CE

IVD

For Professional Use Only

AmpliSens® *MTC*-FEP

PCR kit

Instruction Manual

AmpliSens®

EC REP

Ecoli s.r.o., Studenohorska 12
841 03 Bratislava 47
Slovak Republic
Tel.: +421 2 6478 9336
Fax: +421 2 6478 9040

Federal Budget Institute of
Science “Central Research
Institute for Epidemiology”
3A Novogireevskaya Street
Moscow 111123 Russia

TABLE OF CONTENTS

1. INTENDED USE

AmpliSens® ***MTC*-FEP** PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Mycobacterium tuberculosis (MBT)* DNA – *Mycobacterium tuberculosis complex (MTC),* including *M.tuberculosis, M.bovis, M.africanum, M.microti, M.canetti, M.pinipedii* – in clinical materials, paraffin units, cultures of microorganisms and environmental objects by using end-point hybridization-fluorescence detection of amplified products.

The results of PCR analysis are taken into account in complex diagnostics of disease.

2. PRINCIPLE OF PCR DETECTION

Mycobacteria tuberculosis detection by the polymerase chain reaction (PCR) is based on the amplification of a pathogen genome specific region using special *Mycobacteria tuberculosis* primers. In **F**luorescent **E**nd-**P**oint PCR, the amplified product is detected by using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. A multichannel rotor-type fluorometer is specially designed to detect fluorescence emission from the fluorophores in a reaction mixture after PCR. It allows detection of the accumulating product without re-opening the reaction tubes after the PCR run. **AmpliSens®** ***MTC*-FEP** 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®** ***MTC*-FEP** 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 or a chemically modified polymerase (TaqF). Wax melts and reaction components mix only at 95 °C. Chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

AmpliSens® ***MTC*-FEP** PCR kit includes enzyme uracil-DNA glycosylase (UDG) to reduce the risk of contamination.

For optimization of *Mycobacterium tuberculosis* research report, an integrated procedure of DNA extraction for quantitative detection, identification to species, and determination of resistance to antitubercular therapy can be carried out.

3. CONTENT

AmpliSens® ***MTC*-FEP** PCR kit is produced in 1 form:

AmpliSens® *MTC*-FEP PCR kit variant FEP, **REF** B57-FEP-CE.

AmpliSens® *MTC*-FEP PCR kit includes:

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FEP *MTC*	colorless clear liquid	0.28	2 tubes
PCR-buffer-Flu	colorless clear liquid	0.28	1 tube
PCR-mix-Background	colorless clear liquid	0.5	1 tube
Mineral oil for PCR	colorless viscous liquid	2.0	1 dropper bottle
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
Enzyme UDG	colorless clear liquid	0.03	1 tube
Positive Control DNA *MTC* / STI ($C+_{MTC/STI}$)	colorless clear liquid	0.1	1 tube
TE-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-) *	colorless clear liquid	1.6	1 tube
Internal Control STI-87 (IC) **	colorless clear liquid	1.0	1 tube
RNA-buffer ***	colorless clear liquid	1.2	1 tube

* must be used in the extraction procedure as Negative Control of Extraction.

** add 10 µl of Internal Control STI-87 (IC) during the DNA extraction procedure directly to the sample/lysis mixture (DNA-sorb-C, **REF** K1-6-50-CE or DNA-sorb-B, **REF** K1-2-50-CE or RIBO-prep, **REF** K2-9-Et-50-CE).

*** used for elution during DNA extraction (for RIBO-prep, **REF** K2-9-Et-50-CE).

AmpliSens® *MTC*-FEP 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.
- Desktop centrifuge with a rotor for 2-ml tubes.
- PCR box.
- Personal thermocyclers (for example, Maxygene (Axygen, USA) or equivalent).

- Fluorometer (for example, ALA-1/4 (Biosan, Latvia) or equivalent).
- Personal computer.
- Disposable polypropylene microtubes for PCR (0.5- or 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, 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 samples and unused reagents in compliance with local authorities' requirements.
- Special precautions, in compliance with local authorities' requirements, should be taken when working in laboratories of antituberculosis institutions.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices. Infected material and disposable plastic water that was in contact with infected material must be treated with chlorine-containing solutions.
- 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

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.

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.

6.1. Material sampling

1. *Bronchoalveolar lavage (BAL) and bronchoalveolar lavage fluid (BALF), liquor,* are collected to disposable hermetically screwed polypropylene vessels (for preventing adhesion of the cells on their internal surface) with a volume no less than 5 ml.
2. *Sputum and urine (medium portion)* is collected to disposable graduated screwed vessels with a wide neck with a volume no less than 50 ml.
3. *Fasting morning whole blood and pleural fluid* is collected to tubes (for example, Vacuette®) with EDTA spraying or its solute. Close the tube and turn it upside down and back several times.
4. *Menstrual blood* is collected to dry disposable test tubes using a Kafka cap.
5. *Synovial fluid* is collected to dry disposable test tubes.
6. *Prostate gland secretion* is collected to sterile disposable 1.5-ml tubes after massage of the prostate gland. If, after massage of the prostate gland, it is impossible to get the secretion, use the first portion of urine, which contents the prostate gland secretion.
7. *Tissue (biopsy, surgical) material* is collected to tubes (for example, Vacuette®) with EDTA or to disposable tubes with 0.2 ml of sterile saline or PBS.
8. Paraffin units are cut by using microtome. Then cut out a fragment of tissue by disposable scalpel, remove paraffin by using xylene, remove xylene by series of ablution with decrease of ethanol concentration (similarly to standard histolytic conducting).
9. *Cultures of microorganisms* grown on selective solid nutrient media for *Mycobacteria tuberculosis* are collected to glass tubes as working with turbidity standard by resuspending in saline. *Cultures of microorganisms*, grown on selective liquid nutrient media, are used in original vial.
10. *Washing fluids from environmental objects* are collected by a tent with a wad wetted in saline. The square of washing from a flat surface is 5-10 cm^2. The working part of the tent is to be transferred to the 1.5-ml tube with 0.5 ml of sterile saline. The top of the tent is to be broken and removed.

The samples (except for urine) are stored at 2–8 °C for 3 days, at ≤ –16 °C for 1 year. For archiving (more than 1 year), store the samples at ≤ –68 °C.

Urine is stored at 2–8 °C for 6 hours. Freeze urine for a long storage. Repeated freeze is possible.

Do not freeze blood.

Transport the samples in a thermocontainer for no more than 3 days.

6.2. Preparation of samples

1. Mix *BAL* or *BALF* by turning upside down and back. Transfer 1 ml of the sample to a 1.5-ml Eppendorf tube using a pipette with a tip with aerosol barrier, mark it, and centrifuge at 10000 g for 10 min. Carefully remove the supernatant using a tip with aerosol barrier and leaving about 100 µl of the sample.
2. Add “Mucolysin” to the vessel with *sputum* (5 : 1, v/v) and then add and 3-5 sterile porcelain or glass beads to this mixture. Stir the vessel periodically for 20–30 min. Transfer 100 µl of the sample to 1.5-ml tube Eppendorf using a pipette with a tip with aerosol barrier and mark it.
3. Mix *urine* by turning the vessel upside down and back. Using a pipette with a tip with aerosol barrier, transfer 5–10 ml of the sample to a screwed tube, mark it, and centrifuge at 10000 g for 10 min (or at 3000 g for 20 min). Carefully remove the supernatant using a tip with aerosol barrier and leaving about 100 µl of the sample (if the pellet is visible, remove the supernatant leaving just a pellet).
4. Add “Mucolysin” to the vessel with *synovial fluid* (1 : 1, v/v). Stir the vessel periodically during 20-30 min.
5. Transfer *tissue material* to a disposable Petri dish. Mince the fragment (10 mm^3 or 10 µl) with a disposable scalpel. If a 12-well Multispin MSC-6000 vortex/centrifuge is used, transfer fragments of tissue to 2-ml disposable screwed polypropylene conical tubes with loops and add 2 or 3 sterile glass beads. If a TissueLyser LT homogenizer is used, transfer tissue fragments to 2-ml disposable screwed tubes and add 1 or 2 sterile metal beads. If porcelain mortars and pestles are used, transfer tissue fragments to a mortar and add an equal volume of PBS or sterile saline. Homogenize the sample.
6. Resuspend *cultures of microorganisms grown on <u>solid</u> nutrient medium (SNM)* in a sterile saline or PBS using turbidity standard No. 5 ($5x10^8$ microbial bodies per 1 ml (m.b./ml)) or McFarland No. 0.5, 1 or 2. Use 5 µl of this suspension. Take a 1-ml aliquot of *cultures of microorganisms grown on <u>liquid</u> nutrient medium (LNM)* and centrifuge it at 1000 g for 10 min. Discard the supernatant.

7. Use 100-µl aliquots of *washing fluids from environmental objects*.

Table 1

The volume of samples used for treatment and DNA extraction

Material	Aliquot volume for treatment	Aliquot volume for DNA extraction
Sputum	All sample	0.1 ml
BAL or BALF	1 ml	0.1 ml
Urine	5–10 ml	0.1 ml
Liquor	1 ml	0.1 ml
Synovial fluid	1 ml	0.1 ml
Prostate gland secretion	1 ml	0.1 ml
SNM	1.5–6 x 10^8 m.b./ml	0.05 ml
LNM	1 ml	0.1 ml
Blood	0.1 ml	
Menstrual blood	0.1 ml	
Tissue	10–25 µl	
Washing fluids from environmental objects	0.1 ml	

It is necessary to prevent the repeated sample extraction and reserve the sample aliquot in accordance with storage regulations.

7. WORKING CONDITIONS

AmpliSens® *MTC*-FEP 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 (for clinical material, cultures of microorganisms, and environmental objects).
- RIBO-prep, **REF** K2-9-Et-50-CE (for clinical material, cultures of microorganisms, and environmental objects).
- DNA-sorb-C, **REF** K1-6-50-CE (for human tissues).

Extract DNA according to the manufacturer's instructions.

Add 100 µl of Negative Control (C–) to the tube labeled C–.

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 tubes (for DNA samples, 3 control samples and 2 background tubes).
2. Prepare the tubes and mark them as **Background**. For each sample mix **10 µl** of **PCR-mix-1-FEP *MTC*** and **15 µl** of **PCR-mix-Background**. Thoroughly vortex the mixture.

It is recommended to sediment drops from walls of tubes by short centrifugation (1–3 s) before placing them in the thermocycler.

3. Prepare reaction mix. Sequentially add **10 µl** of **PCR-mix-1-FEP *MTC***, **5 µl** of **PCR-buffer-Flu, 0.5 µl** of **polymerase (TaqF)** and **0.5 µl** of **enzyme UDG** for one reaction. Vortex the reaction mixture.
4. Transfer **15 µl** of the reaction mixture to each tube for clinical and control samples.
5. Add above **1 drop** of **mineral oil for PCR** (about **25 µl**) to the surface of reaction mix if a thermocycler without constant-temperature cover is used.
6. Add **10 µl** of **DNA samples** obtained from clinical or control samples at the DNA extraction stage using tips with aerosol barrier.

Do not add sorbent into the reaction mixture if DNA-sorb-B or DNA-sorb-C are used.

7. Carry out the control amplification reactions:

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

C+ -Add **10 µl** of **Positive Control DNA *MTC* / STI** to the tube labeled C+ (Positive Control of Amplification).

For carrying out decontamination of the reaction mixture incubate the prepared tubes at room temperature for 10–30 min.

8.2.2 Amplification

Run the following program in the thermocycler (see Table 2). When the temperature reaches 95 °C (pause mode), insert tubes into the thermocycler cells and press the button to continue.

Table 2

Amplification program “65 MTC”

	Thermocyclers with active temperature adjustment			Thermocyclers with block temperature adjustment			
Step	Temperature, °C	Time	Cycles	Step	Temperature, °C	Time	Cycles
1	**95**	15 min	1	1	**95**	15 min	1
2	**95**	15 s	45	2	**95**	20 s	45
	65	20 s			**65**	20 s	
	72	15 s			**72**	20 s	
3	**10**	Storage		3	**10**	Storage	

When amplification is finished, take the tubes off the thermocycler and insert them into the fluorescence detector.

9. DATA ANALYSIS

Detection is conducted using a fluorescence detector. Create a template indicating the number of samples and tubes Background and typing the names of the control samples.

Please read the fluorescence detector Operating Manual before use of this kit.

Program the detector according to the manufacturer’s manual, **Guidelines** and **Bulletin**.

The fluorescent signal intensity is detected in two channels:

1. the signal from the ***Mycobacterium tuberculosis*** DNA amplification product is detected in the FAM channel (or analogous, depending on the detector model);
2. the signal from the **IC** amplification product is detected in the HEX channel (or analogous, depending on the detector model).

Principle of interpretation:

- *Mycobacterium tuberculosis* DNA is **detected** in a sample if its signal in the FAM channel is greater than the defined threshold value of the positive result.
- *Mycobacterium tuberculosis* DNA is **not detected** in a sample if the signal in the FAM channel is less than the defined threshold value of the negative result while the signal in the HEX channel is greater than the defined threshold value.
- The result is **invalid** if the signal of a sample in FAM and HEX channels is less than defined threshold values for these channels.
- The result is **equivocal** if the signal of a sample in the FAM channel is greater than the defined threshold value of the negative result but less than the threshold value of the positive result (the signal is between thresholds).

Table 3

Interpretation of results for the samples

FAM	HEX	Validity	Interpretation
> threshold of positive result	**>** or **<** threshold	valid	*M.tuberculosis complex* is detected
< threshold of negative result	**>** threshold	valid	*M.tuberculosis complex* is not detected
Between thresholds of positive and negative results	**>** threshold	invalid	equivocal (repeat material sampling)
< threshold of negative result	**<** threshold	invalid	invalid (repeat material sampling)

If the result is invalid or equivocal, the PCR should be repeated once again. If the equivocal result is repeated, carry out the procedure starting from the DNA extraction stage.

3. The result of the analysis is considered reliable only if the results obtained for C–, C+ and NCA are correct (see Table 4).

Table 4

Results for controls

Control	Stage for control	Result of automatic interpretation	
		FAM channel (samples)	**HEX channel** (IC)
C–	DNA extraction	**<** threshold of negative result	**>** threshold
C+	Amplification	**>** threshold of negative result	**>** threshold
NCA	Amplification	**<** threshold of positive result	**<** threshold

10. TROUBLESHOOTING

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

- If the signal for C+ is less than the threshold of negative result in the FAM channel or less than threshold in the HEX channel, repeat amplification of these samples. If the result is the same repeat DNA extraction of these samples.
- If the signal for C- or NCA is more than the threshold of positive result in the FAM channel, it indicates reagent or sample contamination. The analyses should be repeated for these samples starting from the DNA extraction stage.
- If the signal for C- is less than the threshold in the HEX channel, it indicates incorrect DNA extraction. The analyses should be repeated for these samples starting from the DNA extraction stage.
- If the signal for the samples is less than the threshold of negative result in the FAM channel and less than the threshold in the HEX channel, repeat amplification of these samples. If the result is the same, repeat DNA extraction of these samples.

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

11. TRANSPORTATION

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

12. STABILITY AND STORAGE

All components of the **AmpliSens® *MTC*-FEP** PCR kit (except for polymerase (TaqF), enzyme UDG, and PCR-mix-1-FEP *MTC*) are to be stored at 2–8 °C when not in use. All components of the **AmpliSens® *MTC*-FEP** 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.

Polymerase (TaqF), enzyme UDG, and PCR-mix-1-FEP *MTC* are to be stored at temperature from minus 24 to minus 16 °C when not in use.

PCR-mix-1-FEP *MTC* is to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

Nucleic extraction kit	Material	Sensitivity, mb/ml
		M.tuberculosis **(H37 Ra strain)**
RIBO-prep	PBS, sputum, BAL	$5x10^2$
	Urine	$1x10^3$
	Washing fluids from environmental objects[1]	$2.5x10^2$ copy/ml
DNA-sorb-B	PBS, sputum	$5x10^2$
	BAL, urine	$1x10^3$
	Washing fluids from environmental objects	$2.5x10^2$ copy/ml
DNA-sorb-C	10 % homogenate of different tissues (lungs, lymph nodes, kidney, liver, brain, spleen)	$1x10^2$

13.2. Specificity

The analytical specificity of **AmpliSens® *MTC*-FEP** PCR kit is ensured by selection of specific primers and probes as well as strict reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. The analytical specificity of **AmpliSens® *MTC*-FEP** PCR kit, which was found to be 100%, was checked by testing 67 reference strains and clinical isolates:

[1] Analysis can be performed without DNA extraction if washing fluids from environmental objects are added immediately to the reaction mixture for carrying out PCR analysis

- 16 bacteria representative of the *mycobacterium tuberculosis complex* (*M.tuberculosis, M.bovis, M.bovis BCG, etc.)*;
- 23 nontuberculosis mycobacteria (*M.avium, M.fortuitum, M.gordonae, M.intracellulare, M.kansasii, M.marinum, M.paratuberculosis, M.phlei, M.scrofulaceum, M.xenopi, M.smegmatis, M.ulcerans, M.terrae, etc.*);
- Bacteria of other groups (*Brucella abortus*, *B.melitensis, B.ovis, and B.suis; Campylobacter jejuni; Chlamydia suis; Chlamydophila abortus and Ch.felis; Cryptococuss neoformans; Enterobacter cloaca* and *E.faecalis; Enterococcus faecalis; Escherichia coli*; *Klebsiella pneumoniae; Listeria monocytogenes; Moraxella catarrhalis; Neisseria cinerea, N.elongata, N.flava, N.gonor, N.mucosa, N.sicca*, and *N.subflava*; *Pantoea agglomerans; Pasteurella tularensis; Proteus vulgaris* and *P.mirabilis; Pseudomonas aeruginosa*; *Salmonella enteritidis* and *S.typhi; Shigella flexneri* and *Sh.sonne; Staphylococcus aureus;* different clinical isolates of *S.aureus* MRSA, *S.faecalis, S.saprophyticus*; and different clinical isolates of *Streptococcus* A, B, C, G, S.*oralis,* and *S.pneumonia*).

The analytical specificity of **AmpliSens® *MTC*-FEP** PCR kit was estimated by the absence of positive result of the non-tuberculosis bacteria DNA amplification and by the presence of positive result of the *Mycobacterium tuberculosis complex* DNA amplification.

The clinical specificity of **AmpliSens® *MTC*-FEP** PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook “Sampling, Transportation, 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.

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® *MTC*-FEP** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Sufficient for
LOT	Batch code		Expiration Date
IVD	*In vitro* diagnostic medical device		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limitation	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**C+**	Positive control of amplification
EC REP	Authorised representative in the European Community	**IC**	Internal control
	Caution		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
01.07.11 RT	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
20.12.11 LA	13.1. Sensitivity	The name of the strain, *M.tuberculosis* (H37 Ra strain), was added