2. Guidelines to **AmpliSens® *Bacillus anthracis*-FRT** PCR kits 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 developed by Federal Budget Institute of Science "Central Research Institute for Epidemiology".

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of **AmpliSens® *Bacillus anthracis*-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code		Sufficient for
IVD	*In vitro* diagnostic medical device		Expiration Date
VER	Version		Consult instructions for use
	Temperature limitation		Keep away from sunlight
	Manufacturer	**NCA**	Negative control of amplification
	Date of manufacture	**C–**	Negative control of extraction
EC REP	Authorised representative in the European Community	**C+*Bacillus anthracis* pXO1, C+*Bacillus anthracis* pXO2**	Positive Control of Amplification
FBIS CRIE	Federal Budget Institute of Science "Central Research Institute for Epidemiology"	**IC**	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.12.10	Cover page	The phrase “For Professional Use Only” was added
	Content	New sections “Working Conditions” and “Transportation” were added
		The “Explanation of Symbols” section was renamed to “Key to Symbols Used”
	Stability and Storage	The information about the shelf life of reagents before and after the first use was added
		Information that PCR-mix-1-FRT *Bacillus anthracis* is to be kept away from light was added
	Key to Symbols Used	The explanation of symbols was corrected
	Text	Positive Control STI (CS+) was changed to Positive Control STI-88 (CS+)
		Positive Control DNA *Bacillus anthracis* pXO1 (C1+) and Positive Control DNA *Bacillus anthracis* pXO2 (C2+) were changed to Positive Control DNA *Bacillus anthracis* pXO1 (C+$_{\textit{Bacillus anthracis}\ \text{pXO1}}$) and Positive Control DNA *Bacillus anthracis* pXO2 (C+$_{\textit{Bacillus anthracis}\ \text{pXO2}}$), respectively
21.06.11 LA	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”