IVD For *in Vitro* Diagnostic Use

CE

Yersinia pestis Real-TM

Handbook

Real Time PCR kit for qualitative detection of *Yersinia pestis*

REF B79-50FRT

Σ 50

NAME

Yersinia pestis Real – TM

INTENDED USE

Yersinia pestis Real-TM PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Yersinia pestis* DNA in the 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 means of real-time hybridization-fluorescence detection.

PRINCIPLE OF ASSAY

Yersinia pestis detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region by using specific 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. The real-time 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. **Yersinia pestis Real-TM** 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 chemically modified polymerase (TaqF), which is activated by heating at 95 °C for 15 min.

Yersinia pestis DNA detection in clinical samples includes:

(a) *Yersinia pestis* DNA extraction from biological materials simultaneously with the exogenous Internal Control.

(b) Multiplex real-time PCR of a DNA fragment of *Yersinia pestis* and an artificial DNA fragment cloned into phage λ, which is used as a noncompetitive exogenous Internal Control.

Yersinia pestis DNA amplification is detected in the **JOE/Yellow/HEX/Cy3** channel, the noncompetitive exogenous **Internal Control** amplification is detected in the **FAM/Green** channel. The exogenous Internal Control allows monitoring the main steps of PCR analysis (DNA extraction and amplification). The main advantage of a noncompetitive exogenous Internal Control is the extension of the linear detection range and, therefore, an increase in the analytical sensitivity of the test.

MATERIALS PROVIDED

Reagent	*Volume, ml*	*Quantity*
PCR-mix-1-FRT *Y.pestis*	0.6	1 tube
PCR-mix-2-FRT	0.3	1 tube
Polymerase (TaqF)	0.03	1 tube
Pos Control DNA *Y.pestis*/STI (C+)	0.1	1 tube
DNA-buffer	0.5	1 tube
Negative Control (C-)*	1.2	1 tube
Internal Control STI-87 (IC)**	0,6	1 tube

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *add 10 µl of Internal Control to each sample during the DNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

- 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.
- Real Time PCR thermocycler
- Disposable polypropylene microtubes for PCR or PCR-plate
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

STORAGE INSTRUCTIONS

All components of the **Yersinia pestis Real-TM** PCR kit are to be stored at -20°C. All components of the **Yersinia pestis Real-TM** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

The kit can be shipped at 2-8°C but should be stored at -20°C immediately on receipt.

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

STABILITY

Yersinia pestis Real-TM Test 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:

- 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.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Yersinia pestis Real-TM PCR kit is intended to analyze DNA extracted from:

- 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.

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/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.

PROTOCOL (Reaction volume 25 µl):

1. Prepare required quantity of reaction tubes for samples (N) and controls (N+2).
2. Prepare in the new sterile tube for each sample **10*N µl** of **PCR-mix-1**, **5,0*N** of **PCR-mix-2-FRT** and **0,5*N** of **TaqF Polymerase.** Vortex and centrifuge for 2-3 sec.
3. Add **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. Mix by pipetting.
4. Prepare for each panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control (NCA);
   - add **10 µl** of ***Yersinia pestis* C+** to the tube labeled. *C*+;

Yersinia pestis on the JOE (Yellow)/HEX/Cy3 channel, IC is detected on the FAM (Green) channel.

Amplification

Program the real-time instrument according to the manual provided by the manufacturer.

Amplification program for rotor[1] and plate-type instruments[2]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
1	95 °C	15 min	–	1
2	95 °C	5 s	–	5
	60 °C	20 s	–	
	72 °C	15 s	–	
3	95 °C	5 s	–	40
	60 °C	30 s	FAM, HEX/Cy3/JOE	
	72 °C	15 s		

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

INSTRUMENT SETTINGS

Settings for rotor-type instruments (Rotor-Gene 3000/6000, Rotor-Gene Q, etc.)

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct	Eliminate Cycles before
FAM/Green	0.03	10 %	on	5
JOE/Yellow	0.03	10 %	on	5

Note: if the fluorescence curves in JOE channel do not correspond to exponential growth, set the threshold level of negative samples (***NTC threshold***) equal to 15 %.

Settings for plate-type instruments (iCycler iQ, iQ5, etc.)

iCycler iQ, iQ5	When analyzing amplification results, set the following parameters: in ***Base Line cycles*** menu, select ***User Defined***, ***Select all***, ***Edit Range*** and set ***Start Cycle* = 2**, ***Ending Cycle* = 20** (in FAM channel); set ***Start Cycle* = 2**, ***Ending Cycle* = 10** (in JOE channel). In ***Crossing Threshold*** menu, select ***User Defined***, set ***Threshold Position*** for **FAM** channel equal to **50** and for **JOE** channel equal to **100**.

Modular and Plate-type instruments (SaCycle-96, SmartCycler, Mx3005P, ABI 7500/7300,StepOne, LineGeneK)

Channel	Threshold
FAM	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.

Ct boundary values

Sample	Instrument			
	Rotor-Gene 3000/6000/Q		SaCycler-96, iCycler iQ, iQ5, MX, ABI, SC	
	FAM/Green	JOE/Yellow	FAM	HEX/JOE
	IC detection	*Y.pestis* detection	IC detection	*Y.pestis* detection
C–	< 24	-	< 28	-
C+$_{Y.pestis\ /\ STI}$	< 27	< 23	< 31	< 30
Clinical samples	< 27	< 38	< 31	< 39

DATA ANALYSIS

***Yersinia pestis* DNA** amplification product is detected in the **JOE/Yellow/HEX** channel, Internal Control amplification product is detected in the **FAM/Green** channel.

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 analysis results are considered valid, only if the control samples results comply with the following:

1. The sample is considered **positive** if Ct values detected in the FAM/Green and JOE/Yellow/HEX channel are less than the boundary Ct values (≤ 38) for these channels. The fluorescence curve should have a typical sigmoid shape and cross the threshold line in the region of significant fluorescence increase only once.
2. The sample is considered **negative** if its fluorescence curve does not cross the threshold line (Ct value is absent) and does not have the typical shape.

The results of analysis are considered reliable only if the results obtained for Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct.

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity of **Yersinia pestis Real-TM** PCR kit is 10^3 *Yersinia pestis* DNA copies/ml.

*The claimed analytical features of **Yersinia pestis Real-TM** PCR kit are guaranteed only when additional reagent kit (DNA/RNA-prep or DNA-sorb-C) is used.*

Specificity

The analytical specificity of **Yersinia pestis Real-TM** PCR kit is ensured by selection of specific primers and probes as well as 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 **Yersinia pestis Real-TM** PCR kit was confirmed in laboratory clinical tests.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam/Green): retesting of the sample is required.
    - The PCR was inhibited.
        ⇒ Make sure that you use a recommended DNA extraction method and follow the manufacturer's instructions.
    - The reagents storage conditions didn't comply with the instructions.
        ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        ⇒ Check the PCR conditions and for the IC detection select the fluorescence channel reported in the protocol.
    - Improper DNA extraction.
        ⇒ Repeat analysis starting from the DNA extraction stage
    - The IC was not added to the sample during the pipetting of reagents.
        ⇒ Make attention during the DNA extraction procedure.
2. Weak (Ct > 38) signal on the Joe (Yellow)/Cy3/HEX channel: the result is considered equivocal. It is necessary to repeat the analysis twice. If a positive Ct value is detected twice, the sample is considered as positive.
3. Joe (Yellow)/Cy3/HEX 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 among tubes.
        ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
    - Contamination during PCR procedure. All samples results are invalid.
        ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        ⇒ Pipette the Positive controls at the end.
        ⇒ 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	**C–**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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