INSTRUCTION

Reagent kit for *rrs*, *eis*, *gyrA* and *gyrB* genes status detection associated with Mycobacterium tuberculosis complex drug resistance against aminoglycosides and fluoroquinolones, by multiplex PCR-RT

"MTB-RESIST-II-Test"

IVD

Version 3 dated 19.10.2022

Content

List of abbreviations

Abbreviations and designations used in the instruction:

PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
NC	negative control sample
PC	positive control sample
SVC	sampling volume control

Introduction

Target analyte: *rrs, eis, gyrA* and *gyrB* genes polymorphisms associated with Mycobacterium tuberculosis complex:

rrs gene: 1401A>G, 1402C>T and 1484G>T;

eis gene: C-14G, C-14T, C-12T, G-10C, G-10A and G-37T;

gyrA gene: p.G88C, p.A90V, p.S91P, p.D94G, p.D94N, p.D94H, p.D94A and p.D94Y;

gyrB gene: p.D461H, p.D461N, p.N499D, p.E501V and p.A504V.

The target analyte scientific validity lies in its specificity (the detected DNA sequence uniqueness) in relation to the *rrs, eis, gyrA* and *gyrB* genes of Mycobacterium tuberculosis complex molecular genetic polymorphisms.

The use of molecular genetic methods for polymorphisms detection in genes associated with drug resistance is recommended in the Clinical Recommendations "Tuberculosis in Adults", The Ministry of Health of the Russian Federation (Approved in 2020) and in Clinical guidelines "Tuberculosis in children", The Ministry of Health of the Russian Federation (approved in 2020). The list of polymorphisms and their correspondence to drug resistance development are given in details in the Catalogue of mutations in Mycobacterium tuberculosis complex and their association with drug resistance – Supplementary document. World Health Organization. WHO/UCN/GTB/PCI/2021.7 – © WHO 2021.

The reagent kit usage area: infectious diseases clinical laboratory testing.

Indications for use: MTB-RESIST-II-Test reagent kit is designed for patients with approved pulmonary and extrapulmonary tuberculosis for adequate tuberculosis therapy selection.

Contraindications for use: none were identified if used by well-trained personnel and taken into account the intended use.

Population and demographic aspects of the reagent kit usage: no population or demographic usage aspects of the MTB-RESIST-I-Test reagent kit were identified.

Sterility: the kit is not sterile.

1. Intended Use

Intended use: the MTB-RESIST-II-test reagent kit is designed for the *rrs*, *eis*, *gyrA* and *gyrB* genes molecular genetic polymorphisms qualitative detection of associated with drug resistance of Mycobacterium tuberculosis complex against second-line chemotherapeutic drugs — aminoglycosides (*rrs* and *eis* genes) and fluoroquinolones (*gyrA* and *gyrB* genes) using molecular beacons technology with real-time detection of melting temperatures in a DNA sample isolated from clinical material (sputum, bronchoalveolar lavage, bronchial lavage, gastric lavage, pleural fluid, blood, urine, cultures of microorganisms, prostate secretion, tissue (biopsy and surgical) material, synovial fluid, pericardial fluid and cerebrospinal fluid), in patients with a confirmed pulmonary and extrapulmonary tuberculosis diagnosis to select the appropriate therapy.

Functional use: *rrs*, *eis*, *gyrA* and *gyrB* genes status qualitative detection associated with Mycobacterium tuberculosis complex drug resistance against second-line chemotherapeutic drugs — aminoglycosides (*rrs* and *eis* genes) and fluoroquinolones (*gyrA* and *gyrB* genes) in patients with a confirmed pulmonary and extrapulmonary tuberculosis diagnosis for appropriate therapy selection.

Reagent kit potential consumers:

Kit for research use only.

2. Method Principle

Method

Real-time multiplex polymerase chain reaction with DNA probes (molecular beacons) melting temperature detection.

Test sample type

Test material is Mycobacterium tuberculosis complex DNA samples isolated from clinical material: sputum, bronchoalveolar lavage, bronchial washing, gastric washing, pleural fluid, blood, urine, microbiological cultures, prostate secretion, tissue (biopsy and surgical) material, synovial fluid, pericardial fluid and cerebrospinal fluid.

Detection principle

The *rrs*, *eis*, *gyrA* and *gyrB* genes molecular genetic qualitative detection associated with drug resistance to aminoglycosides (*rrs* and *eis* genes) and fluoroquinolones (*gyrA* and *gyrB* genes), Mycobacterium tuberculosis complex by real-time multiplex polymerase chain reaction with melting temperatures detection of DNA probe duplexes (molecular beacons) and the target DNA fragments in a DNA sample isolated from clinical material includes three stages:

1. PCR Setup;
2. DNA PCR amplification with subsequent DNA probes melting;
3. Results interpretation.

DNA samples are used for conducting genomic DNA sections amplification reactions using specific to them primers in a reaction buffer.

5x PCR Buffer contains all the main reagents including a thermostable hot-start DNA polymerase, deoxynucleotide triphosphates and a PCR-optimized buffer.

Oligonucleotide mixtures contain fluorescent-labeled DNA-probes (molecular beacons) that hybridize with the amplified target DNA complementary region. The DNA probes have adapters at the 5` and 3` ends (an adapter at the 5` end is complementary to an adapter at the 3` end), as well as fluorescent dye at one end and quencher at the other. When adaptors form a hybridization complex, dye and quencher converge and fluorescence intensity decreases. A DNA probe and a DNA target form a hybridization complex and as a result the fluorescent dye and quencher get separated and fluorescence intensity increases. DNA probe melting temperature depends on a DNA probe and a target DNA complementary nucleotides number. The highest melting temperature (T_m) is registered

when DNA probes and a target DNA complete the most. A DNA probe and a target DNA hybridization complex melting temperatures analysis allows to draw a conclusion about the DNA probe and the target DNA complementary nucleotides number and about a certain DNA probe correspondence to the target DNA. Conclusion about molecular genetic polymorphisms presence is made based on the analysis. Thus, when interpreting the results, the molecular beacons melting temperatures (T_m) are taken into account in comparison with PC-1, corresponding to the wild-type *Mycobacterium tuberculosis* reference strain - H37Rv strain (GenBank: NC_000962.3).

The kit contains reagents for the *rrs*, *eis*, *gyrA* and *gyrB* genes molecular genetic polymorphisms status qualitative detection associated with drug resistance to aminoglycosides (*rrs* and *eis* genes) and fluoroquinolones (*gyrA* and *gyrB* genes), as well as ICS (Table 1).

ICS allows to evaluate the DNA isolation quality and effectiveness and the possible inhibitors presence in the sample that can lead to obtaining false negative results.

Table 1 - Analyzed targets

Oligonucleotide mixture	**A channel corresponding to a fluorophore**				
	FAM	**HEX**	**ROX**	**Cy5**	**Cy5.5**
A	*rrs* gene: not 1401A>G and 1402C>T	*rrs* gene: 1401A>G	*rrs* gene: 1402C>T	*rrs* gene: 1401A>G + 1402C>T	ICS
B	*rrs* gene: not 1484G>T	*rrs* gene: 1484G>T	□	□	□
C	*eis* gene: not C- 14G, C-14T, C-12T, G- 10C and G-10A	*eis* gene: C-14G	*eis* gene: C-14T	*eis* gene: C-12T	□
D	*eis* gene: not G-37T	*Eis* gene: G-37T	*eis* gene: G-10C	*eis*: gene: G-10A	□
E	*gyrA* gene: not p.G88C, A90V, S91P, D94G, D94N, D94H, D94A and D94Y	*gyrA* gene: p.G88C	*gyrA* gene: p.A90V	*gyrA* gene: p.S91P	□
F	*gyrA* gene: p.D94G	*gyrA* gene: p.D94N	*gyrA* gene: p.D94H	*gyrA* gene: p.D94A	□
G	*gyrB* gene: not p.D461H and D461N	*gyrB* gene: p.D461H	*gyrB* gene: p.D461N	*gyrA* gene: p.D94Y	□
H	*gyrB* gene: not p.N499D, E501V and A504V	*gyrB* gene: p.N499D	*gyrB* gene: p.E501V	*gyrB* gene: p.A504V	□

The analysis takes from 2 hours to 2 hours 45 minutes in total (time for sample preparation is not included).

Method limitations

A possible reason for obtaining a false positive result can be contamination during DNA extraction or during multiplex PCR reaction stages. A false positive result can be detected by a negative control sample.

The reagent kit is not allowed to use after the expiration date.

Do not use the reagent kit if the inner packaging is broken or if the reagent kit appearance does not match the description.

A reagent kit transported or stored in the temperature regime violation is not allowed to use.

3. Reagent Kit Components

The reagent kit comes in 2 configurations:

1. **MTB-RESIST-II-Test-x12 reagent kit configuration** is designed for 12 reactions — 9 test samples, 1 negative and 2 positive control samples (1 cycler run with simultaneous loading of 96 wells);
2. **MTB-RESIST-II-Test-x96 reagent kit configuration** is designed for 96 reactions — 72 test samples, with a sequential cycler start for 96 wells using each multiplex with one negative and two positive control samples in each run (8 cycler runs with a simultaneous loading of 96 wells).

Reagent Kit Components

Table 2 — MTB-RESIST-II-Test-x12 reagent kit configuration components

No	Reagent	Description	Quantity, volume
1	5x PCR buffer	Transparent colorless liquid	1 tube, 480 µl
2	Oligonucleotide mixture A	Transparent liquid, may be lilac	1 tube, 180 µl
3	Oligonucleotide mixture B	Transparent liquid, may be lilac	1 tube, 180 µl
4	Oligonucleotide mixture C	Transparent liquid, may be lilac	1 tube, 180 µl
5	Oligonucleotide mixture D	Transparent liquid, may be lilac	1 tube, 180 µl
6	Oligonucleotide mixture E	Transparent liquid, may be lilac	1 tube, 180 µl
7	Oligonucleotide mixture F	Transparent liquid, may be lilac	1 tube, 180 µl
8	Oligonucleotide mixture G	Transparent liquid, may be lilac	1 tube, 180 µl
9	Oligonucleotide mixture H	Transparent liquid, may be lilac	1 tube, 180 µl
10	PC-1	Transparent colorless liquid	1 tube, 120 µl
11	PC-2	Transparent colorless liquid	1 tube, 120 µl
12	NC	Transparent colorless liquid	1 tube, 300 µl

No	Reagent	Description	Quantity, volume
13	ICS	Transparent colorless liquid	1 tube, 100 µl

Table 3 -MTB-RESIST-II-Testx96 reagent kit configuration components

No	Reagent	Description	Quantity, volume
1	5x PCR buffer	Transparent colorless liquid	2 tubes 1920µl each
2	Oligonucleotide mixture A	Transparent liquid, may be lilac	1 tube, 1440 µl
3	Oligonucleotide mixture B	Transparent liquid, may be lilac	1 tube, 1440 µl
4	Oligonucleotide mixture C	Transparent liquid, may be lilac	1 tube, 1440 µl
5	Oligonucleotide mixture D	Transparent liquid, may be lilac	1 tube, 1440 µl
6	Oligonucleotide mixture E	Transparent liquid, may be lilac	1 tube, 1440 µl
7	Oligonucleotide mixture F	Transparent liquid, may be lilac	1 tube, 1440 µl
8	Oligonucleotide mixture G	Transparent liquid, may be lilac	1 tube, 1440 µl
9	Oligonucleotide mixture H	Transparent liquid, may be lilac	1 tube, 1440 µl
10	PC-1	Transparent colorless liquid	1 tube, 960 µl

No	Reagent	Description	Quantity, volume
11	PC-2	Transparent colorless liquid	1 tube, 960 µl
12	NC	Transparent colorless liquid	2 tubes, 1200 µl each
13	ICS	Transparent colorless liquid	1 tube, 800 µl

NOTE: Operating documentation (instructions for use and quality certificate) is not included in the bill of materials, but is included in the reagent kit delivery scope. To ensure compliance with transportation conditions the reagent kit is placed in a reusable polyurethane foam thermal container filled with ice packs for temporary storage and transportation. The thermal container is put into an individual package with the instruction for use and the quality certificate for every reagent kit batch.

5x PCR Buffer is ready to use. It is a 5x mixture containing all the necessary reagents including recombinant polymerase (modified Taq DNA polymerase), mouse monoclonal antibodies to Taq-polymerase, magnesium ions, Tris-HCl, monovalent cations, non-ionic detergents, stabilizers.

A-H oligonucleotide mixtures are ready to use and contain primers and probes designed to detect specific targets. The oligonucleotide mixture contains in 10% aqueous TE solution (1 mM Tris, 0.1 mM EDTA).

PC-1 (positive control sample-1) is ready to use and is a plasmid DNA mixture with synthetic amplified DNA fragment inserts — *rrs*, *eis*, *gyrA* and *gyrB* genes fragments that do not contain detectable polymorphisms and correspond to the Mycobacterium tuberculosis H37Rv reference strain (GenBank: NC_000962.3) and the internal control sample target sequences in $1x10^7$ copies/ml concentration each. PC-1 contains in a 10% TE-buffer (10 mM Tris, 1 mM EDTA).

PC-2 (positive control sample-2) is ready to use and is a plasmid DNA mixture with synthetic amplified DNA fragment inserts — *rrs*, *eis*, *gyrA* and *gyrB* genes that do not contain polymorphisms (relative to the *Mycobacterium tuberculosis* H37Rv reference strain – GenBank:

NC_000962.3) in genes:

rrs: 1401A>G, 1402C>T and 1484G>T;

eis: C-14G, C-14T, C-12T, G-10C, G-10A, G-37T;

gyrA: p.G88C, p.A90V, p.S91P, p.D94G, p.D94N, p.D94H, p.D94A and p.D94Y;

gyrB: p.N499D, p.E501V, p.A504V, p.D461H and p.D461N;

and internal control sample target sequences at $1x10^7$ copies/mL concentration each. PC-2 contains in a 10% TE-buffer (10 mM Tris, 1 mM EDTA).

NC (negative control sample) is ready to use and is DNase-free deionized water.

ICS (internal control sample) is ready to use and is a plasmid DNA of $1,5x10^6$ copies/ml concentration in a TE-buffer (10 mM Tris, 1 mM EDTA).

The kit contains no substances for medical use, substances of human or animal origin.

4. Reagent Kit Characteristics

4.1. Technical and Performance Characteristics

Table 4 - MTB-RESIST-II-Test reagent kit

Parameter Name	Characteristics and Standards	Control methods according to TS
1. Technical Characteristics		
1.1. Description		
1.1.1. MTB-RESIST-II-Test x 12 reagent kit configuration		
5x PCR buffer	Transparent colorless liquid	Section 7, clause 7.6
Oligonucleotide mixture A	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture B	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture C	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture D	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture E	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture F	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture G	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture H	Transparent liquid, may be lilac	Section 7, clause 7.6
PC-1	Transparent colorless liquid	Section 7, clause 7.6
PC-2	Transparent colorless liquid	Section 7, clause 7.6
NC	Transparent colorless liquid	Section 7, clause 7.6
ICS	Transparent colorless liquid	Section 7, clause 7.6
1.1.2. Reagent kit composition for MTB-RESIST-II-Testx96 configuration		
5x PCR buffer	Transparent colorless liquid	Section 7, clause 7.6
Oligonucleotide mixture A	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture B	Transparent liquid, may be lilac	Section 7, clause 7.6

Oligonucleotide mixture C	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture D	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture E	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture F	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture G	Transparent liquid, may be lilac	Section 7, clause 7.6
Oligonucleotide mixture H	Transparent liquid, may be lilac	Section 7, clause 7.6
PC-1	Transparent colorless liquid	Section 7, clause 7.6
PC-2	Transparent colorless liquid	Section 7, clause 7.6
NC	Transparent colorless liquid	Section 7, clause 7.6
ICS	Transparent colorless liquid	Section 7, clause 7.6
1.2. Packaging	In accordance with clause 1.4 TS 21.20.23-044-97638376-2021	Section 7, clause 7.9
1.3. Marking	In accordance with clause 4 TS 21.20.23-044-97638376-2021	Section 7, clause 7.9
1.4. Packaging	In accordance with clause 5 TS 21.20.23-044-97638376-2021	Section 7, clause 7.9
2. Functional characteristics		
2.1. Positive result with PC-1	Single peaks for each reaction mixture in the following range: **Reaction mixture A:** FAM channel 72±4˚C, HEX channel 71±4˚C, ROX channel 72±4˚C, Cy5 channel 65±4˚C, Cy5.5 channel 65±5˚C **Reaction mixture B**: FAM channel 69±4˚C, HEX channel 65±4˚C, ROX channel is not considered, Cy5 channel is not considered, Cy5.5 channel is not considered **Reaction mixture C**: FAM channel 68±4˚C, HEX channel 62±4˚C, ROX channel 69±4˚C, Cy5 channel 64±4˚C, Cy5.5 channel is not considered **Reaction mixture D**: FAM channel 69±4˚C, HEX channel 68±4˚C, ROX channel 53±4˚C, Cy5 channel	Section 7, clause 7.7.2

	59±4˚C, Cy5.5 channel is not considered **Reaction mixture E**: FAM channel 73±4˚C, HEX channel 69±4˚C, ROX channel 66±4˚C, Cy5 channel 60±4˚C, Cy5.5 channel is not considered **Reaction mixture F**: FAM channel 75±4˚C, HEX channel 70±4˚C, ROX channel 71±4˚C, Cy5 channel 67±4˚C, Cy5.5 channel is not considered **Reaction mixture G**: FAM channel 72±4˚C, HEX channel 69±4˚C, ROX channel 68±4˚C, Cy5 channel 73±4˚C, Cy5.5 channel is not considered **Reaction mixture H**: FAM channel 70±4˚C, HEX channel 69±4˚C, ROX channel 68±4˚C, Cy5 channel 70±4˚C, Cy5.5 channel is not considered	
2.2. Positive result with PC-2	Single peaks for each reaction mixture in the following range: **Reaction mixture A:** FAM channel 67±4˚C, HEX channel 75±4˚C, ROX channel 67±4˚C, Cy5 channel 71±4˚C, Cy5.5 channel 65±5˚C **Reaction mixture B**: FAM channel 62±4˚C, HEX channel 71±4˚C, ROX channel is not considered, Cy5 channel is not considered, Cy5.5 channel is not considered **Reaction mixture C**: FAM channel 53±4˚C, HEX channel 71±4˚C, ROX channel 65±4˚C, Cy5 channel 53±4˚C, Cy5.5 channel is not considered **Reaction mixture D**: FAM channel 69±4˚C, HEX channel 67±4˚C, ROX channel 44±4˚C, Cy5 channel 49±4˚C, Cy5.5 channel is not considered **Reaction mixture E**: FAM channel	Section 7, clause 7.7.2

	70±4˚C, HEX channel 65±4˚C, ROX channel 63±4˚C, Cy5 channel 54±4˚C, Cy5.5 channel is not considered **Reaction mixture F**: FAM channel 73±4˚C, HEX channel 70±4˚C, ROX channel 75±4˚C, Cy5 channel 65±4˚C, Cy5.5 channel is not considered **Reaction mixture G**: FAM channel 65±4˚C, HEX channel 75±4˚C, ROX channel 66±4˚C, Cy5 channel 73±4˚C, Cy5.5 channel is not considered **Reaction mixture H**: FAM channel 67±4˚C, HEX channel 73±4˚C, ROX channel 66±4˚C, Cy5 channel 69±4˚C, Cy5.5 channel is not considered	
2.3. Negative result with NC	No single peaks for each reaction mixture in the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red, and Cy5.5/Crimson channels	Section 7, clause 7.7.2
2.4 Reaction in tubes with SC	No single peaks for each reaction mixture in the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red, and Cy5.5/Crimson channels	Section 7, clause 7.7.2
2.5 Reaction in tubes with SenS-1	Single peaks for each reaction mixture in the range: **Reaction mixture A:** FAM channel 72±4˚C, HEX channel 71±4˚C, ROX channel 72±4˚C, Cy5 channel 65±4˚C, Cy5.5 channel 65±5˚C **Reaction mixture B**: FAM channel 69±4˚C, HEX channel 65±4˚C, ROX channel is not considered, Cy5 channel is not considered, Cy5.5 channel is not considered **Reaction mixture C**: FAM channel 68±4˚C, HEX channel 62±4˚C, ROX channel 69±4˚C, Cy5 channel 64±4˚C, Cy5.5 channel is not considered **Reaction mixture D**: FAM channel 69±4˚C, HEX channel 68±4˚C, ROX channel 53±4˚C, Cy5 channel	Section 7, clause 7.7.2

	59±4˚C, Cy5.5 channel is nor considered **Reaction mixture E**: FAM channel 73±4˚C, HEX channel 69±4˚C, ROX channel 66±4˚C, Cy5 channel 60±4˚C, Cy5.5 channel is not considered **Reaction mixture F**: FAM channel 75±4˚C, HEX channel 70±4˚C, ROX channel 71±4˚C, Cy5 channel 67±4˚C, Cy5.5 channel is not considered **Reaction mixture G**: FAM channel 72±4˚C, HEX channel 69±4˚C, ROX channel 68±4˚C, Cy5 channel 73±4˚C, Cy5.5 channel is not considered **Reaction mixture H**: FAM channel 70±4˚C, HEX channel 69±4˚C, ROX channel 68±4˚C, Cy5 channel 70±4˚C, Cy5.5 channel is not considered	
2.6 Reaction in tubes with SenS-2	Single peaks for each reaction mixture in the range: **Reaction mixture A:** FAM channel 67±4˚C, HEX channel 75±4˚C, ROX channel 67±4˚C, Cy5 channel 71±4˚C, Cy5.5 channel 65±5˚C **Reaction mixture B**: FAM channel 62±4˚C, HEX channel 71±4˚C, ROX channel is not considered, Cy5 channel is not considered, Cy5.5 channel is not considered **Reaction mixture C**: FAM channel 53±4˚C, HEX channel 71±4˚C, ROX channel 65±4˚C, Cy5 channel 53±4˚C, Cy5.5 channel is not considered **Reaction mixture D**: FAM channel 69±4˚C, HEX channel 67±4˚C, ROX channel 44±4˚C, Cy5 channel 49±4˚C, Cy5.5 channel is not considered **Reaction mixture E**: FAM channel 70±4˚C, HEX channel 65±4˚C, ROX channel 63±4˚C, Cy5 channel 54±4˚C, Cy5.5 channel is not considered **Reaction mixture F**: FAM channel 73±4˚C, HEX channel 70±4˚C, ROX channel 75±4˚C, Cy5 channel 65±4˚C, Cy5.5 channel is not considered	Section 7, clause 7.7.2

	Reaction mixture G: FAM channel 65±4˚C, HEX channel 75±4˚C, ROX channel 66±4˚C, Cy5 channel 73±4˚C, Cy5.5 channel is not considered **Reaction mixture H**: FAM channel 67±4˚C, HEX channel 73±4˚C, ROX channel 66±4˚C, Cy5 channel 69±4˚C, Cy5.5 channel is not considered	

In case of the reagent kit failure, functional deviation that may affect the kit safety or the kit analytical characteristics change immediately stop using the product and inform the manufacturer (see section 14 of the instruction).

4.2 Analytical efficiency characteristics

4.2.1 Analytical specificity

MTB-RESIST-II-Test reagent kit is specific to Mycobacterium tuberculosis complex *rrs, eis, gyrA* and *gyrB* genes DNA target fragments including *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. canettii, M. caprae, M. microti*.

There were no nonspecific positive amplification results when the following DNAs were present in a sample:

non-tuberculosis complex mycobacteria *(M. avium, M. abscessus, M. septicum, M. fortuitum, M. gordonae, M. intracellulare, M. kansasii, M. marinum, M. smegmatis, M. xenopi, M. ulcerans, M. terrae), and Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae, Chlamydophila pneumoniae, Streptococcus pyogenes, Mycoplasma pneumoniae, Streptococcus pneumoniae, Legionella pneumophila* cytomegalovirus, herpes simplex virus types 1 and 2 (at least $1x10^6$GE/mL concentration)

4.2.2. Analytical sensitivity: detection limit

At least 2000 genomic DNA copies per 1ml of biomaterial in case of DNA extraction from a 100µl sample and 50µl eluate.

4.2.3. Accuracy under repeatability conditions

To assess accuracy under repeatability conditions, standard enterprise samples were examined in 10 repetitions:

ESS-PC-1 is a plasmid DNAs mixture with synthetic inserts of amplified mycobacterial genomic DNA regions (*rrs, eis, gyrA* and *gyrB* genes regions) that do not contain the detectable polymorphisms and correspond to the nucleotide sequence of the reference Mycobacterium

tuberculosis strain H37Rv (GenBank: NC_000962.3) and the ICS target sequences at $1x10^7$ copies/ml concentration each in a 10% TE buffer (10 mM Tris, 1 mM EDTA), manufactured by TestGene LLC. **Contains 100% of normal DNA copies** (*rrs*, *eis*, *gyrA* and *gyrB* wild-type genes (without mutations));

ESS-PC-2 is a plasmid DNA mixture with synthetic inserts of amplified mycobacterial genomic DNA regions — *rrs*, *eis*, *gyrA* and *gyrB* genes fragments that contain polymorphisms (relatively to the *Mycobacterium tuberculosis strain* H37Rv — GenBank: NC_000962.3) in genes:

rrs: 1401A>G, 1402C>T and 1484G>T;

eis: C-14G, C-14T, C-12T, G-10C, G-10A, G-37T;

gyrA: p.G88C, p.A90V, p.S91P, p.D94G, p.D94N, p.D94H, p.D94A and p.D94Y;

gyrB: p.N499D, p.E501V, p.A504V, p.D461H and p.D461N;

and the ICS target sequences at the $1x10^7$ copies/ml concentration each in a 10% TE buffer (10 mM Tris, 1 mM EDTA) manufactured by TestGene LLC. **Contains 100% of DNA copies with mutations**.

Repeatability data are obtained within the laboratory for specific equipment and within a specific reagent kit batch.

To precise the accuracy under repeatability the sample arithmetic mean, dispersion, standard deviation, and variation index coefficient are calculated based on the data obtained in control samples repetitions.

Essay results showed that the variation index under repeatability is not higher than 3%.

4.2.4. Accuracy under reproducibility conditions

The test system reproducibility assessment is carried out similarly to the accuracy under repeatability conditions calculation. However, different batches of the reagent kit are used for testing and testings are carried out in different laboratories, by different operators, on different days, via different PCR cyclers (Reproducibility test Block 1, Reproducibility test Block 2, Reproducibility test Block 3, Reproducibility test Block 4).

When conducting accuracy testing under reproducibility conditions variation index was not higher than 5%.

4.3. Clinical Effectiveness

204 clinical samples were used for clinical essay conduction (sputum, bronchoalveolar lavage, bronchial washing, gastric washing, pleural fluid, blood, urine, microbiological cultures, prostate secretion, tissue (biopsy and surgical) material, synovial fluid, pericardial fluid and cerebrospinal fluid) of patients diagnosed with pulmonary and extrapulmonary tuberculosis.

For cross-reactivity assessment in clinical trials the MTB-RESIST-II-Test reagent kit was also used for testing **12 samples** of non-tuberculosis mycobacteria *(M. avium, M. abscessus, M. septicum, M. fortuitum, M. gordonae, M. intracellulare, M. kansasii, M. marinum, M. smegmatis, M. xenopi, M. ulcerans, M. terrae)* and **37 samples** that did not contain the analytes under study but contained confirmed positive presence of *Streptococcus pneumoniae, Staphylococcus aureus, Klebsiella pneumoniae, Haemophilus influenza, Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila, Pseudomonas aeruginosa, Escherichia coli, Streptococcus pyogenes,* Human herpesvirus 5, Human herpesvirus 1, Human herpesvirus 2.

This samples were collected according to **GOST R 51352-2013** requirements and taking into account the International guideline **CLSI EP09-A3** recommendations.

For MTB-RESIST-II-Test-x12 and MTB-RESIST-II-Test-x96 reagent kit configurations DNA extraction from clinical material was carried out using:

– for DNA extraction from sputum, blood, urine and prostate secretion: NA-Extra reagent kit for RNA/DNA extraction from clinical material according to TS 21.20.23-013-97638376-2019 manufactured by TestGene LLC (registration certificate No RZN 2021/15428 dated 24.09.2021);

– for DNA extraction from bronchoalveolar lavage, bronchial lavage waters, gastric lavage waters, pleural fluid, cultures of microorganisms, tissue (biopsy and surgical) material, synovial fluid, pericardial fluid and cerebrospinal fluid — Ribo-Sorb reagent kit for RNA /DNA extraction from clinical material according to TS 9398-004-01897593-2008 produced by the Federal Budget Institute of Epidemiology, Central Research Institute of Epidemiology of Rospotrebnadzor (registration certificate No. FSR 2008/03993 dated

22.02.2019).

Every sample was tested in two rounds using the MTB-RESIST-II-Test reagent kit manufactured by TestGene LLC and the kit for differential detection of drug-resistant/sensitive to fluoroquinolones, amikacin/kanamycin, capriomycin Mycobacterium tuberculosis complex DNA by real-time polymerase chain reaction method Polyprob LU TB-2 according to TS 9398-003-26124018-2015, Niarmedic Plus, LLC, Russian Federation (registration certificate № RZN 2018/7891 dated 25.06.2021).

Cyclers recommended by the reagent kit manufacturer that were used for PCR testing:

- DTprime Detecting Cycler (DNA-Technology, LLC, Russia);
- CFX 96 Cycler (Bio-Rad, USA);
- QuantStudio 5 Cycler (Thermo Fisher Scientific, USA).

All the 204 samples were tested by MTB-RESIST-II-Test reagent kit in two rounds (408 observations). For all the four cyclers the *rrs*, *eis*, *gyrA* and *gyrB* genes polymorphisms qualitative determination results coincided with the results of the Polyprob LU TB-2 reagent kit, NIARMEDIC PLUS LLC.

Diagnostic characteristics confidence intervals (CI) will be calculated using the Clopper-Pearson Confidence Interval method (Clopper-Pearson Confidience Interval; Clopper C., Pearson E. The Use of Confidence or Fiducial Limits Illustrated in the Case of the Binomial. Biometrika. 1934. Vol. 26(4). P. 404–413. doi:10.2307/2331986). The reagent kit diagnostic characteristics were calculated with 95% confidence coefficient.

The reagent kit diagnostic characteristics study results in relation to each tested clinical material and the *rrs, eis, gyrA* and *gyrB* genes molecular genetic polymorphisms are shown in the Table 5.

Table 5- Clinical efficiency

Test sample type	Tested gene	Positive samples observation number	Negative samples observations number	Diagnostic sensitivity with 95% confidence probability	Diagnostic sensitivity with 95% confidence probability
	rrs	14	50	(100% (95% diagnostic interval: 76,84%-100%)	(100% (95% diagnostic interval: 92,89%-100%)
	eis	24	40	100% (95% diagnostic interval: 85,75%-100%)	(100% (95% diagnostic interval: 91,19%-100%)

Sputum	*gyrA*	18	46	(100% (95% diagnostic interval: 81,47%-100%)	(100% (95% diagnostic interval: 81,47%-100%)
	gyrB	14	52	(100% (95% diagnostic interval: 76,84%-100%)	(100% (95% diagnostic interval: 93,15%-100%)
Bronchoalveolar lavage	*rrs*	10	26	100% (95% diagnostic interval: 69,15%-100%)	(100% (95% diagnostic interval: 86,77%-100%)
	eis	14	26	(100% (95% diagnostic interval: 76,84%-100%)	(100% (95% diagnostic interval: 86,77%-100%)
	gyrA	8	20	(100% (95% diagnostic interval: 63,06%-100%)	(100% (95% diagnostic interval: 83,16%-100%)
	gyrB	6	28	(100% (95% diagnostic interval: 83,16%-100%)	(100% (95% diagnostic interval: 87,66 %-100%)
Bronchial washing	*rrs*	16	34	100% (95% diagnostic interval: 79,41%-100%)	100% (95% diagnostic interval: 89,72%-100%)
	eis	12	36	100% (95% diagnostic interval: 73,54%-100%)	(100% (95% diagnostic interval: 90,26%-100%)
	gyrA	18	32	(100% (95% diagnostic interval: 81,47%-100%)	(100% (95% diagnostic interval: 89,11%-100%)
	gyrB	8	42	(100% (95% diagnostic interval: 63,06%-100%)	(100% (95% diagnostic interval: 91,59%-100%)
Gastric washing	*rrs*	14	22	(100% (95% diagnostic interval: 76,84%-100%)	(100% (95% diagnostic interval: 84,56%-100%)
	eis	8	26	(100% (95% diagnostic interval: 63,06%-100%)	(100% (95% diagnostic interval: 86,77%-100%)

	gyrA	8	26	(100% (95% diagnostic interval: 63,06%-100%)	(100% (95% diagnostic interval: 86,77%-100%)
	gyrB	6	28	(100% (95% diagnostic interval: 83,16%-100%)	(100% (95% diagnostic interval: 87,66 %-100%)
Pleural fluid	*rrs*	10	18	100% (95% diagnostic interval: 69,15%-100%)	(100% (95% diagnostic interval: 81,47%-100%)
	eis	4	24	(100% (95% diagnostic interval: 39,76%-100%)	100% (95% diagnostic interval: 85,75%-100%)
	gyrA	10	18	100% (95% diagnostic interval: 69,15%-100%)	(100% (95% diagnostic interval: 81,47%-100%)
	gyrB	8	20	(100% (95% diagnostic interval: 63,06%-100%)	(100% (95% diagnostic interval: 83,16%-100%)
Blood	*rrs*	20	40	(100% (95% diagnostic interval: 83,16%-100%)	(100% (95% diagnostic interval: 91,19%-100%)
	eis	12	44	100% (95% diagnostic interval: 73,54%-100%)	100% (95% diagnostic interval: 91,96%-100%)
	gyrA	22	34	(100% (95% diagnostic interval: 84,56%-100%)	100% (95% diagnostic interval: 89,72%-100%)
	gyrB	8	48	(100% (95% diagnostic interval: 63,06%-100%)	(100% (95% diagnostic interval: 92,60%-100%)
Urine	*rrs*	14	34	(100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 89,72%-100%)
	eis	12	32	100% (95% diagnostic interval: 73,54%-100%)	100% (95% diagnostic interval: 73,54%-100%)
	gyrA	10	32	100% (95% diagnostic interval: 69,15%-100%)	100% (95% diagnostic interval: 89,11%-100%)
	gyrB	14	30	(100% (95% diagnostic interval: 76,84%-100%)	(100% (95% diagnostic interval: 88,43%-100%)
Microbiological cultures	*rrs*	4	8	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
	eis	4	8	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
	gyrA	6	8	(100% (95% diagnostic interval: 83,16%-	(100% (95% diagnostic interval: 63,06%-

				100%)	100%)
	gyrB	2	10	(100% (95% diagnostic interval: 15,81%-100%)	100% (95% diagnostic interval: 69,15%-100%)
Prostate secretion	*rrs*	6	12	(100% (95% diagnostic interval: 83,16%-100%)	100% (95% diagnostic interval: 73,54%-100%)
	eis	2	16	(100% (95% diagnostic interval: 15,81%-100%)	100% (95% diagnostic interval: 79,41%-100%)
	gyrA	8	10	(100% (95% diagnostic interval: 63,06%-100%)	100% (95% diagnostic interval: 69,15%-100%)
	gyrB	4	14	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 76,84%-100%)
Tissue (biopsy and surgical) material	*rrs*	8	20	(100% (95% diagnostic interval: 63,06%-100%)	(100% (95% diagnostic interval: 83,16%-100%)
	eis	12	16	100% (95% diagnostic interval: 73,54%-100%)	100% (95% diagnostic interval: 79,41%-100%)
	gyrA	12	16	100% (95% diagnostic interval: 73,54%-100%)	100% (95% diagnostic interval: 79,41%-100%)
	gyrB	4	24	(100% (95% diagnostic interval: 39,76%-100%)	100% (95% diagnostic interval: 85,75%-100%)

Synovial fluid	*rrs*	2	12	(100% (95% diagnostic interval: 15,81%-100%)	100% (95% diagnostic interval: 73,54%-100%)
	eis	4	10	(100% (95% diagnostic interval: 39,76%-100%)	100% (95% diagnostic interval: 69,15%-100%)
	gyrA	4	10	(100% (95% diagnostic interval: 39,76%-100%)	100% (95% diagnostic interval: 69,15%-100%)
	gyrB	4	10	(100% (95% diagnostic interval: 39,76%-100%)	100% (95% diagnostic interval: 69,15%-100%)
Pericardial fluid	*rrs*	2	6	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 83,16%-100%)
	eis	2	8	(100% (95% diagnostic interval: 15,81%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
	gyrA	4	6	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 83,16%-100%)
	gyrB	2	8	(100% (95% diagnostic interval: 15,81%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
Cerebrospinal fluid	*rrs*	6	8	(100% (95% diagnostic interval: 83,16%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
	eis	4	8	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
	gyrA	4	10	(100% (95% diagnostic interval: 39,76%-100%)	100% (95% diagnostic interval: 69,15%-100%)
	gyrB	2	12	(100% (95% diagnostic interval: 15,81%-100%)	100% (95% diagnostic interval: 73,54%-100%)

5. Risks associated with the reagent kit use

The risk zone includes the following hazards:

1. The kit reagents functional properties loss due to transportation, storage or usage under inappropriate conditions;

2. Clinical material contamination with inhibiting substances in concentrations exceeding the permissible ones;

3. Reaction mixtures and test DNA samples contamination with contents from PC-1, PC-2 tubes or with PCR products;

4. Testing with a poor-quality DNA sample (low concentration and/or poor purification);

5. Failure to comply with the requirements for sample preparation, analysis and disposal due to unqualified personnel work;

6. Usage of an unusable kit (after the expiration date or in case of damaged package).

No risks have been identified in the risk zone area.

Total residual risk of using the reagent kit for *rrs, eis, gyrA* and *gyrB* genes status detection associated with Mycobacterium tuberculosis complex drug resistance against aminoglycosides and fluoroquinolones, by multiplex PCR-RT "MTB-RESIST-II-Test is acceptable; the benefit of its usage exceeds the risk.

6. Safety Precautions

All components and reagents included in MTB-RESIST-II-Test reagent kit belong to low-hazard substances. Precautions against any special, unusual environmental risks when using or selling the product are not provided.

The reagents included in the MTB-RESIST-II-Test reagent kit have low vapor pressure and exclude the possibility of inhalation poisoning.

The reagents included in the MTB-RESIST-II-Test reagent kit are non-toxic, as they are prepared by mixing separate non-toxic components.

Personnel should ensure and comply with the biological safety rules and work requirements for the organization and conduct it in order to prevent contamination with nucleic acids and (or) amplicons of the tested samples, premises and equipment.

The work should be carried out in a laboratory performing clinical material molecular-biological (PCR) testing in accordance with sanitary and epidemiological requirements.

The following requirements should always be met when working:

- Remove unused reagents in accordance with sanitary and epidemiological requirements for the management of medical waste.

ATTENTION! When removing waste after amplification (tubes containing PCR products), it is not allowed to open the tubes and spill the contents, as this may lead to contamination of a laboratory area, equipment and reagents with PCR products.

- use the kit strictly for its intended purpose, according to this instruction;
- only specially trained personnel are allowed to work with the kit (a specialist with higher medical education who has been trained in licensed qualification courses to work with Pathogenic Biological Agents (PBA) of pathogenicity groups III and IV and to conduct PCR testing, as well as a laboratory assistant with secondary special medical education);
- do not use the kit after the expiration date;
- avoid contact with skin, eyes and mucous membrane. In case of contact, immediately flush the affected area with water and seek medical assistance.

The necessary precautions are not provided for the magnetic fields effects, external electrical influences, electrostatic discharges, pressure or pressure changes, overloads, or sources of thermal ignition.

The kit contains no substances of human or animal origin with a potential infectious nature, therefore, precautions against any special, unusual risks during product use or sale are not provided.

7. Required Equipment and Materials

Equipment

1. Class II and III biological safety cabinet;
2. Vortex
3. A set of electronic or automatic variable volume dispensers;
4. Refrigerator for 2°C... 8°C with a freezer for less than -16 °C;
5. Freezer for -2°C... -40°C.
6. Cycler[1] with real-time fluorescence detection via channels corresponding to FAM, HEX, ROX, Cy5 and Cy5.5: CFX96 (BioRad, USA), DTprime (DNA-Technology LLC, Russian Federation), QuantStudio 5 (Thermo Fisher Scientific, USA).

The software for the cyclers manufactured by DNA-Technology LLC should be not older than the 7.7.5.23 version, 7.7.5.44[2] version is recommended.

Materials and reagents not included in the kit:

ATTENTION! When working with a DNA, it is necessary to use only disposable sterile plastic consumables with a "DNase-free" label.

1. Disposable pipette tips with aerosol barrier up to 1000 µl, 200 µl, 20 µl and 10 µl (for example, Axygen, USA);
2. 1.5ml disposal Eppendorf type sterile tubes;
3. Thin-walled disposable tubes with an optically transparent lid (when using plate type cyclers): 0.1 or 0.2 ml[3] PCR tubes, or 0.1 ml or 0.2ml PCR tubes in strips or PCR plates with an optically transparent film (e.g., Axygen, USA), compatible with the used cycler;
4. Isolation gown coat and disposable talc-free gloves;
5. Container with disinfectant;
6. Test tube rack for 0.1ml or 0.2ml tubes or for 0.1ml or 0.2ml tube strips (e.g., InterLabService, Russian Federation);
7. Reagent kit for DNA extraction from clinical material (see section 8.2 of the instruction);

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[1] The cyclers must be maintained, calibrated and used in accordance with the manufacturer's recommendations. The kit usage in an uncalibrated device may have an impact on the performance of the test.

[2] As software is updated, the recommended software version may change. The latest recommended software version can be downloaded on the http://www.dna-technology.ru/po/ website.

[3] Ensure that the PCR tubes are compatible with the cycler being used.

8. For sputum and synovial fluid testing: use mucous material pretreatment reagent MUKOLIZIN

8. Test samples

Test sample type

Test material is Mycobacterium tuberculosis complex DNA samples isolated from clinical material: sputum, bronchoalveolar lavage, bronchial washing, gastric washing, pleural fluid, blood, urine, microbiological cultures, prostate secretion, tissue (biopsy and surgical) material, synovial fluid, pericardial fluid and cerebrospinal fluid.

8.1 Clinical material collection procedure

Biological material should be collected and packed by healthcare providers specially trained to follow biological safety requirements and rules when working and collecting material suspected of being infected with microorganisms of the pathogenicity group III.

Test Material Collection

The material must be collected before chemotherapy is started.

Sputum. Should be collected in disposable wide-mouth at least 50ml volume screw cap containers. The biomaterial sample recommended volume is from 3ml to 5ml. Sputum retest (up to three times within three days) should be conducted to increase informativeness.

Bronchoalveolar lavage, gastric washing, bronchial washing, cerebrospinal fluid. Collect in disposable tightly screwed containers with a minimum 5ml volume.

Blood, pleural fluid, pericardial fluid. Better to collect in vacuum tubes with EDTA preservative. After the biomaterial collection it is recommended to turn the tube over several times to mix the preservative.

Urine (middle morning urine portion or the whole morning urine portion). Collect in sterile disposable wide-mouth least 50ml volume screw cap containers after thoroughly cleaning external genitalia. Urine testing for the mycobacteria should include a mandatory triplicate testing.

Prostate secretion. Prostate secretion is collected after prostate massage through rectum. A doctor performs a massage making several vigorous movements from the base to the top. Prostate secretion is collected in a disposable sterile dry plastic 2ml tube after a prostate massage. The test tube should be tightly closed with a lid, avoiding gaps and the lid inner part wrinkling, and labeled.

If the clinical material volume is not enough for the DNA extraction conduction (100µl) it is necessary to adjust the volume with saline solution. If it is impossible to obtain prostate secretion immediately after prostate massage, collect 15-25ml of the urine first portion (which contains prostate secretion).

Synovial fluid. Collect into disposable tightly screwed containers.

Tissue (biopsy and surgical) material. Should be collected into disposable vacuum tubes with EDTA preservative or disposable 1.5ml screw cap tubes containing 0.2ml of sterile saline solution.

Microbiological cultures. If the nutrient medium is dense resuspend the colony into 0,2ml of sterile saline solution or use liquid medium directly.

Initial clinical material transportation, storage and disposal conditions

- Sputum, tissue (biopsy and surgical) material, microbiological cultures: at 2°C... 8°C — up to 3 days; at -18°C... - 22°C — up to 1 week.
- Bronchoalveolar lavage and bronchial washing, prostate secretion, urine, synovial, pleural, pericardial, cerebrospinal fluids, gastric washing: at 2°C... 8°C — up to 1 day, at -18°C... -22°C — up to 1 week.
- Blood: at 2°C... 8°C — not longer than 12 hours.

Do not freeze blood. Double freezing and thawing of the other clinical material are allowed.

8.1. Material pre-processing

The aliquot volume for DNA extraction is at least 100µl if the biomaterial is liquid or 10-20mm^3 of solid tissue homogenate. The aliquot should be placed into a 1.5ml Eppendorf type tube. All tubes containing testing samples must be labeled. Recommended elution volume is 50µl.

Sputum and synovial fluid. Pretreatment with MUKOLIZIN pretreatment reagent is required according to the instructions for the used nucleic acid isolation kit.

Bronchial washing, gastric washing, pericardial fluid, bronchoalveolar lavage, cerebrospinal fluid. Mix by turning over and transfer 1ml of the sample into a 1.5ml Eppendorf type tube. Label the tube. Centrifuge for 10 minutes at 10,000g. Remove the supernatant using a vacuum aspirator with a trap flask, leave the required for extraction sample amount.

Blood, pleural fluid. No preparation required.

Urine (middle morning urine portion or the whole morning urine portion). Shake the container with the urine. Transfer 10ml of urine into a sterile screw cap tube using a tip with a filter, centrifuge for 5 minutes at 10,000g or for 20 minutes at 3 000g. Remove the supernatant using a vacuum aspirator with a trap flask. Add transport medium to the sediment to obtain the final 0.2-1.0ml volume (depending on the volume required for extraction). If there is no visible sediment after centrifugation, do not remove the supernatant completely, leave about 0.2–1.0ml. Thoroughly mix the contents using vortex.

Prostate secretion. If the clinical material volume is not enough for the DNA extraction conduction (100µl) it is necessary to adjust the volume with saline solution. If it is impossible to obtain the secretion, collect 15-25ml of the urine first portion (which contains prostate secretion) right after the prostate massage. In that case follow the previous material pre-processing instruction point.

Tissue (biopsy and surgical) material. A 10-20mm^3 sample pre-homogenization by any available method is required. Shake the tube for 3-5 seconds using Vortex.

Microbiological cultures. In case of using microorganisms grown on solified medium, use 5-10µl of adjusted with sterile saline solution suspension to the required volume for DNA extraction. In case of using microorganisms grown on liquid medium centrifuge 500-1000 µl of the aliquot for 5 minutes at 3 000g and then remove the supernatant and adjust the volume with sterile saline solution to the required for DNA extraction volume.

8.2. DNA extraction from biological material

The following reagent kits are recommended for DNA sample extraction from clinical material:

– <u>if sputum, blood, urine and prostate secretion are used as clinical material:</u> reagent kit for DNA/RNA extraction from clinical material NA-Extra, manufactured by TestGene LLC, Russian Federation

– <u>if bronchoalveolar lavage, bronchial lavage waters, gastric lavage waters, pleural fluid, culture of microorganisms, tissue (biopsy and surgical) material, synovial fluid, pericardial fluid and cerebrospinal fluid are used as clinical material:</u> Ribo-Sorb reagent kit for RNA/DNA extraction from clinical material, produced by the Federal Budget Institute

of Epidemiology, Central Research Institute of Epidemiology of Rospotrebnadzor

– During DNA extraction it is required to strictly follow the protocol and the instruction for use for the applied reagent kit.

10µl of ICS from the MTB-RESIST-II-Test reagent kit should be added to each test sample before extraction.

NC sample also undergoes 100µl DNA extraction with 10µl ICS addition. If the reagent kit manufacturer's instructions for DNA extraction allow to use larger sample volume, adjust the NC volume to the required volume with saline or with TE-buffer.

Conditions for test DNA samples possible storage

– at +2...+8°C — up to 24 hours,

– at -18 … -22 °C — up to a month,

– at - 80°C — for a long time.

ATTENTION! Before the assay conduction it is necessary to make sure that the sample contains tuberculosis complex mycobacteria DNA using the appropriate reagent kit.

8.3. Interfering substances and restrictions on the tested material use

The potentially interfering substances effect on the MTB-RESIST-II-Test reagent kit performance has been examined for potentially interfering substances that may originate from the following external and internal sources:

1) substances used in a patient treatment (e.g., medicines);

2) substances found in specific sample types — in this case a clinical sample contamination with a biological agent (hemoglobin, hyaluronic acid) can inhibit a PCR if not sufficiently purified during the DNA isolation procedure;

The interfering substances concentrations that are expected to be recorded during normal use of the MTB-RESIST-II-Test reagent kit are shown in the Table 6.

Table 6 - interfering substances

Type	Substance	Active component	Concentration max.
Endogenic	Biological agents	hemoglobin	260 µg/ml
		Hyaluronic acid	50 µg/ml
Exogenic	Antituberculosis agent	isoniazid	0.02 mg/ml
	Antibiotic, rifampicin	rifampicin	0.02 mg/ml
	Antibiotic, aminoglycoside.	streptomycin	0.2 mg/ml
	Antibiotic, aminoglycoside.	kanamycin	0.2 mg/ml
	Antibiotic, aminoglycoside.	amikacin	0.2 mg/ml
	Antituberculosis agent	ethambutol	0.02 mg/ml
	Antituberculosis agent	pyrazinamide	0.05 mg/ml
	Fluoroquinolones antibacterial agent	ofloxacin	0.04 mg/ml
	Fluoroquinolones antibacterial agent	ciprofloxacin	0.05 mg/ml
	Antituberculosis agent	protionamide	0.05 mg/ml
	Aminosalicylic acid derivative antibiotic. Antituberculosis agent	capreomycin	0.2 mg/ml
	Antibiotic. Antituberculosis agent	cycloserine	0.05 mg/ml

The tests evaluated the potentially interfering substances effect on the assay result obtained with the test reagent kit. According to the study results, exogenous substances (drugs used in the diagnosed with pulmonary and extrapulmonary tuberculosis patients treatment) and endogenous substances (substances found in specific types of biological samples – blood, hyaluronic acid) do not interfere with the work of the kit.

Limitations on test material use:

- test material usage is not allowed under storage and transportation conditions violation (temperature, duration, repeated freezing and thawing);
- test material cannot be used under the requirements violation for the pretreatment procedure;

– it is not allowed to use samples contaminated with extraneous biological material.

– it is not allowed to use the samples that do not meet the requirements.

If the analyzed DNA sample contains several strains of Mycobacterium tuberculosis complex carrying different polymorphisms of the analyzed gene regions it is possible to obtain invalid results.

9. Kit Components Preparation for Testing

The kit does not need to be installed, assembled, adjusted, calibrated for commissioning.

ATTENTION! When working with a DNA, it is necessary to use only disposable sterile plastic consumables with a "DNase-free" label. It is mandatory to use a separate pipette tip with an aerosol barrier for each reaction component.

ATTENTION! Reaction mixture components should be mixed according to the Table 5 in PCR tubes before test conduction.

Kit Components Preparation for Testing

1. Mix thoroughly the tube contents with the DNA isolated for test, PCR buffer, oligonucleotide mixtures A-H, PC-2 and NC, turn over each tube 10 times or mix using vortex at low speed for 3-5 seconds, then remove the drops from the test tube lids by short centrifugation.

2. Select the required number of 0.1ml or 0.2ml PCR tubes (with optically transparent lids or walls, depending on the used cycler type) according to the calculation: 8 x (test samples number + 1 PC-1 + 1 PC-2 + 1 NC) (Table 7).

Before preparing the reactions, PCR cabinet, equipment and materials contained in it should be wet cleaned using disinfectants suitable for use in PCR laboratories, and exposed to UV-radiation for 20-30 minutes.

Table 7 -Tubes labeling principle

	Test samples			PC-1	PC-2	NC
	1	2	n			
Reaction mixture A	○	○	○	○	○	○
Reaction mixture B	○	○	○	○	○	○
Reaction mixture C	○	○	○	○	○	○
Reaction mixture D	○	○	○	○	○	○
Reaction mixture E	○	○	○	○	○	○
Reaction mixture F	○	○	○	○	○	○

Reaction mixture G	○	○	○	○	○	○
Reaction mixture H	○	○	○	○	○	○

10. Testing procedure

PCR testing includes following steps:

1. PCR Setup
2. DNA PCR amplification with in real time DNA probe melting temperature analysis;
3. Test sample results interpretation
4. Results Interpretation (fully described in Chapter 11).

A) PCR-test preparation

(is carried out in pre-PCR area — a room for reagent dispensing and preparation for PCR amplification)

Total reaction amount — 25µl.

ATTENTION! It is not allowed to change the reaction amount.

Every reaction mixture (A, B, C, D, E, F, G and H) preparation requires:

- 5x PCR-buffer — 5µl,

- Corresponding oligonucleotide mixture — 15µl,
- Sample (DNA test sample, PC-1, PC-2 or NC) — 5 µl.

The reaction tubes should be prepared in the following order:

1. Label 0.1ml or 0.2ml test tubes or a plate for PCR.
2. Prepare the reaction mixture A in a separate disposable sterile 1.5ml or 2.0ml Eppendorf type test tube: (n+4)x5µl of 5xPCR-buffer and (n+4)x15µl of the oligonucleotide mixture A, where n stands for the test samples number.
3. Prepare the reaction mixture B in a separate disposable sterile 1.5ml or 2.0ml Eppendorf type test tube: (n+4)x5µl of 5xPCR-buffer and (n+4)x15µl of the oligonucleotide mixture B, where n stands for the test samples number.
4. Prepare the reaction mixture C in a separate disposable sterile 1.5ml or 2.0ml Eppendorf type test tube: (n+4)x5µl of 5xPCR-buffer and (n+4)x15µl of the oligonucleotide mixture C, where n stands for the test samples number.
5. Prepare the reaction mixture D in a separate disposable sterile 1.5ml or 2.0ml Eppendorf type test tube: (n+4)x5µl of 5xPCR-buffer and (n+4)x15µl of the oligonucleotide mixture D, where n stands for the test samples number.
6. Prepare the reaction mixture E in a separate disposable sterile 1.5ml or 2.0ml Eppendorf type test tube: (n+4)x5µl of 5xPCR-buffer and (n+4)x15µl of oligonucleotide mixture E, where n stands for the test samples number.
7. Prepare the reaction mixture F in a separate disposable sterile 1.5ml or 2.0ml Eppendorf type test tube: (n+4)x5µl of 5xPCR-buffer and (n+4)x15µl of oligonucleotide mixture F, where n stands for the test samples number.
8. Prepare the reaction mixture G in a separate disposable sterile 1.5ml or 2.0ml Eppendorf type test tube: (n+4)x5µl of 5xPCR-buffer and (n+4)x15µl of oligonucleotide mixture G, where n stands for the test samples number.
9. Prepare the reaction mixture H in a separate disposable sterile 1.5ml or 2.0ml Eppendorf type test tube:

(n+4)x5µl of 5xPCR-buffer and (n+4)x15µl of oligonucleotide mixture G, where n stands for the test samples number.

10. Add 20µl of prepared reaction mixtures A-H into corresponding tubes (Table 6).

11. Add 5µl of extracted DNA into the corresponding tubes (Table 6). Do not add the DNA into the PC-1, PC-2 and NC tubes.

12. Add 5µl of PC-1 into the 8 corresponding tubes.

13. Add 5µl of PC-2 into the 8 corresponding tubes.

14. Add 5µl of NC-1 that has passed the isolation stage into the 8 corresponding tubes.

15. Centrifugate the test tubes during 1-3 seconds to remove the drops from the walls. Use a microcentrifuge-vortex.

B) Real-Time DNA PCR amplification with DNA probes melting temperature detection

(performed in the PCR area — PCR amplification room)

1. Install tubes in the real-time PCR device reaction module. It is recommended to install the tubes in the center of the thermoblock to ensure that the tubes are pressed evenly by the heating lid.

2. Program the device to perform the corresponding PCR program according to the instructions for the used device. Analysis type: PCR amplification with further melting curves analysis with fluorescence signal detection. PCR protocol is specified in the Tables 5-6 depending on the used cycler.

ATTENTION! If you use QuantStudio 5 and other similar cyclers it is necessary to adjust optical filters before starting the amplification protocol. Click the “Action” button in the “Method” tab, then select “Optical filter settings” in the pop-up menu and in the «Melt Curve Filter» tab choose just the following filter combinations: x1 - m1, x2 - m2, x3 – m3, x4 - m4, x5 - m5, x6 - m6.

3. Specify the samples number and identifiers, mark the tubes location on the thermoblock matrix in accordance with their installation.

4. Make sure that the FAM, HEX, ROX, Cy5 and Cy5.5 detection channels are applied for the optical measurement parameter amplification program for the reaction mixture A and the FAM, HEX, ROX and Cy5 channels for the reaction mixtures B-H.

5. Start amplification with melting curve analysis.

6. Proceed to analyze the data at the end of the program.

Table 8 — PCR protocol and melting temperature analysis for the CFX96 (BioRad, USA) and QuantStudio 5 (Thermo Fisher Scientific, USA) cyclers.

Stage	Temperature, °C	Time, min.:sec.	Detection channels	Total cycles amount
1	95	05:00	□	□
2	95	00:15	□	50
	64	00:45	□	
3	Melting at 35°C... 85°C, increment 0.5°C	00:10	FAM, HEX, ROX, Cy5, Cy5.5	100

Table 9 — PCR and melting temperature analysis protocols for DTprime cycler (manufactured by DNA-Technology LLC, Russia)

Stage	Temperature, °C	Time, min.:sec.	Detection channels	Total cycles amount
1	95	05:00	□	□
2	95	00:15	□	50
	64	00:45	□	
3	Melting at 35°C... 85°C, increment 1.0°C	00:25	FAM, HEX, ROX, Cy5, Cy5.5	50

B) Results Registration and Interpretation.

Result interpretation is carried out according to the melting point values (T_m) that correspond to the highest fluorescence level in the detection channels corresponding to the FAM, HEX, ROX, Cy5 and Cy5.5 fluorophores for the reaction mixture A and FAM, HEX, ROX and Cy5 for the reaction mixtures B-H.

Single melting peaks with melting temperature (T_m) in the range 40˚C…80 ˚C should be recorded for all the tested samples in the FAM, HEX, ROX, Cy5, and Cy5.5 channels for the reaction mixture A. Additional peaks with lower fluorescent signal intensity comparing to the target peak are acceptable.

Test sample results interpretation starts only after the correct PC-1, PC-2 and NC passage.

ATTENTION! If DTprime cycler is used the "-dF/dT" option should be applied. Automatic peak detection for all detection channels may not be technically available. In this case use "Temperature marker" setting and manual melting temperature (Tm) detection.

ATTENTION! When using CFX96 and similar cyclers, threshold line should be set in such a way that one peak is detected in the temperature range 40˚C… 80˚C (additional peaks with a lower fluorescent signal intensity presence is acceptable).

Test sample result Interpretation

Single melting peaks should be detected for PC-1 and PC-2 (additional peaks presence with a lower fluorescent signal intensity is acceptable) in the temperature range mentioned in the Table 10. The actual melting temperatures may vary and depend on the used cycler model.

If the melting peaks cannot be differentiated by the cycler software, but they are clearly differentiated visually, the melting temperatures can be determined manually.

There should be no melting peaks for NC in the FAM, HEX, ROX and Cy5 channels (see Table 10).

Table 10 — PC-1, PC-2 and NC results

Control sample	T_m in detection channels, corresponding to fluorophores, ˚C				
	FAM	HEX	ROX	Cy5	Cy5.5
Reaction mixture A					
NC	no melting peaks				□
PC-1	72±4	71±4	64±5	65±4	65±5
PC-2	67±4	75±4	59±5	71±4	65±5
Reaction mixture B					
NC	no melting peaks				□
PC-1	69±4	65±4	□	□	□
PC-2	62±4	71±4	□	□	□
Reaction mixture C					
NC	no melting peaks				□
PC-1	68±4	62±4	69±4	64±4	□
PC-2	53±4	71±4	65±4	53±4	□
Reaction mixture D					
NC	no melting peaks				□
PC-1	69±4	68±4	53±4	59±4	□
PC-2	69±4	67±4	44±4	49±4	□
Reaction mixture E					
NC	no melting peaks				□
PC-1	73±4	69±4	66±4	60±4	□
PC-2	70±4	65±4	63±4	54±4	□
Reaction mixture F					
NC	no melting peaks				□
PC-1	75±4	70±4	71±4	67±4	□
PC-2	73±4	70±4	75±4	65±4	□
Reaction mixture G					
NC	no melting peaks				□
PC-1	72±4	69±4	68±4	73±4	□
PC-2	65±4	75±4	66±4	73±4	□
Reaction mixture H					
NC	no melting peaks				□
PC-1	70±4	69±4	68±4	70±4	□
PC-2	67±4	73±4	66±4	69±4	□

When obtaining NC values that differ from those mentioned in the Table 10, the entire assay results are considered unreliable. In that case, special measures should be taken to eliminate possible contamination.

If the values obtained for PC-1 or PC-2 differ from those mentioned in the Table 10 repeat amplification of the entire sample batch. If reobtained PC-1, PC-2 values differ from those mentioned in the Table 10, it is necessary to replace the reagents.

D) Results interpretation

Single melting peaks with corresponding temperature (T_m) characteristics in the range from 40˚C to 80˚C should be registered for all test samples in the FAM, HEX, ROX, Cy5 and Cy5.5 channels for the reaction mixture A. If it is impossible to detect melting temperature (T_m) peaks using a cycler software but they can be clearly differentiated visually the melting temperatures can be determined manually.

Melting peak absence in some of the channels is allowed during interpretation. This may be associated with a certain polymorphism presence and a matrix concentration. If more than 70% of melting peaks are absent the result for all the reactions with a test sample is considered invalid (low Mycobacterium tuberculosis complex DNA concentration in the sample, inhibitors absence or other closely related to Mycobacterium tuberculosis complex species presence — *M. avium*, *M. abscessus* etc.)

ICS detection is conducted in the Cy5.5 channel of the reaction mixture A. A peak with a 60°C to 70°C melting point should be registered in this channel. ICS positive passage indicates the nucleic acid extraction effectiveness and PCR inhibitors absence in the reaction. If there is no reaction with the ICS as well as no reactions in FAM, HEX, ROX and Cy5 channels, the results should be considered invalid, and a second test starting with DNA extraction should be conducted for the test sample. If there is no reaction with ICS, but there are reactions in the FAM, HEX, ROX and Cy5 channels, the results should be considered reliable. If the result is invalid again, biomaterial from this patient should be collected for the second time.

The sample melting temperatures are analyzed comparing to a PC-1 of a reaction mixture in each channel according to the formula:

$$\mathbf{\Delta T_m = T_{m(PC\text{-}1)} - T_{m(sample)}.}$$

For example, calculation for a reaction mixture A:

$\Delta T_{m(FAM)} = T_{m(PC\text{-}1\ FAM)} - T_{m(sample\ FAM)}$;

$\Delta T_{m(HEX)} = T_{m(PC\text{-}1\ HEX)} - T_{m(HEX\ sample)}$;

$\Delta T_{m(ROX)} = T_{m(PC\text{-}1\ ROX)} - T_{m(ROX\ sample)}$;

$\Delta T_{m(Cy5)} = T_{m(PC\text{-}1\ Cy5)} - T_{m(Cy5\ sample)}$ (and further by analogy for all the reaction mixtures)

The interpretation is carried out in accordance with the Tables 11-17, where the obtained ΔT_m values are analyzed.

Conclusion about the Mycobacterium tuberculosis complex drug resistance against chemotherapeutic drugs is made depending on the identified polymorphisms.

Table 11 - Results interpretation principle for the *rrs* gene in the reaction mixture A

	Reaction mixture				Result
	A				
	FAM	HEX	ROX	Cy5	
	normal	1401A>G	1402C>T	1401A>G + 1402C>T	
	ΔT_m				
1.	≥ -2	≥ -2; there may be no melting peak in one of the channels			1401A>G and 1402C>T polymorphisms in the *rrs* gene not detected
2.	not considered	< -2 in one of the channels; there may be no melting peak in one of the channels			polymorphism in the *rrs* gene corresponding to the channel with the lowest ΔT_m is detected, except $\Delta Tm(HEX) < 0$ and $\Delta Tm(Cy5) < 0$: in that case only 1401A>G polymorphism is specified
3.	< -2	≥ -2 in all the channels; there may be no melting peak in one of the channels			invalid result for the *rrs* gene in the reaction mixture A
4.	not considered	no melting peaks in two or more channels			invalid result for the *rrs* gene in the reaction mixture A

"not considered" - the ΔT_m values are not taken into account during interpretation.

Table 12 - Results interpretation principle for the *rrs* gene in the reaction mixture B

	Reaction mixture				Result
	B				
	FAM	HEX	ROX	Cy 5	
	normal	1484G>T	–	–	
	ΔT_m				
1.	≥ -2	≥ -2 or absent	not considered	not considered	1484G>T polymorphism in the *rrs* gene not detected
2.	≥ -2 or absent	< -2			1484G>T polymorphism in the *rrs* gene is detected
3.	< -2	< -2			invalid result for the *rrs* gene in the reaction mixture B
4.	absent				invalid result for the *rrs* gene in the reaction mixture B

"not considered" - T_m values are not taken into account during interpretation.

Table 13 - Results interpretation principle for the *eis* gene in the reaction mixtures C and D

	Reaction mixture						Result
	C				D		
	FAM	HEX	ROX	Cy5	ROX	Cy5	
	normal	C-14G	C-14T	C-12T	G-10C	G-10A	
	ΔT_m						
1.	≥ -2	≥ -2, there may be no melting peak in one of the channels					C-14G, C-14T, C-12T, G-10C and G-10A polymorphisms in the *eis gene* not detected
2.	≥ -2	< -2 in one of the channels; there may be no melting peak in two of the channels					Polymorphism in the *eis* gene corresponding to a channel with the lowest ΔT_m is detected
3.	< -2	≥ -2 for all the channels; there may be no melting peaks in two of the channels					Invalid result for the *eis* gene
4.	not considered	no melting peak in three or more channels					Invalid result for the *eis* gene

"not considered" - the ΔT_m values are not taken into account during interpretation.

Table 14 - Results interpretation principle for the *eis* gene in the reaction mixture D

	Reaction mixture		Result
	D		
	FAM	HEX	
	normal	G-37T	
	ΔTm		
1.	≥ -2	≥ -2 or absent	G-37T polymorphism in the *eis* gene not detected
2.	≥ -2 or absent	< -2	G-37T polymorphism in the *eis* gene detected
3.	< -2	< -2	Invalid result for the *eis gene* in a reaction mixture D
4.	absent		Invalid result for the *eis gene* in a reaction mixture D

"not considered" - T_m values are not taken into account during interpretation.

Table 15 - Results interpretation principle for the *gyrA* gene in the reaction mixtures E, F, and G

	Reaction mixture									Result
	E				F				G	
	FAM	HEX	ROX	Cy5	FAM	HEX	ROX	Cy5	Cy5	
	normal	p.G88C	p.A90V	p.S91P	p.D94G	p.D94N	p.D94H	p.D94A	p.D94Y	
	ΔT_m									
1.	≥ -2	≥ -2, there may be no melting peak in three of the channels								p.G88C, p.A90V, p.S91P, p.D94G, p.D94N, p.D94H, p.D94A and p.D94Y polymorphisms in the *gyrA* gene not detected
2.	not considered	< -2 in one of the channels; there may be no melting peak in three of the channels								polymorphism in the *gyrA* gene corresponding to the channel with the lowest ΔT_m is detected
3.	< -2	≥ -2 in all the channels; there may be no melting peak in three of the channels								invalid result for the *gyrA* gene
4.	not considered	there may be no melting peak in four channels or more								invalid result for the *gyrA* gene

"not considered" - the ΔT_m values are not taken into account during interpretation.

Table 16 - Results interpretation principle for the *gyrB* gene in the reaction mixture G

	Reaction mixture			Result
	G			
	FAM	HEX	ROX	
	normal	p.D461H	p.D461N	
	ΔT_m			
1.	≥ -2	≥ -2, there may be no melting peak in one of the channels		p.D461H and p.D461N polymorphisms in the *gyrB* gene not detected
2.	not considered	<-2 in one of the channels; there may be no melting peak in one of the channels		polymorphism in the *gyrB* gene corresponding to the channel with the lowest ΔT_m value was detected
3.	< -2	≥ -2 in both channels		invalid result for the *gyrB* gene
4.	no melting peaks in two or more channels			invalid result for the *gyrB* gene

"not considered" - the ΔT_m values are not taken into account during interpretation.

Table 17 - Results interpretation principle for the *gyrB* gene in the reaction mixture H

	Reaction mixture				Result
	H				
	FAM	HEX	ROX	Cy5	
	normal	p.N499D	p.E501V	p.A504V	
	ΔT_m				
1.	≥ -2	≥ -2; there may be no melting peaks in one of the channels			p.N499D, p.E501V and p.A504V polymorphisms in the *gyrB* gene not detected
2.	≥ -2	< -2 in one of the channels; there may be no melting peak in one of the channels			polymorphism in the *gyrB* gene corresponding to the channel with the lowest ΔT_m value was detected
3.	< -2	≥ -2 in all the channels			invalid result for the *gyrB* gene
4.	no melting peaks in two or more channels				invalid result for the *gyrB* gene

"not considered" - the ΔT_m values are not taken into account during interpretation.

The reasons for obtaining an invalid result may be low DNA concentration, inhibitors' presence in the DNA sample obtained from clinical material; incorrect analysis protocol execution; non-compliance with the PCR temperature regime; several strains of Mycobacterium tuberculosis complex presence in the sample, carrying different mutations in the analyzed *rrs*, *eis*, *gyrA* and *gyrB* genes regions, et al.

If the result is invalid the conclusion is not issued. It is necessary to recollect biomaterial from a patient and retest it. If an invalid result repeats, retest with another manufacturer's reagent kit or using another method.

Table 18 – Association of*rrs* and *eis* gene polymorphisms with aminoglycoside resistance

Polymorphism	**Resistance to aminoglycosides:**			
	kanamycin	**amikacin**	**capreomycin**	**streptomycin**
rrs gene				
1401A>G (a1401g)	++	++	++	?
1402C>T (c1402t)	+	+	++	?
1484G>T (g1484t)	++	++	++	?
eis gene				
C-14G	–[A]	–[A]	–	–
C-14T	++	++	–	–
C-12T	++	–	–	–
G-10C	?	?	–	–
G-10A	++	?	–	–
G-37T	++	?	–	–

Designations: "++" — a polymorphism association with resistance against a corresponding drug; "+" — a polymorphism association with moderate resistance against a corresponding drug; "?" — non-specified amount of polymorphism in resistance development; "-" — no polymorphism association with resistance (according to the Catalogue of mutations in Mycobacterium tuberculosis complex and their association with drug resistance — Supplementary document. World Health Organization. WHO/UCN/GTB/PCI/2021.7 — © WHO 2021;
«[A]» — according to Pholwat S. et al. *eis* Promoter C14G and C15G Mutations Do Not Confer Kanamycin Resistance in Mycobacterium tuberculosis // Antimicrobial Agents and Chemotherapy. 2016. Vol. 60 (12). P. 7522–7523).

Table 19 – Polymorphism association in the *gyrA* and *gyrB* genes with resistance to fluoroquinolones

Polymorphism	**Resistance to fluoroquinolones:**	
	levofloxacin	**moxifloxacin**
gyrA gene		
p.G88C	++	++
p.A90V	++	++
p.S91P	++	++
p.D94G	++	++
p.D94N	++	++
p.D94H	++	++
p.D94A	++	++
p.D94Y	++	++
gyrB gene		
p.D461H	?	?
p.D461N	+	+
p.N499D	+	+
p.E501V	+	+
p.A504V	+	+

Designations: "++" — a polymorphism association with resistance against a corresponding drug; "+" — a polymorphism association with moderate resistance against a corresponding drug; "?" — non-specified amount of polymorphism in resistance development; "-" — no polymorphism association with resistance (according to the Catalogue of mutations in Mycobacterium tuberculosis complex and their association with drug resistance — Supplementary document. World Health Organization. WHO/UCN/GTB/PCI/2021.7 — © WHO 2021;

Obtained analysis result diagnostic value

Obtained positive or negative result of *rrs, eis, gyrA* and *gyrB* genes status detection associated with Mycobacterium tuberculosis complex drug resistance against second-line chemotherapeutic drugs — aminoglycosides (*rrs* and *eis* genes) and fluoroquinolones (*gyrA* and *gyrB* genes) can be used by a qualified specialist (a doctor) taking into account clinical findings and other investigations, to select appropriate therapy for patients with a confirmed pulmonary and extrapulmonary tuberculosis diagnosis according to the clinical recommendations "Tuberculosis in adults" (age group: adults; approved in 2022), "Tubesculosis in children" (age group: children; approved in 2022), Russian Society of Phthisiatricians, National Association of Non-Profit Phthisiatricians Organizations"Association of Phthisiatricians"" (approved by the Ministry of Health of Russia).

11. Storage, Transportation and Usage Conditions

Storage

MTB-RESIST-II-Test reagent kit should be stored in the manufacturer's packaging at -18°C...-22°C during the entire kit shelf life. It is allowed to store at 2°C...8°C up to 5 days. It is allowed to freeze / thaw MTB-RESIST-II-Test reagent kit up to 5 times max.

Atmospheric pressure is not under control because it does not affect the product quality.

Reagent kit stored under storage conditions violation cannot be used.

Transportation

MTB-RESIST-II-Test reagent kit can be transported by all types of covered vehicles in accordance with the transportation rules applicable for the vehicle type. MTB-RESIST-II-Test reagent kit transportation is allowed at -18°C... -22°C during the entire shelf-life period. Transportation is allowed at 2°C... 8°C up to 5 days.

Atmospheric pressure is not under control because it does not affect the product quality.

To ensure compliance with transportation conditions throughout the entire transportation period, the reagent kit should be placed in a reusable polyurethane foam thermal container filled with ice packs for temporary storage and transportation. Ice packs type, volume and their number in a thermal container and the thermal container size varies according to the

transportation duration and conditions.

Reagent kits transported under the temperature conditions violation cannot be used.

Shelf life

MTB-RESIST-II-Test shelf life is 12 months from the acceptance date by the manufacturer's Quality Control Department (QCD) under all the transportation, storage and usage conditions. A reagent kit with an expired shelf life cannot be used.

Opened kit components shelf life

12 months from the acceptance date by the manufacturer's QCD if stored at -18°C... -22°C.

Ready for usage kit components shelf life

One hour under conditions that prevent drying of the components as well as contamination by extraneous biological material.

12. Disposal

Reagent kits that have become unusable including the ones with expired shelf life, are subject to disposal in accordance with sanitary and epidemiological requirements for the management of medical waste.

According to medical waste classification the kits belong to Class A (epidemiologically safe waste, which is similar in composition to solid household waste).

Unused reagents are collected in a single-use labeled packaging of any color (except yellow and red) in accordance with sanitary and epidemiological requirements for the management of medical waste.

Used tubes and materials are disposed of in accordance with the requirements for disinfection, pre-sterilization, cleaning and sterilization of medical devices.

Liquid components (reagents, chemical agents) are disposed by draining into a sewer with a reagent preliminary dilution with tap water 1:100 and removing the packages remains as industrial or household garbage.

MTB-RESIST-II-Test consumer packaging is subject to mechanical destruction with the residues removal as industrial or household garbage.

Personnel carrying out the reagent kit destruction must comply with the safety rules for carrying out one or another destruction method.

Warranty, Contacts

The manufacturer guarantees the MTB-RESIST-II-Test reagent kit quality and safety during the shelf-life period in compliance with the product transportation and storage requirements, as well as in compliance with the usage rules. In case of complaints about the reagent kit quality, undesirable events or incidents, submit information to:

Limited Liability Company TestGene (TestGene, LLC),
9, 44 Inzhenerny Proezd, office 13, Ulyanovsk, 432072, Russian Federation
www.testgene.com

Technical Support Service:

Phone number: +7 927 981 58 81
E-mail: help@testgen.ru