



For *in Vitro* Diagnostic Use

TABLE OF CONTENTS

1. INTENDED USE3
 2. PRINCIPLE OF PCR DETECTION.3
 3. CONTENT.3
 4. ADDITIONAL REQUIREMENTS.....4
 5. GENERAL PRECAUTIONS.....4
 6. SAMPLING AND HANDLING5
 7. PROTOCOL.7
 8. DATA ANALYSIS.....8
 9. TROUBLESHOOTING.9
 10. STABILITY AND STORAGE.....10
 11. SPECIFICATIONS.....10
 12. REFERENCES.....10
 13. QUALITY CONTROL11
 14. EXPLANATION OF SYMBOLS11

AmpliSens® *Bacillus anthracis*-FRT

PCR kit

Instruction Manual

AmpliSens®



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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 vegetative and cryptogamic forms of *Bacillus anthracis* DNA in biological material and environmental compartments 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.

2. PRINCIPLE OF PCR DETECTION.

Bacillus anthracis 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 usually linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. The real-time PCR monitoring of the 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, which contain the Internal Control (IC). It must be used in the isolation procedure in order to control the isolation process of each individual sample and to identify possible reaction inhibition.

AmpliSens® *Bacillus anthracis*-FRT PCR kit uses “hot-start”, which greatly reduces frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using wax layer. Wax melting and reaction mix components occur 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 FEP includes:

Reagent	Description	Volume (ml)	Quantity
PCR-mix-1-FRT <i>Bacillus anthracis</i>	colorless clear liquid	0.008	55 tubes
PCR-mix-2-FL	colorless clear liquid	0.77	1 tube
Positive Control DNA <i>Bacillus anthracis</i> pXO1 (C1+)	colorless clear liquid	0.1	1 tube
Positive Control DNA <i>Bacillus anthracis</i> pXO2 (C2+)	colorless clear liquid	0.1	1 tube
Positive Control STI (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 isolation procedure as Negative Control of Extraction.

** add 10 µl of Internal Control during the DNA isolation 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 isolation 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 for temperature between 2 and 8 °C
- Deep-freezer with temperature not more than minus16°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 is 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 isolation kit from biological material and environmental compartments.

The following material is used for analysis:

- Water (from water body, wastewater, drinking water) – 10-20 ml.
- Soil.
- Washouts from air filters.
- Powdery substances (cattle food, meal etc)

Human material:

- Whole blood – 5 ml. Blood is taken fasting into Vacuette® tube, with 6% EDTA calculating 50 µl of EDTA per 1 ml of blood. Closed tube with blood is to be mixed carefully by overturning.
- Exudate from lesion foci (in case of skin form), is placed into 200 µl of 0,9 % sodium chloride sterile solution (it can be used without preliminary treatment).
- Sputum is to be treated with “Mucolysin” reagent **REF** 180, according to “Mucolysin”

instruction manual. If the analysis is to be repeated the rest of the sputum is to be frozen.

Animal material:

- Whole blood – 5 ml. Blood is taken into Vacuette® tube, with 6% EDTA calculating 50 µl of EDTA per 1 ml of blood. Closed tube with blood is to be mixed carefully by overturning.
- Cattle milk – without preliminary treatment.
- Parenchymal organs and lymph nodes.



Biological material is delivered to the laboratory in container with ice during a day.

Material's preliminary preparation:

Water and washouts from air filters.

10-20 ml of water is centrifuged at 8000g(10000 rpm at rotor's radius 70 mm or 3 000 rpm at rotor's radius 150 mm) during 15 min. Supernatant is to be removed carefully, reserving 100 µl. The residue is to be resuspended in 100 µl volume and transferred into tubes of 1,5 ml volume.

Soil:

Transfer 0.4-1.0 g (near 1.0 ml) of soil into 5 ml volume tubes with tightly closed caps by individual spatula. Add 3 ml of sterile 0.9 % sodium chloride solution, mix carefully and stand during 5 min. Then transfer 1 µl of obtained solution into 1.5 ml volume tubes with tightly closed caps and precipitate the coarse-dispersion fraction by centrifuging during 2-3 min at 300 g (2000 rpm at rotor's radius 70 mm)

Use clarified supernatant.

Powdery substances.

Powdery substances (near 0.05 cm³) are to be dissolved in 150 µl of sterile 0.9 % sodium chloride solution. Obtained solution is used in working.

If the substances aren't dissolved in water they should be treated as soil samples.

Parenchymal organs.

Triturate the pieces with size of not less than 1 cm³ and lymph nodes (as a whole) in sterile porcelain mortar, add near 100 µl of sterile 0.9 % sodium chloride solution and mix carefully. Suspension is settled at the room temperature during 2-3 min then upper phase is transferred into 1.5 ml volume 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 temperature (37±1) °C during 2,5 h.

2. Treatment by penicilline.

Add fresh made solution into the tubes (to end concentration - 1000 unit/ml) and incubate 15 min more at temperature (37±1) °C.

3. Transfer 1 ml of suspension into 1.5 ml volume tubes with tightly closed caps by automatic pipette with aerosol barrier tips. Centrifuge at 12000 rpm during 10 min. Remove supernatant, add 100 µl of 0.9 % sodium chloride solution, then resuspend. Thermostat at temperature (110±5) °C during 10 min.

4. Lysis Solution from “DNA-sorb-B”, **REF** K1-2-50-CE (if stored at 2-8 °C) should be heated to 65 °C until ice crystals disappear. Add 300 µl of Lysis Solution into each tube with 100 µl of decontaminated material and incubate at 65 °C for 15 min. Further analysis is performed according “DNA-sorb-B”, **REF** K1-2-50-CE Protocol.

7. PROTOCOL

7.1. DNA Isolation

It's recommended to use the following nucleic acid extraction kits:

- “DNA-sorb-B”, **REF** K1-2-50-CE.



Carry the DNA isolation according to the manufacturer’s instructions.

7.2. Preparing the PCR

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

7.2.1 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. Using tips with aerosol barrier add **10 µl** of **DNA** obtained from clinical or control samples at the DNA extraction stage into prepared tubes.

4. Carry out the control amplification reactions:

- NCA** -Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).
- C1+** -Add **10 µl** of **Positive Control DNA *Bacillus anthracis* pXO1** to the tube labeled C1+ (Positive Control of Amplification).
- C2+** Add **10 µl** of **Positive Control DNA *Bacillus anthracis* pXO2** to the tube labeled C2+ (Positive Control of Amplification).
- CS+** Add **10 µl** of **Positive Control STI** to the tube labeled CS+ (Positive Control of Amplification).

7.2. 2. Amplification

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

AmpliSens-1 RG program

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

3. Fluorescence detection is in Cycling 2 step (**60°C**) in FAM/Green, JOE/Yellow and ROX/Orange fluorometer channels.
4. Make the adjustment of the fluorescence channel sensitivity according to Appendix 1.

8. DATA ANALYSIS

See **Appendix 1** 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 isolation	Neg	Neg	Pos (< Z*)	OK
NCA	Amplification	Neg	Neg	Neg	OK
C1+	Amplification	Pos (< Y*)	Neg	Neg	OK
C2+	Amplification	Neg	Pos (< X*)	Neg	OK
CS+	Amplification	Neg	Neg	Pos (< N*)	OK

*For X, Y values see Appendix 1.

- The sample is considered to be positive for DNA *Bacillus anthracis* pXO1+ and pXO2+ presence, if Ct value on FAM/Green and JOE/Yellow channels is not less than Y and X respectively, regardless of Ct value on ROX/Orange channel.
- The sample is considered to be positive for DNA *Bacillus anthracis* pXO1+ presence, if Ct value on FAM/Green channel is less than Y, regardless of Ct value on ROX/Orange channel.
- The sample is considered to be positive for DNA *Bacillus anthracis* pXO2+ presence, if Ct value on JOE/Yellow channel is less than X, regardless of Ct value on ROX/Orange channel.
- The sample is considered to be negative, if Ct value on FAM/Green and JOE/Yellow channels is absent and a on ROX/Orange channel Ct value does not exceed Z.

9. TROUBLESHOOTING

Results of analysis are not being registered in the following cases:

- If no signal is detected for Positive Controls of amplification, it can indicate the incorrect programming of the temperature profile of used Instrument or other mistakes during of the PCR reaction carrying. PCR should be repeated.
- If Ct value on FAM/Green channel exceeds Y and Ct value on ROX/Orange channel does not exceed Z then PCR should be repeated. The result of analysis is considered to be positive if it is the same or if the Ct value on FAM/Green channel is less than Y.
- If Ct value on JOE/Yellow channel exceeds X, and Ct value on ROX/Orange channel does not exceed Z, then PCR should be repeated. The result of analysis is considered to be positive if it is the same or if the Ct value on JOE/Yellow channel is less than X
- If Ct value is absent on FAM/Green and JOE/Yellow channels and Ct value on ROX/Orange channels exceeds Z or absent, PCR and detection should be repeated. If the same result is obtained PCR should be repeated from the isolation stage.
- If the Ct value is present for the Negative Control on JOE/Yellow and/or FAM/Green channel

and for Negative Control of Amplification (DNA-buffer) on any channel it indicates the contamination of reagents or samples. In such case the results of analysis are considered to be irrelevant. Test analysis must be repeated and measures to detect and eliminate the source of contamination are to be taken.

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

10. STABILITY AND STORAGE

All components of the **AmpliSens® *Bacillus anthracis*-FRT** PCR kit are to be stored at the temperature between 2 °C and 8 °C, when not in use. All components of the **AmpliSens® *Bacillus anthracis*-FRT** PCR kit are to be stable until labeled expiration date.



All components of the **AmpliSens® *Bacillus anthracis*-FRT** PCR kit are to be stored away from the light.

11. SPECIFICATIONS

11.1. Sensitivity

Analytical Sensitivity of **AmpliSens® *Bacillus anthracis*-FRT** PCR kit is not less than 1×10^3 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 reagents kit "DNA-sorb-B" (manufactured by Federal State Institution of Science Central Research Institute of Epidemiology) is used.

11.2. Specificity

Specificity of **AmpliSens® *Bacillus anthracis*-FRT** PCR kit is assured by selection of specific primers and probes, as well as the selection of strict reaction conditions. The primers and probes were checked for possible homologies to all in gene banks published sequences by sequence comparison analysis. Specificity of **AmpliSens® *Bacillus anthracis*-FRT** PCR kit was confirmed in laboratory clinical trials.




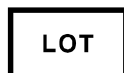




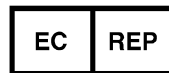


12. REFERENCES

- Handbook "Sampling, transportation, storage of clinical material for PCR diagnostics", developed by Federal State Institution of Science Central Research Institute of Epidemiology of Federal Service for Surveillance on Consumers' Rights Protection and Human Well-Being, Moscow, 2008.

13. QUALITY CONTROL

In compliance with Federal State Institution of Science “Central Research Institute of 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.

14. EXPLANATION OF SYMBOLS

	Manufacturer		Temperature limitation
	Use by		Batch code
	For <i>in Vitro</i> Diagnostic Use		Version
	Catalogue number	NCA	Negative Control of Amplification
	Contains sufficient for <n> tests		Authorised representative in the European Community.
	Consult instructions for use		Caution, consult accompanying documents
C+	Positive Control of Amplification	C-	Negative control of Extraction
IC	Internal Control		