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For Professional Use Only

AmpliSens® *Yersinia pestis*-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® ***Yersinia pestis*****-FRT** PCR kit is an *in vitro* nucleic acid amplification test for detection of *Yersinia pestis* DNA in biological material 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

Yersinia pestis DNA detection in human biological material (blood; node, vesicle, pustule, and carbuncle aspirate samples; sputum; oropharyngeal swabs; urine; feces; lymph nodes; liver, spleen, lungs, adrenal, and brain tissues; as well as pathologically changed tissues and organs), animal material (blood, feces, parenchymal organs, brain tissues, and pathologically changed tissues and organs), fleas, ticks, bird pellets, and soil by polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific *Yersinia pestis* primers. In real-time PCR, the amplified product is detected using 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. **AmpliSens®** ***Yersinia pestis*****-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®** ***Yersinia pestis*****-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 chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT

AmpliSens® ***Yersinia pestis*****-FRT** PCR kit is produced in 1 form:

AmpliSens® *Yersinia pestis*-FRT PCR kit variant FRT **REF** R-B79(RG,iQ,Dt)-CE.

AmpliSens® ***Yersinia pestis*****-FRT** PCR kit variant FRT includes:

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FRT *Yersinia pestis*	colorless clear liquid	0.6	1 tube
RT-PCR-mix-2-FEP/FRT	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
Positive Control DNA *Yersinia pestis* / STI ($C+_{Y.pestis / STI}$)	colorless clear liquid	0.1	1 tube
DNA-buffer	colorless clear liquid	0.6	1 tube
Internal Control STI-87 (IC)*	colorless clear liquid	0.6	1 tube

* add 10 µl of Internal Control STI-87 (IC) during the DNA extraction procedure directly to the sample/lysis mixture (see RIBO-prep, **REF** K2-9-Et-50-CE protocol).

AmpliSens® *Yersinia pestis*-FRT PCR kit is intended for 60 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.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia); Rotor-Gene Q (Qiagen, Germany); iCycler iQ5 (Bio-Rad, USA), or equivalent).
- Disposable polypropylene tubes for PCR (0.2-ml; for example, Axygen, USA).
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ – 16 °C.
- Waste bin for used tips.

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 and handle amplicons away from all other reagents.
- 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 another suitable disinfectant.
- Avoid contact with the skin, eyes, and mucosa. If skin, eyes, or mucosa contact, immediately flush with water and seek medical attention.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then 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® ***Yersinia pestis*****-FRT** PCR kit is intended for analysis of DNA extracted with DNA extraction kits from clinical material.

The following material is used for analysis:

- Fleas;
- Ticks;
- Bird pellets;

- Soil;
- Human biological material:
  - Whole blood;
  - Aspirates from buboes (vesicle, pustule, or carbuncle) is taken with a 5-ml syringe. Cleanse the skin area to be punctured first with 70% ethanol, then with a tincture of iodine (5 %), and then once again with 70 % ethanol. Insert the needle into the center of the bubo, then pull the hub as much as possible and slowly eject the needle. Since buboes are located between solid tissues, the volume of exudate withdrawn with the syringe is usually small and often fills only the needle bore. After removing the needle from the bubo, take 0.5 ml of 15 mM NaCl through it and empty the content into an Eppendorf tube. If it is impossible to take a sample of material, inject 0.3–0.5 ml of a sterile isotonic NaCl solution into the bubo. If the bubo bursts, take the material separately from the solid peripheral area and from the fistula discharge. Analyze both portions separately.
  - Sputum;
  - Oropharyngeal swabs are taken with a sterile probe from buboes located in the head and neck area;
  - Urine.
- Animal material
  - Blood;
  - Lymph nodes; liver, spleen, lung, adrenal, and brain tissues; and pathologically changed tissues and organs;
  - Feces.

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

The samples are to be stored at 2–8 °C for 1 day or at ≤ –16 °C for 6 months.

Only one freeze–thaw cycle of clinical material is allowed.

Pretreatment of material:

1. **Fleas**

   One test sample may contain up to 30 fleas. Insects should be ground in a homogenizer or a sterile mortar with pestle. Add 0.5 ml of a sterile 0.9 % NaCl solution or PBS and mix thoroughly. Centrifuge the suspension at 3000 rpm for 2 min (500 g, 50 mm radius rotor), then transfer 100 µl of the top phase into 1.5-ml tubes and use at

the disinfection stage.

2. **Ticks**

   A pooled sample of imagoes may contain up to 3 blood-filled ticks and 10 hungry ticks; a pooled sample of nymphs, up to 10 blood-filled ticks and 30 hungry ticks; and a pooled sample of larvae, up to 30 blood-filled ticks. Pierce the blood-filled imagoes with a needle to release the blood. When using a closed-type homogenizer, this procedure can be omitted. Grind the sample using a homogenizer or a sterile mortar and pestle. Add 1 ml of a sterile 0.9 % NaCl or PBS and mix thoroughly. Centrifuge the suspension at 3000 rpm for 2 min (500 g, 50 mm radius rotor) and transfer 50 µl of the top phase to 1.5-ml tubes for DNA extraction.

3. **Human and animal feces**

Preparation of 10–20 % suspension:

- Add 2 ml of saline or PBS to 5-ml tubes with tightly sealing caps.
- Add 0.5–1.0 g (~ 0.5–1.0 ml) of feces to each tube using the single tips with aerosol barriers (or a disposable scapula). Mix thoroughly to obtain a homogenous suspension. If it is necessary to store the samples, add glycerol to the suspension (final concentration, 20 %), mix it, and store at ≤ –16 °C.
- Preparation of fecal bacterial fraction:

  Transfer 1 ml of the suspension into 1.5-ml tubes with tightly sealing caps and centrifuge at 8000 g for 5 min. Use 100 µl of the clarified fraction taken from the boundary of the liquid transparent and solid fecal fractions to extract DNA.

4. **Bird pellets**

   Thoroughly grind bone marrow fragments from bone remains from bird pellets using a homogenizer or a sterile mortar and pestle. Add sterile 0.9 % NaCl or PBS (at least 500 µl) to obtain a 10 % suspension and mix thoroughly. Allow the suspension to settle at room temperature for 2–3 min and then transfer the top phase into 1.5-ml tubes. Use 100 µl of the suspension to extract DNA.

5. **Soil**

   Transfer 0.4–1.0 g (~ 1.0 ml) of soil into 5-ml tubes with tightly sealing caps using disposable spatula. Add 3 ml of 0.9 % NaCl, mix thoroughly, and allow to settle for 5 min. Transfer 1 ml of solution from tubes with settled ground into 1.5-ml tubes with tightly closed cap. Precipitate the coarsely dispersed fraction by centrifuging at 300 g for 2–3 min (2300 rpm, 50 mm radius rotor). Use 100 µl of the clarified supernatant to extract DNA.

6. **Blood**

Take a whole blood specimen in the morning after overnight fasting to a tube with 6 % EDTA (ratio, 1:20). Invert the closed tube several times. Transfer 1.5 ml of the whole blood with EDTA into an Eppendorf tube. Centrifuge at 800 rpm for 10 min (380 g, 50 mm radius rotor). Transfer the top layer of plasma with leucocytes (500–600 µl) into another Eppendorf tube and centrifuge it at 8000 rpm for 5 min. Transfer the supernatant (leaving ~200 µl of liquid above the cell pellet) into a container with a disinfectant. Use the cell pellet and 200 µl of the supernatant to extract DNA.

If blood clots from the heart and large vessels of animals are used for analysis, the procedure of sampling is the same as for organs.

7. Sputum

Pretreatment is performed in accordance with the instruction manual for the Mucolysin reagent. Use 50 µl of the sample for DNA extraction.

8. Oropharyngeal swabs

Oropharyngeal swabs are taken using sterile dry probes with cotton tips from the surface of tonsils, palatine arches, and the posterior oropharynx. After sampling, transfer the part of the probe with the cotton swab to a sterile disposable tube containing 500 µl of the transport medium for storage and transportation of respiratory swabs (or sterile saline or PBS). Break the end of probe or cut it with sterile scissors so that the cap of the tube could be sealed tightly. Close the tube with the solution and the probe fragment. Before starting work, the probe should be removed from the tube and discarded into disinfectant. Use 100 µl of the sample to extract DNA.

9. Urine

Collect urine into a clean container. When storage is required, transfer the urine sample into a 20-ml centrifuge tube or an Eppendorf tube, add glycerol (10 % of the sample volume), and mix. Urine samples with glycerol can be stored at ≤ –20 °C for 1 week or at ≤ –70 °C for a long time.

When using a refrigerated centrifuge for 20-ml tubes (4 °C, 8000 g) the following pretreatment procedure is used:

— Centrifuge the sample at 8000–9000 g for 10 min. Discard the supernatant (except for 1 ml of liquid above the cell pellet) into a container with disinfectant. Transfer the cell pellet and 1 ml of the supernatant to an Eppendorf tube. Centrifuge the sample at 8000 g for 10 min once again. Discard the supernatant (900 µl) into a container with disinfectant. Use the pellet and 100 µl of the supernatant to extract DNA. If the urine sample contains excess salts, only the supernatant (100 µl) is transferred into an Eppendorf tube and used to extract DNA.

— In the absence of a centrifuge for 20-ml tubes with a speed of 8000 g, concentrate bacteria from only 1 ml of urine as specified above. Use the pellet and 100 µl of the supernatant to extract DNA.

10.Organs

Thoroughly grind fragments of organs (no less than 0.5 cm^3 in size) and lymph nodes (whole) in a homogenizer or a sterile mortar with pestle. Add sterile 0.9 % NaCl (no less than 500 µl) or PBS and mix thoroughly. Allow the suspension to settle at room temperature for 2–3 min and then transfer the top phase into 1.5-ml tubes. Use 50 µl of the suspension to extract DNA.

Before disinfection and DNA extraction, the pretreated material can be stored at ≤ –20 °C for 1 month or at ≤ –70 °C for a long time.

7. WORKING CONDITIONS

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

8. PROTOCOL

8.1. DNA extraction

For a complete PCR assay, use the following nucleic acid extraction kit:

- RIBO-prep, **REF** K2-9-Et-50-CE.

Extract DNA from all types of biological material as described in the instruction manual to the RIBO-prep reagent kit with some modifications specified below (item 8.1.2).

Prior to DNA extraction, test material must be disinfected.

8.1.1 Disinfection of test material

Add sodium merthiolate to the samples prepared for DNA extraction to a final concentration of 0.01% (dilution, 1:10000). The recommended volumes of test samples are specified in Section 6 (Pretreatment of material). Incubate the samples at 56 °C for 30 min, then add 300 µl of Solution for Lysis containing 6M guanidine thiocyanate and incubate at 65 °C for 15 min.

Thus disinfected material is then used for DNA extraction.

8.1.2 DNA extraction with RIBO-prep

DNA extraction from all types of biological material

Add **10 µl** of **Internal Control STI-87 (IC)** and **300 µl** of **Solution for Lysis** to the tube labeled **C–** (Negative Control of Extraction).

After adding **Internal Control STI-87 (IC)** and heating at 65 °C for 5 min, centrifuge the tubes at 5,000 rpm for 5 s to be sure there are no drops on the caps.

Centrifuge the tubes at 13,000 rpm for 2 min:

- after adding **500 µl** of **Washing Solution 3**;
- after adding **200 µl** of **Washing Solution 4**.

8.2. Preparing PCR

8.2.1 Preparing tubes for PCR

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

1. Prepare the required number of tubes with the **reaction mixture**. Take into account that each run includes at least three control points: Negative Control of Extraction (NCE) and Positive and Negative Controls of Amplification (C+$_{Y.pestis\ /\ STI}$ and NCA). Take excess reagents for one extra reaction.
2. Mix in a new tube **PCR-mix-1-FRT *Yersinia pestis***, **RT-PCR-mix-2 FEP/FRT**, and **polymerase (TaqF)**, calculating per <u>each</u> reaction:
   - **10 µl PCR-mix-1-FRT *Yersinia pestis*;**
   - **5 µl RT-PCR-mix-2-FEP/FRT;**
   - **0.5 µl polymerase (TaqF).**
3. Prepare the required number of tubes for amplification of DNA from clinical and control samples.
4. Add **15 µl** of the prepared mixture into each tube.

Do not store the prepared mixture.

5. Using tips with aerosol barrier, add **10 µl** of **DNA samples** obtained from clinical or control samples at the DNA extraction stage into the tubes with the reaction mixture. Carefully mix the contents of the tubes by pipetting.
6. Carry out the control amplification reactions:

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

C+$_{Y.pestis\ /\ STI}$ - Add **10 µl** of **Positive Control DNA *Yersinia pestis* / STI** to the tube labeled C+$_{Y.pestis\ /\ STI}$ (Positive Control of Amplification).

C– - Add **10 µl** of the sample that was extracted from the sample labeled C– (see Section 8.1.2).

Amplification should be started immediately after mixing the reaction mixture with DNA samples and controls.

8.2.2. Amplification

1. Program the real-time PCR instrument according to manufacturer's manual and Guidelines [2].
2. Create a temperature profile on your PCR instrument as follows:

Table 1

Amplification program

	Rotor-type instruments[1] and plate-type instruments[2]		
Step	***Temperature, °C***	***Time***	***Cycles***
1	95	15 min	1
2	95	5 s	5
	60	20 s	
	72	15 s	
3	95	5 s	40
	60	30 s *fluorescent signal detection*	
	72	15 s	

3. Fluorescent signal is detected in the channels designed for the FAM and JOE fluorophores on the 2nd step (60 °C) of stage 2.
4. Insert tubes into the reaction module of the instrument.

It is necessary to insert a test tube into well No. 1.

5. Run the amplification program with fluorescence detection.
6. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

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 fluorescent signal intensity is detected in two channels:

– The signal from the amplification product of the Internal Control STI-87 DNA is detected in the FAM channel;

– The signal from the *Yersinia pestis* DNA amplification product is detected in the JOE channel.

The results are interpreted by the crossing (or not-crossing) of the fluorescence curve with a threshold line and shown as the presence (or absence) of a Ct (threshold cycle) value in the result grid.

[1] For example, Rotor-Gene 3000, Rotor-Gene 6000, or equivalent.
[2] For example, iCycler iQ5, or equivalent.

9.1. Interpretation of results

Principle of interpretation:

- *Yersinia pestis* DNA is **detected** in a sample if its Ct value detected in the result grid in the JOE channel is less than the specified boundary Ct value. Moreover, the fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.
- *Yersinia pestis* DNA is **not detected** in a sample if its Ct value detected in the result grid in the FAM channel is less than the specified boundary Ct value, whereas the Ct value in the JOE channel is not detected.
- The result is **invalid** if the Ct value of a sample in the JOE channel is absent whereas the Ct value in the FAM channel is either absent or greater than the specified boundary Ct value. Repeat the PCR test for such a sample starting from the DNA extraction stage.

Boundary Ct values are specified in the *Important Product Information Bulletin* enclosed in the PCR kit. See also the Guidelines manual [2].

The result of the analysis is considered reliable only if the results for Positive and Negative Controls of Amplification as well as Negative Control of Extraction are correct (Table 2).

Table 2

Results for controls

Control	Stage for control	Ct value in channel	
		JOE	FAM
NCE	DNA extraction	Neg	Pos (< boundary Ct value*)
NCA	Amplification	Neg	Neg
C+$_{Y.pestis\ /\ STI}$	Amplification	Pos (< boundary Ct value*)	Pos (< boundary Ct value*)

10. TROUBLESHOOTING

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

1. If the Ct value of the Positive Control of Amplification (C+$_{Y.pestis\ /\ STI}$) in the JOE channel is absent or greater than the specified boundary Ct value, PCR should be repeated for all samples where the *Yersinia pestis* DNA was not detected.
2. If the Ct value of the Negative Control of Extraction (NCE) is detected in the JOE channel, PCR should be repeated for all samples where *Yersinia pestis* DNA was detected.
3. If the Ct value is present for the Negative Control of Amplification (NCA) in FAM and/or JOE channels, PCR should be repeated for all samples where *Yersinia pestis* DNA was

detected, with simultaneously running NCA in triplicate.

11. TRANSPORTATION

AmpliSens® ***Yersinia pestis*-FRT** PCR kit should be transported at 2–8 °C for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens®** ***Yersinia pestis*-FRT** PCR kit (except for PCR-mix-1-FRT *Yersinia pestis*, RT-PCR-mix-2-FEP/FRT, and polymerase (TaqF)) are to be stored at 2–8 °C when not in use. All components of the **AmpliSens®** ***Yersinia pestis*-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 *Yersinia pestis*, RT-PCR-mix-2-FEP/FRT, and polymerase (TaqF) are to be stored at temperature from minus 24 to minus 16 °C when not in use.

PCR-mix-1-FRT *Yersinia pestis* is to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

The analytical sensitivity of **AmpliSens®** ***Yersinia pestis*-FRT** PCR kit is specified in the table below.

Biological material (volume of sample)	RNA/DNA extraction kit	PCR kit	Analytical sensitivity	Pretreatment of biological material
- fleas (30 specimens homogenized in 500 µl of PBS, sample volume 100 µl); - *Dermacentor reticulatus* ticks (pool of 10 specimens, sample volume 50 µl); - blood (200 µl); - urine (100 µl); - sputum (50 µl); - feces (100 µl of 10 % suspension); - 10 % suspension of liver tissue, lymph nodes (50 µl)	RIBO-prep	PCR kit variant FRT	1 x 10^3 copies/ml	Indicated sensitivity can be reached only if the specified pretreatment instructions are followed and the specified specimen volume is used

13.2. Specificity

The analytical specificity of **AmpliSens®** ***Yersinia pestis*-FRT** PCR kit was assessed using the following microorganisms: *Yersinia enterocolitica* (326 strains),

Y.pseudotuberculosis (145 strains); *Shigella sonne*, *Sh.flexneri*; *Salmonella typhi*, *S.enteritidis*; *Klebsiella pneumonia*; *Esherichia coli* NCTC 9001; *Enterococcus faecalis*; *Staphylococcus aureus*, *St.saprophyticus*; *Pseudomonas aeruginosa*; *Proteus mirabilis*; and *Enterobacter cloacae*.

No false-positive results were observed during analysis of DNA of the above-mentioned microorganisms as well as DNA of ticks, mosquitoes, birds, and humans.

The clinical specificity of **AmpliSens® *Yersinia pestis*-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® *Yersinia pestis*-FRT PCR Kit for *Yersinia pestis* DNA Detection in Biological Material by the Polymerase Chain Reaction (PCR) with Hybridization-Fluorescence Detection”.

15. QUALITY CONTROL

In accordance with Federal Budget Institute of Science “Central Research Institute for Epidemiology” ISO 13485-Certified Quality Management System, each lot of **AmpliSens® *Yersinia pestis*-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
RUO	Research use only		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
FBIS CRIE	Federal Budget Institute of Science “Central Research Institute for Epidemiology”	**C+**	Positive control of amplification
		IC	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
09.07.11 LA	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
01.07.12 lvl	Title page, Key to symbols used	Symbol IVD *in vitro* diagnostic medical device was changed to RUO research use only
	Sensitivity	Analytical sensitivity was changed from 5 x 10^3 copies/ml to1 x 10^3 copies/ml