INSTRUCTION FOR USE

Reagent kit for *rpoB, katG and inhA* gene polymorphisms status detection associated with Mycobacterium tuberculosis complex drug resistance against the first-line chemotherapeutic agents (rifampicin, isoniazid) by multiplex PCR-RT

MTB-RESIST-I-test

IVD

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Content

List of abbreviations

Abbreviations and designations used in the instruction:

PCR	multiplex polymerase chain reaction
DNA	deoxyribonucleic acid
MTB	Mycobacterium tuberculosis (latin)
ICS	internal control sample
NC	negative control sample
PCS	positive control sample
SenC	Control sample for specificity detection
SC	specificity control sample

Introduction

Infectious diseases caused by tuberculosis complex mycobacteria (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canettii*, *M. caprae*, *M. microti*, etc.) are one of the life quality reduction causes and often lead to death. The pathogen's drug-resistance timely detection is important for adequate tuberculosis therapy selection.

Conclusion about drug resistance using molecular genetic methods is based on the mutations detection in genes associated with drug resistance to certain groups of agents.

Molecular genetic methods usage to determine drug resistance is an initial patient examination stage and does not exclude the need to use traditional culture methods to investigate the tuberculosis complex mycobacteria drug sensitivity. Mutation detection associated with drug resistance in the tuberculosis complex mycobacteria DNA with sensitivity determination to isoniazid and rifampicin is an initial stage of tuberculosis patients examination[1,2,3,4].

Target analytes: specific parts if a genomic tuberculosis complex mycobacteria DNA— gene fragments:

rpoB (β subunit of a DNA polymerase) — 510–533th codons polymorphisms are detected but not differentiated (according to the nomenclature *Escherichia coli*[5]), at the same time the test system is not sensitive to possible synonymous mutations in codons 513 (p.Q513Q, c.1539A>G) and 514 (p.F514F, c.1542C>T) that have no clinical significance; polymorphisms differentiated with high probability: D516V, D516Y, 526th codon, L533R, L533P, S531L

[1] Clinical guidelines. Tuberculosis in adults. The Ministry of Health of the Russian Federation. Approved in 2020.

[2] Methodological recommendations for respiratory tuberculosis diagnosis and treatment improvement. Approved by the Order of the Ministry of Health of the Russian Federation № 951 dated 29.12.2014

[3] Federal clinical recommendations on diagnosis and treatment of tuberculosis in HIV-infected patients. M.- Tver: Publishing house "Triada", LLC, 2014. p. 56.

[4] WHO consolidated guidelines on drug-resistant tuberculosis treatment. Geneva: World Health Organization, 2019. License: CC BY-NC-SA 3.0 IGO. 104 p. (ISBN 978-92-4-155052-9)

[5] Telenti A., Imboden P., Marchesi F., Lowrie D., Cole S., Colston M.J., Matter L., Schopfer K., Bodmer T. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis* // Lancet. 1993. Vol. 341. № 8846. P. 647–650.

katG (catalase-peroxidase) — polymorphisms leading to S315T, S315N, S315R, S315I amino acid replacement are differentiated;

inhA (inhibin α-subunit) — a polymorphism is differentiated in the the *inhA* gene promoter region C-15T; polymorphisms of the region -20… +6 are also detected but not differentiated.

Scientific validity of a target analyte

According to the Clinical Guidelines Tuberculosis in Adults, 2020 (approved by the Ministry of Health of the Russian Federation):

Detection of drug resistance-associated mutations in tuberculosis complex mycobacteria DNA (Mycobacterium tuberculosis complex) with the sensitivity detection to isoniazid and rifampicin or at least rifampicin is recommended as a priority component of the examination in patients with tuberculosis. Testing is conducted twice if the first test result is negative as well as in case of a positive result and simultaneous absence of clinical and radiological signs of tuberculosis disease.

Comments: the molecular genetic testing methods main advantage is that they are "fast" and highly sensitive, and allow to obtain results in a short time (1-2 days), unlike culture testing methods (10-90 days) and have a high sensitivity of 75% (microscopic method — 50%). Conclusion about the tuberculosis complex mycobacteria presence in the diagnostic material is made based on Mycobacterium tuberculosis DNA detection. Molecular genetic methods positive results do not determine the bacterial isolation status as well as microscopic and culture testing methods.

Decision about drug resistance (DR) using molecular genetic testing methods is based on mutations detection in genes, associated with drug resistance. The molecular genetic testing method important advantage is a rapid and reliable detection of tuberculosis in patients with MDR-MBT. It allows to separate patient streams and timely prescribe the IV chemotherapy regimen. Molecular genetic testing methods usage for DR determination is an initial step in patient examination and does not preclude the need for traditional culture methods for MBT drug susceptibility testing."

Target analyte scientific validity lies in the association of the studied *rpoB*, *katG* and *inhA* gene polymorphisms with the tuberculosis complex mycobacteria drug resistance development against the first-line chemotherapeutic drugs — rifampicin (*rpoB*), isoniazid (*katG* and *inhA*) and their analogs.

The *rpoB* gene detected polymorphisms comply with the codons

510-533, corresponding to the RRDR[6] (rifampicin resistance determining region), that defines resistance to rifampicin. More than 30 clinically relevant polymorphisms (nucleotide substitutions, deletions and insertions) of the region are known. The *katG* gene differentiated polymorphisms: S315T (its replacement is the most frequent), S315N, S315R, S315I are associated with strains drug resistance to isoniazid [7,8,9].

Differentiated polymorphism in the *inhA* gene promoter: C-15T[7,8,9 is the] most frequent. There may be rare polymorphisms (e.g., A-16G, T-8A, T-8C) that are not differentiated during the assay but that may confer intermediate drug resistance to isoniazid in the detected area from -20 to +6.

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

Indications and contraindications for use:

Indications for use: reagent kit MTB-RESIST-I-Test is recommended for use in patients with confirmed pulmonary and extrapulmonary tuberculosis for appropriate 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.

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[6] Goldstein B.P. Resistance to rifampicin: a review // The Journal of Antibiotics. 2014. Vol. 67. P. 625–630.

[7] Technical report on critical concentrations for drug susceptibility testing of isoniazid and the rifamycins (rifampicin, rifabutin and rifapentine). Geneva: World Health Organization, 2021. License: CC BY-NC-SA 3.0 IGO. 107 p. (ISBN 978-92-4- 001728-3)

[8] Unissa A.N., Doss C.G.P., Kumar T., Sukumar S., Lakshmi A.R., Hanna L.E. Analysis of interactions of clinical mutants of catalase-peroxidase (*KatG*) responsible for isoniazid resistance in *Mycobacterium tuberculosis* with derivatives of isoniazid // Journal of Global Antimicrobial Resistance. 2017. Vol. 11. P. 57–67.

[9] Salina T.U., Morozova T.I. Prevalence of mutations in Mycobacterium tuberculosis genes encoding drug resistance to isoniazid and rifampicin in tuberculosis patients of different age groups
// Tuberculosis and Lung Diseases. 2019. Vol. 97, № 4. P. 12-18

1. Intended use

Intended use: MTB-RESIST-I-Test reagent kit is designed for the status qualitative determination of *rpoB, katG and inhA* genes molecular genetic polymorphisms associated with drug resistance against the first-line chemotherapeutic drugs — rifampicin, isoniazid and their analogs, using molecular beacon technology with real-time detection in a sample of Mycobacterium tuberculosis complex DNA, isolated from clinical material (sputum, bronchoalveolar lavage, bronchial lavage, gastric lavage, pleural fluid, blood, urine, microbial cultures, prostate secretion, tissue (biopsy and surgical) material, synovial fluid, pericardial fluid and cerebrospinal fluid) in patients with confirmed pulmonary and extrapulmonary tuberculosis for appropriate therapy selection.

Functional use: obtained results can be used for an appropriate chemotherapy regimen prescription for tuberculosis treatment.

Reagent kit potential consumers:

Kit for research use only.

2. Method Principle

Method

Real-time multiplex polymerase chain reaction with melting temperatures of DNA probes (molecular beacons) 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

Quantitative detection of the *rpoB*, *katG* and *inhA* genes molecular genetic polymorphisms associated with drug resistance to chemotherapeutic drugs — rifampicin and isoniazid, tuberculosis complex mycobacteria using real-time multiplex polymerase chain reaction with the DNA probes melting temperatures detection (molecular beacons) 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.

The PCR buffer contains all essential reagents, including thermostable hot-start DNA polymerase, deoxynucleotide triphosphates, and an optimized buffer.

The oligonucleotide mixtures contain fluorescent-labeled oligonucleotide DNA probes that hybridize with the complementary region of the amplified target DNA. 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 are separated and fluorescence intensity increases.

The DNA probes melting temperature depends on the number of a DNA probe complementary nucleotides and a target DNA. The highest melting temperature (T_m) is recorded when the DNA probes to the target DNA complete the most.

The DNA probes melting temperatures analysis (hybridization complex of a DNA probe and a target DNA) allows to draw a conclusion about the complementary nucleotides number of the DNA probe and the target DNA and about a certain DNA probe correspondence to the target DNA. Conclusion about molecular genetic polymorphisms presence is made based on the analysis.

The kit contains reagents for qualitative detection of *rpoB*, *katG* and *inhA* gene polymorphisms associated with drug resistance against such chemotherapeutic drugs as rifampicin and isoniazid, as well as ICS (Table 1).

Table 1 - Targets to be analyzed

Oligonucleotide mixture	**Channel corresponding to fluorophore**				
	FAM / Green	**HEX / Yellow**	**ROX / Orange**	**Cy5 / Red**	**Cy5.5 / Crimson**
A	***rpoB***: codons 529–533	***rpoB***: codons 523–528	***rpoB***: codons 516–522	***rpoB***: codons 510–515	
B	***katG***: codon 315 – standard	***katG***: p.S315T1	***katG***: p.S315T2	***katG***: p.S315N	
C	***inhA***: standard -20 - +6	***inhA***: C-15T	***katG***: p.S315R	***katG***: p.S315I	ICS

ICS (internal control sample) allows to evaluate DNA extraction quality and efficiency and inhibitors possible presence in the sample that can lead to false negative results.

Method limitations

Assay conduction using clinical material without mycobacterium tuberculosis complex DNA.

A possible reason for obtaining a false positive result is contamination at the DNA extraction or multiplex PCR reaction stages. A

false positive result can be detected by a negative control sample.

Package integrity violation during transportation.

An expired kit usage or a kit storage conditions violation.

Storage conditions violation when transporting samples.

Presence of several Mycobacterium tuberculosis complex strains carrying different mutations of the analyzed *rpoB*, *katG* and *inhA* genes regions can be a possible reason for obtaining a false positive result.

Multiplex PCR reaction takes from 125 to 165 minutes (time for sample preparation is not included) depending on a used cycler.

3. Reagent Kit Components

MTB-RESIST-I-Test reagent kit comes only in one kit configuration.

Number of analyzed samples

The reagent kit comes in one configuration (Table 2) and is intended for 96 reactions that equate to detection of 29 test samples, negative and positive samples in a single run of a cycler for 96 wells or 8 single runs of test samples with negative and positive control samples in each run.

Table 2 -MTB-RESIST-I-Test reagent kit composition

No.	Reagent	Description	Quantity, volume
1	5x PCR Buffer	Transparent colorless liquid	1 test tube, 480 µl
2	Oligonucleotide mixture A	Transparent colorless liquid, may be purple	1 test tube, 1440 µl
3	Oligonucleotide mixture B	Transparent colorless liquid, may be purple	1 test tube, 1440 µl
4	Oligonucleotide mixture C	Transparent colorless liquid, may be purple	1 test tube, 1440 µl
5	PC-1	Transparent colorless liquid	1 test tube, 120 µl
6	PC-2	Transparent colorless liquid	1 test tube, 120 µl
7	NC	Transparent colorless liquid	1 test tube, 800 µl
8	ICS	Transparent colorless liquid	1 test tube, 300 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 batch of the reagent kit.

Reagent Kit Components

5x PCR Buffer is ready to use. It contains all necessary reagents including a thermostable hot-start DNA polymerase, deoxynucleotide triphosphates and a PCR-optimized buffer.

Oligonucleotide mixtures are ready to use and contain primers and probes designed for specific targets detection. See the Table 1.

The passage of the ICS reaction is evaluated by the presence of a melting peak on the Cy5.5/Crimson channel of the reaction mixture C at the 60°C... 70°C range. A positive reaction indicates the efficiency of nucleic acid extraction and the absence of PCR inhibitors in the reaction. If there is no reaction with an ICS and no reaction in the FAM/Green,

Hex/Yellow, ROX/Orange and Cy5/Red channels, the result should be considered invalid, and a second test starting with DNA extraction should be conducted for such test sample. If the result is invalid again, biomaterial from this patient should be collected for the second time.

Positive control 1 (PC-1) is ready for use and is a mixture of plasmid DNAs with synthetic inserts of amplified DNA fragments — *rpoB*, *katG* and *inhA* genes regions not containing the detectable polymorphisms and corresponding to the H37Rv *Mycobacterium tuberculosis* nucleotide sequence (GenBank: NC_000962.3).

Positive control sample 2 (PC-2) is ready to use and is a mixture of plasmid DNAs with synthetic inserts of amplified DNA fragments — *rpoB*, *katG* and *inhA* genes fragments containing the polymorphisms (relative to the H37Rv *Mycobacterium tuberculosis* nucleotide sequence (GenBank: NC_000962.3) in genes:

rpoB: D516V, S522L, H526Y, L533P;

katG: S315T;

inhA: C-15T.

Negative control sample (NC) is ready to use. It is DNase-free deionized water.

Internal control sample (ICS) is ready to use. It is a plasmid DNA.

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 3 - MTB-RESIST-I-Test reagent kit composition

Parameter Name	Characteristics and Standards	Control methods according to TS
1. Technical Characteristics		
1) Description		
5x PCR Buffer	Transparent colorless liquid	Section 7, clause 7.6
Oligonucleotide mixture A	Transparent colorless liquid, may have a shade of lilac color	Section 7, clause 7.6
Oligonucleotide mixture B	Transparent colorless liquid, have a shade of lilac color	Section 7, clause 7.6
Oligonucleotide mixture C	Transparent colorless liquid, may have a shade of lilac color	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- 032-97638376-2020	Section 7, clause 7.12
1.3. Marking	In accordance with clause 1.5 TS 21.20.23- 032-97638376-2020	Section 7, clause 7.12
1.4. Packaging	In accordance with clause 1.6 TS 21.20.23- 032-97638376-2020	Section 7, clause 7.12
2. Functional characteristics		
2.1. Positive result with PC-1	Single peaks are recorded in the range of 50˚C to 80˚C in the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red channels, as well as Cy5.5/Crimson for a reaction mixture C	Section 7, clause 7.8.2
2.2. Positive result with PC-2	Single peaks are recorded in the range of 50˚C to 80˚C are recorded in the FAM/Green, HEX/Yellow channels,	Section 7, clause 7.8.2

	ROX/Orange, Cy5/Red, as well as Cy5.5/Crimson for a reaction mixture C	
2.3. Negative result with NC	No single peaks in the range of 50˚C to 80˚C in the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson channels.	Section 7, clause 7.8.2
2.4. Passage of the reaction in tubes with SC	No single peaks in the range of 50˚C to 80˚C in the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red. Peaks are recorded in the range of 60°C to 70°C in the Cy5.5/Crimson channel for a reaction mixture C	Section 7, clause 7.8.2
2.5. Passage of the reaction in tubes with a SenC-1	Single peaks are recorded in the range of 50˚C to 80˚C in the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red channels as well as in the Cy5.5/Crimson channel for a reaction mixture C	Section 7, clause 7.8.2
2.6. Passage of the reaction in tubes with a SenC-2	Single peaks are recorded in the range of 50˚C to 80˚C are recorded in the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red channels, as well as in the Cy5.5/Crimson channel for a reaction mixture C	Section 7, clause 7.8.2
2.7 Passage of the reaction in NC+ICS	No single peaks in the range of 50˚C to 80˚C in the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red channels. Peak sare recorded in the range of 60°C to 70°C in the Cy5.5/Crimson channel for a reaction mixture C.	Section 7, clause 7.8.2
2.8 Estimation of an indicator ΔTm (Tm(PCS)-1) – Tm(PCS)-2))	Estimation of the indicator ΔT_m ($T_{m(PCS-1)} - T_{m(PCS-2)}$): **for a reaction mixture A:** $\Delta T_{m(rpoB)}$ for FAM, HEX, ROX and Cy5 > 2. **for a reaction mixture B:** $\Delta T_{m(katG)}$ for FAM and Cy5 > 0; $\Delta T_{m(katG)}$ for HEX < -2; $\Delta T_{m(katG)}$ for ROX < -2; The lowest value of $\Delta T_{m(katG)}$ should be for the HEX channel.	Section 7, clause 7.8.2

	for a reaction mixture C: $\Delta T_{m(inhA)}$ for FAM > 0; $\Delta T_{m(inhA)}$ for HEX < 0; $\Delta T_{m(katG)}$ for ROX and Cy5 > 0.	
2.9 Test for polymorphisms correct detection	The result obtained for the qualitative determination of *rpoB, katG and inhA* gene polymorphisms should correspond to the result established for the standard enterprise sample (ESS) by the Sanger sequencing method.	Section 7, clause 7.8.2

NOTE: During control PCR testing as SenC and SC are used:

- as standard control sample for determining sensitivity (SenC-1 and SenC-2) is used a mixture of plasmids with synthetic inserts of a mycobacterium genomic DNA fragment in concentration of 5000 copies per 1ml of each one and a bacteriophage genome fragment in $1*10^7$ copies/ml concentration in 10% TE buffer (10 mM Tris, 1 mM EDTA);

- a specificity control sample (SC) is a mixture of human genomic DNA isolated from Jurkat cell line in 1,000 copies per 5µl (200,000 copies/ml) concentration.

4.2 Analytical efficiency characteristics

4.2.1 Analytical specificity

MTB-RESIST-I-Test is specific to target gene fragments of *rpoB*, *katG* and *inhA* DNA Mycobacterium tuberculosis complex, including M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. canettii, M. caprae, M. microti.

Analytical specificity of target *rpoB, katG* and *inhA* gene fragments was approved *in silico* via the BLAST Resource (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*).

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

At least 5000 copies of genomic DNA per 1ml of biomaterial in case of DNA extraction from a 100µl sample and 50µl of 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 DNA mixture with synthetic amplified mycobacteria DNA fragments inserts (fragments of rpoB, katG and inhA genes that do not contain detectable polymorphisms and correspond to the H37Rv Mycobacterium tuberculosis nucleotide sequence (GenBank: NC_000962.3) — POS_RP_W, POSkat_WT, POSinh_15W) and a bacteriophage genome fragment (pMS2) at 10% TE-buffer (10 mM Tris, 1 mM EDTA) at $1x10^7$ concentration of copies per 1 ml produced by TestGen LLC. **Contains 100% of normal DNA copies** (rpoB, katG and inhA wild-type genes (no mutations));
- **ESS-PC-2** is a plasmid DNA mixture with synthetic amplified mycobacteria DNA fragments inserts that contain D516V, S522L, H526Y, L533P polymorphisms of the *rpoB* gene, S315T1 of the *katG* gene, C-15T of the *inhA* gene (relative to the H37Rv Mycobacterium tuberculosis nucleotide sequence — GenBank: NC_000962.3) and a bacteriophage genome fragment (pMS2) at 10% TE-buffer (10 mM Tris, 1 mM EDTA) of $1x10^7$ concentration of copies per 1 ml produced by TestGen LLC. **Contains 100% of DNA copies with mutations**.

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

To precise the accuracy under repeatability the arithmetic mean of the sample, dispersion, standard deviation, and coefficient of variation index 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 assessment of the test system reproducibility is carried out similarly to the calculation of accuracy under repeatability conditions. 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

161 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, washings from environmental objects) of patients diagnosed with pulmonary and extrapulmonary tuberculosis.

For cross-reactivity assessment in clinical trials the MTB-RESIST-I-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 do 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* heterologous microorganisms.

DNA extraction from clinical samples was performed using the extraction kits recommended in the MTB-RESIST-I-Test kit instructions for use:

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

for DNA extraction from 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, flushes from environmental objects — 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 reagent kit for rpoB, katG and inhA gene polymorphisms status detection associated with Mycobacterium tuberculosis complex drug resistance against the first-line chemotherapeutic drugs (rifampicin, isoniazid) by multiplex PCR-RT MTB-RESIST-I-Test manufactured by TestGene LLC and AmpliTube-RV-Differentiation kit, Syntol LLC; Russia.

Cyclers recommended by the manufacturer of the reagent kit and used for PCR testing:

- DTprime Detecting Cycler (DNA-Technology, NPO, Russia);
- CFX 96 Cycler (Bio-Rad, USA);
- Rotor-Gene Q Cycler (Qiagen, Germany);
- QuantStudio 5 Cycler (Thermo Fisher Scientific, USA).

All the 160 samples were tested by MTB-RESIST-I-Test reagent kit in two rounds (320 observations). For all the four cyclers the results of qualitative determination of rpoB, katG and inhA genes polymorphisms coincided with the results of AmpliTube-RV-Differentiation kit, Syntol LLC; Russia.

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

The results of the reagent kit diagnostic characteristics study in relation to each tested clinical material and analyzed molecular genetic polymorphisms of the *rpoB, katG and inhA* genes are shown in Table 5.

Table 5- Clinical efficiency

Test sample type	Tested gene	Number of positive samples observations	Number of negative samples observations	Diagnostic sensitivity with 95% confidence probability	Diagnostic specificity with 95% confidence probability
Sputum	rpoB	24	24	100% (95% diagnostic interval: 85,75%-100%)	100% (95% diagnostic interval: 85,75%-100%)
	katG	12	36	100% (95% diagnostic interval: 73,54%-100%)	(100% (95% diagnostic interval: 90,26%-100%)

	inhA	14	34	(100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 89,72%-100%)
Bronchoalveolar lavage	rpoB	14	14	(100% (95% diagnostic interval: 76,84%-100%)	(100% (95% diagnostic interval: 76,84%-100%)
	katG	8	20	(100% (95% diagnostic interval: 63,06%-100%)	(100% (95% diagnostic interval: 83,16%-100%)
	inhA	10	18	100% (95% diagnostic interval: 69,15%-100%)	(100% (95% diagnostic interval: 81,47%-100%)
Bronchial washing	rpoB	14	24	(100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 85,75%-100%)
	katG	12	26	100% (95% diagnostic interval: 73,54%-100%)	(100% (95% diagnostic interval: 86,77%-100%)
	inhA	14	24	(100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 85,75%-100%)
Gastric washing	rpoB	12	14	100% (95% diagnostic interval: 73,54%-100%)	(100% (95% diagnostic interval: 76,84%-100%)
	katG	10	16	100% (95% diagnostic interval: 69,15%-100%)	100% (95% diagnostic interval: 79,41%-100%)
	inhA	6	20	(100% (95% diagnostic interval: 54,07 %-100%)	(100% (95% diagnostic interval: 83,16%-100%)
Pleural fluid	rpoB	8	16	(100% (95% diagnostic interval: 63,06%-100%)	100% (95% diagnostic interval: 79,41%-100%)
	katG	10	14	100% (95% diagnostic interval: 69,15%-100%)	(100% (95% diagnostic interval: 76,84%-100%)
	inhA	6	18	(100% (95% diagnostic interval: 54,07 %-100%)	(100% (95% diagnostic interval: 81,47%-100%)
Blood	rpoB	28	34	(100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 89,72%-100%)
	katG	22	40	(100% (95% diagnostic interval: 84,56%-100%)	(100% (95% diagnostic interval: 91,19%-100%)
	inhA	22	40	(100% (95% diagnostic interval: 84,56%-100%)	(100% (95% diagnostic interval: 91,19%-100%)
Urine	rpoB	14	16	(100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 79,41%-100%)
	katG	12	18	100% (95% diagnostic interval: 73,54%-100%)	(100% (95% diagnostic interval: 81,47%-100%)

	inhA	10	20	100% (95% diagnostic interval: 69,15%-100%)	(100% (95% diagnostic interval: 83,16%-100%)
Microbiological cultures	rpoB	4	8	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
	katG	4	8	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
	inhA	6	6	(100% (95% diagnostic interval: 54,07 %-100%)	(100% (95% diagnostic interval: 54,07 %-100%)
Prostate secretion	rpoB	4	6	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 54,07 %-100%)
	katG	4	6	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 54,07 %-100%)
	inhA	4	6	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 54,07 %-100%)
Tissue (biopsy and surgical) material	rpoB	8	10	(100% (95% diagnostic interval: 63,06%-100%)	100% (95% diagnostic interval: 69,15%-100%)
	katG	4	14	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 76,84%-100%)
	inhA	10	8	100% (95% diagnostic interval: 69,15%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
Synovial fluid	rpoB	6	4	(100% (95% diagnostic interval: 54,07 %-100%)	(100% (95% diagnostic interval: 39,76%-100%)
	katG	2	8	(100% (95% diagnostic interval: 15,81%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
	inhA	2	8	(100% (95% diagnostic interval: 15,81%-100%)	(100% (95% diagnostic interval: 63,06%-100%)
Pericardia fluid	rpoB	2	4	(100% (95% diagnostic interval: 15,81%-100%)	(100% (95% diagnostic interval: 39,76%-100%)
	katG	2	4	(100% (95% diagnostic interval: 15,81%-100%)	(100% (95% diagnostic interval: 39,76%-100%)
	inhA	4	2	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 15,81%-100%)
Cerebrospinal fluid	rpoB	4	6	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 54,07 %-100%)
	katG	4	6	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 54,07 %-100%)
	inhA	4	6	(100% (95% diagnostic interval: 39,76%-100%)	(100% (95% diagnostic interval: 54,07 %-100%)

5. Risks associated with the reagent kit use

The border risk zone includes the following:

Loss of functional properties of reagents included in the kit due to transportation, storage or operation under inappropriate conditions;

Clinical material contamination with inhibitory substances in concentrations exceeding the permissible ones;

Contamination of reaction mixtures and tested RNA samples with contents from the PCS-1, PCS-2 tubes or with PCR products;

Performing an assay using a low-quality DNA sample (low concentration and/or poor purification);

Failure to meet the requirements for sample preparation, testing and disposal due to the actions of unqualified personnel;

Usage of an unusable kit (after the expire date or in case of packaging violation).

No risks have been identified in the risk zone area.

Total residual risk of using the reagent kit for qualitative detection of *rpoB, katG* and *inhA* genes polymorphisms associated with drug resistance to rifampicin and isoniazid in Mycobacterium tuberculosis complex by multiplex PCR-RT MTB-RESIST-I-Test is acceptable; the benefit of its usage exceeds the risk.

6. Safety Precautions

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

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

Personnel should ensure and comply with the rules of biological safety 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 effects of magnetic fields, 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 that have potential infectious nature. So, precautions against any special, unusual risks when using or selling the product are not provided.

7. Required Equipment and Materials

Work with the reagent kit is carried out in working area 3 (for preparing reactions)

Multiplex PCR Equipment:

1. PCR-biological safety cabinet, Class II and III (e.g., BMB-II – "Laminar-C-1,2", Laminar Systems, Russian Federation).
2. Vortex (e.g., TETA-2, Biokom, Russian Federation).
3. Set of electronic or automatic variable volume dispensers (e.g., Eppendorf, Germany).
4. Refrigerator for +2°C to +8 °C with a freezer for max. -16°C.
5. Cycler[10] with real-time fluorescence detection via channels corresponding to FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson fluorophores in the melting curve analysis mode: CFX96 (BioRad, USA), DTprime (DNA-Technology LLC, Russian Federation), Rotor-Gene Q (Qiagen, Germany), QuantStudio 5 (Thermo Fisher Scientific, USA).

Materials and reagents not included in the kit:

ATTENTION! It is required to use only disposable sterile plastic consumables that have a special "DNase-free" label when working with a DNA.

1. Disposable tips with an aerosol barrier up to 1000 µl, 200 µl, 20 µl and 10 µl (e.g., Axygen, USA);
2. 1.5ml Disposal Eppendorf type sterile tubes;
3. Thin-walled disposable PCR test tubes with an optically transparent lid (while using plate-type cyclers) or optically transparent walls (while using rotary-type cyclers): 0.1 ml or 0.2ml PCR tubes[11], 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 a used cycler;

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

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

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).

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

8. Test samples

Test sample type

Test material are 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 pathogenicity group III.

Material Collection for Testing

The material must be collected before chemotherapy is started.

Sputum. Should be collected in disposable wide-mouth screw cap containers with a volume of at least 50ml. The recommended volume of biomaterial sample 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 volume of 5ml.

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 portion of morning urine or the whole morning urine portion). Collect in sterile disposable wide-mouth screw cap containers of at least 50ml volume 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 the prostate massage. The test tube should be tightly closed with a lid, avoiding gaps and wrinkling of the inner part of the lid, and labeled.

If the volume of the clinical material 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. In case of dense nutrient medium resuspend the colony into 0,2ml of sterile saline solution or use liquid medium directly.

Conditions for transportation, storage and disposal of initial clinical material

Sputum, tissue (biopsy and surgical) material, microbiological cultures: at 2°C... 8°C — not longer than 3 days; at -18°C... - 22°C — not longer than 1 week.

Bronchoalveolar lavage and bronchial washing, prostate secretion, urine, synovial, pleural, pericardial, cerebrospinal fluids, gastric washing: at 2°C... 8°C — not longer than 1 day, at -18°C... -22°C — not longer than 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.

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 is 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. Using a vacuum aspirator with a trap flask, remove the supernatant, leave the required for extraction sample amount.

Blood, pleural fluid. No preparation required.

Urine (morning urine middle 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 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 volume of the clinical material 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 first portion of urine (which contains prostate secretion) right after the prostate massage. In that case follow the previous point of the material pre-processing instruction.

Tissue (biopsy and surgical) material. Pre-homogenization of a 10-20mm^3 sample by any available method is required.

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 volume for DNA extraction.

8.2 DNA extraction from biological material

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

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

- 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: 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-I-Test reagent kit should be added to each sample tested before extraction.

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

Conditions for tested DNA samples possible storage

- at +2...+8°C — no longer than 24 hours,
- at -18 … -22 °C — no longer than 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 mycibacteria DNA using the appropriate reagent kit.

8.3. Interfering substances and restrictions on the tested material use

The effect of potentially interfering substances on the MTB-RESIST-I-Test reagent kit performance has been tested 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 biologic agent (hemoglobin, hyaluronic acid) can inhibit a PCR with insufficient purification during the DNA isolation procedure;

The interfering substances concentrations that are expected to be recorded during normal use of the MTB-RESIST-I-Test reagent kit:

Type	Substance	Active component	Concentration
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
Exogenic	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, Antituberculosis agent	capreomycin	0.2 mg/ml
	Antibiotic. Antituberculosis agent	cycloserine	0.05 mg/ml

Based on an assay results these substances have no interfering effect on the kit performance and do not lead to PCR inhibition at concentrations not exceeding the permissible limits.

It is necessary to follow the clinical material collection rules to reduce the PCR inhibitors number.

Limitations on the use of the test material:

- usage of test material is not allowed if storage and transportation conditions are violated (temperature, duration, repeated freezing and thawing);

- usage of samples contaminated with extraneous biological material is not allowed;

- An assay can be conducted using only containing mycobacterium tuberculosis complex DNA clinical material.

9. Kit Components Preparation for Testing

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

ATTENTION! It is required to use only disposable sterile plastic consumables that have a special "DNase-free" label when working with a DNA. It is required to use an individual pipette tip with an aerosol barrier for each reaction component.

ATTENTION! The reaction mixture components should be mixed right before performing the test.

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

1. Thoroughly mix the test tubes contents with an extracted for the assay DNA, 5x PCR-buffer, oligonucleotide mixtures A, B and C, PC-1, PC-2 and NC samples, turning each tube over 10 times or mixing using vortex at low speed during 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 detecting cycler type) using the calculation: 3 x (the test samples number) + 1 PC + 1 NC-2 + 1 NC) (Table 6).

Table 6 - Tubes labeling principle

	Test samples			**PC-1**	**PC-2**	**NC**
	1	**2**	**n**			
Reaction mixture A	+	+	+	+	+	+
Reaction mixture B	+	+	+	+	+	+
Reaction mixture C	+	+	+	+	+	+

10. Testing procedure

PCR testing includes following steps:

1. PCR Setup;
2. DNA PCR amplification with subsequent DNA probes melting;
3. Results Interpretation (fully described in Chapter 11).

A) PCR-test preparation

(carries 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 and C) preparation requires:

1. 5x PCR-buffer — 5µl,
2. Corresponding oligonucleotide mixture — 15µl,
3. Sample (test DNA sample, PC-1, PC-2, 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 oligonucleotide mixture, where n stands for the tested 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 oligonucleotide mixture, where n stands for the tested samples number.
4. 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 oligonucleotide mixture, where n stands for the tested samples number.
5. Add 20µl of the prepared reaction mixtures A, B and C into each PCR tube (Table 6).
6. Add 5µl of extracted DNA into corresponding tubes for the test samples. Do not add DNA into the PC-1, PC-2 and NC tubes.
7. Add 5µl of PC-1 into the 3 corresponding tubes.
8. Add 5µl of PC-2 into the 3 corresponding tubes.
9. Add 5µl of NC that has passed the isolation stage into the 3 corresponding tubes.

10. 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 hybridization fluorescence detection of amplification products;

(performed in the PCR area — PCR amplification room)

1. Install tubes in the reaction module of the real-time PCR device. 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 and detect the fluorescent signal, according to the instructions for the used device. Analysis type: PCR amplification with further melt curves analysis with fluorescence signal detection. PCR protocol is specified in Tables 7-9 depending on the used cycler.

ATTENTION! In case of usage 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.

ATTENTION! In case of use Rotor-Gene Q and similar cyclers signal level optimization is required. When creating a melting protocol choose "Perform optimization at the 1st detection step" in the "Signal level optimization" tab for all channels and specify the start signal range from 2 Fl to 10 Fl. "Signal level optimization before the start in all test tubes" should be inactivated.

3. Specify the samples number and identificators, mark the tubes layout on the thermoblock matrix according to their layout.

4. Make sure that the FAM/Green, HEX/Yellow, ROX/Orange and Cy5/Red detection channels are applied to the amplification program optical measurement parameters for reaction mixtures A and B, and FAM/Green, HEX/Yellow, ROX/Orange and Cy5.5/Crimson for a reaction mixture C.

5. Start amplification with melt curve analysis.

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

Table 7 — PCR Protocol and melting temperature analysis for cyclers except Rotor-Gene Q, DTlite and Dtprime

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/Green, HEX/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	100

Table 8 — PCR and melting temperature analysis protocols for DTlite and DTprime 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 1.0°C	00:25	FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	50

Table 9 — PCR and melting temperature analysis protocols for Rotor-Gene Q and similar 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	00:05	FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	100

11. Results Registration and Interpretation.

Results registration is carried out upon a PCR completion.

Interpretation recommendations

Result interpretation is carried out according to the melting point values (T_m) corresponding to the highest fluorescence level in the detection channels corresponding to the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red fluorophores for all the reaction mixtures and FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson for the reaction mixture C (Table 1).

Single melting peaks with corresponding temperature characteristics (T_m) in the range from 45˚C to 80 ˚C should be recorded for all the samples investigated using the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson channels for the reaction mixture C. Additional peaks with lower fluorescent signal intensity comparing to the target peak are acceptable.

Test samples results interpretation starts only when the PC and NC have been carried out correctly.

ATTENTION! In case of use Rotor-Gene Q and similar cyclers the "Heavy" digital filter setting should be applied for the graph. Use "Auto-scale" function for each fluorophore of each reaction mixture. Threshold line should be set in such a way that one peak is detected in the temperature range from 45 ˚C to 80 ˚C (presence of additional peaks with a lower intensity of the fluorescent signal is acceptable).

ATTENTION! When using DTprime and similar amplifiers, the "-dF/dT" option should be applied. Automatic peak detection for all detection channels may not be technically available. In this case use

ATTENTION!

When using CFX96 and similar amplifiers, threshold line should be set in such a way that one peak is detected in the temperature range from 45˚C to 80˚C (presence of additional peaks with a lower intensity of the fluorescent signal is acceptable).

Results interpretation in control samples

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

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

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

Table 10 — PC-1, PC-2 and NC results

Control sample	T_m values in detection channels, corresponding to fluorophores, ˚C				
	FAM / Green	HEX / Yellow	ROX / Orange	Cy5 / Red	Cy5.5 / Crimson
Reaction mixture A					
NC	No melting peaks				□
PC-1	70–75	60–68	63–69	61–67	□
PC-2	66–73	52–58	54–59	55–61	□
Reaction mixture B					
NC	No melting peaks				□
PC-1	72–76	68–73	63–69	69–75	□
PC-2	61–68	71–77	66–71	68–73	□
Reaction mixture C					
NC	No melting peaks				62–68
PC-1	70–80	66–72	61–67	63–69	62–68
PC-2	63–73	68–76	58–64	61–67	62–68

When obtaining values for NC 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 shown in the Table 10 repeated amplification of the entire sample batch is required. When reobtaining values for PC-1, PC-2 that differ from those indicated in the Table 10, it is necessary to replace the reagents.

Result interpretation in tested clinical samples

Single melting peaks with corresponding temperature characteristics (T_m) in the range from 50˚C to 80 ˚C should be registered for all test samples in the FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson channels for a reaction mixture C. If it is impossible to detect melt temperature peaks (T_m) using a cycler software but they can be clearly differentiated visually the melting temperatures can be determined manually.

The Cy5.5/Crimson channel of a reaction mixture C is used to detect the internal control sample. A peak with melting temperature in the range of 60°C to 70°C should be registered in this channel. Positive ICS results indicate nucleic acid extraction efficiency and PCR inhibitors absence in the reaction. If there is no reaction in the FAM/Green, Hex/Yellow, ROX/Orange and Cy5/Red channels for each of the reaction mixtures, the sample result 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/Green, Hex/Yellow, ROX/Orange and Cy5/Red channels, the results should be considered reliable. If the result is invalid again, biomaterial from this patient should be collected for the second time.

For the *rpoB gene* (reaction mixture A): the obtained melting peaks of the samples are analyzed in comparison with PC-1: ΔT_m values in each channel are calculated according to the formula:

$\Delta T_{m(rpoB)} = T_{m(PC-1)} - T_{m(sample)}$.

Interpretation is carried out in accordance with the Table 11. For reaction mixture A the results in each channel are taken into account separately.

It is concluded that the strain is drug resistant to rifampicin if molecular genetic polymorphisms are detected, while taking into account[12]:

- for the FAM channel (codons 529–533) if $\Delta T_{m(rpoB)} = 10,5 \pm 1,5$ ˚C: it indicates that S531L polymorphism is most likely to be present;

- for the FAM channel (codons 529–533) if $\Delta T_{m(rpoB)} = 3 \pm 0,9$ ˚C: it indicates that polymorphism in codon 533 (e.g., L533R or L533P) is most likely to be present; at the same time the sample's T_m should correspond to T_m in PCS -2 ± 2 ˚C;

[12] The most probable polymorphisms are indicated with regard to their incidence rate

- for the HEX channel (codons 523–528) if $\Delta T_{m(rpoB)} > 9$ ˚C: it indicates that polymorphism in codon 526 (e.g., H526Y) is most likely to be present; in case of presence of H526Y polymorphism the sample's T_m should correspond to T_m in PCS -2 ± 2 ˚C;

- for the Cy5 channel (codons 523–528) if $\Delta T_{m(rpoB)} = 6 \pm 1$ ˚C: it indicates that polymorphism in codon 516 (e.g., D516V or D516Y) is most likely to be present; in case of presence of D516V polymorphism T_m of the sample should correspond to T_m in PCS -2 ± 2 ˚C.

For the *katG gene* (reaction mixtures B and C): the obtained melting peaks of the samples are analyzed in comparison with PC-1: the ΔT_m values in each channel and each reaction mixture are calculated according to the formula:

$\Delta T_{m(katG)} = T_{m(PC-1)} - T_{m(sample)}$**.**

Interpretation is carried out in accordance with the Table 12. **If molecular genetic polymorphisms are detected it is concluded that the strain is drug resistant to isoniazid** (regardless of the results for *the inhA gene*)**.**

If there are no melting peaks or it is impossible to accurately detect them in a sample for one or more fluorophores, the result is considered invalid, except as described in Table 12.

For the ***inhA* gene (reaction mixture C)**: the obtained melting peaks of the samples are analyzed in comparison with PC-1: the ΔT_m values in each channel are calculated according to the formula:

$\Delta T_{m(inhA)} = T_{m(PC-1)} - T_{m(sample)}$**.**

Interpretation is carried out in accordance with the Table 13. If the C-15T molecular genetic polymorphism is detected it is concluded that the strain is drug resistant to isoniazid (regardless of the results for *the katG gene*).

If there are no melting peaks or it is impossible to accurately detect them in a sample for one or more fluorophores, the result is considered invalid.

Table 11 - Results interpretation principle for the *rpoB* gene (reaction mixture A)

	Reaction mixture A				Result
	FAM / Green	HEX / Yellow	ROX / Orange	Cy5 / Red	
	gene regions corresponding to fluorophores				results in each of the channels and the corresponding regions are accounted separately
	codons 529–533	codons 523–528	codons 516-522	codons 510–516	
$\Delta T_{m(rpoB)}$, °C	**≤ 2**	**≤ 2**	**≤ 2**	**≤ 2**	no molecular genetic polymorphisms have been identified
	> 2	**> 2**	**> 2**	**> 2**	molecular genetic polymorphisms associated with **drug resistance to rifampicin** were identified in the corresponding region [13]. The results for each channel are counted separately
	absent	**absent**	**absent**	**absent**	The analysis result for the *rpoB* gene is invalid
	Additional information about the detected polymorphisms:				
	10,5 ± 1,5	not considered	not considered	not considered	the most expectable polymorphism: S531L
	3 ± 0,9	not considered	not considered	not considered	the most expectable polymorphisms: L533R / L533P
	not considered	> **9**	not considered	not considered	the most expectable polymorphisms of the 526th codon
	not considered	not considered	not considered	**6** ± 1	the most expectable polymorphisms: D516V / D516Y

Note: "absent" - melting peak in a test sample or in a PC is absent and therefore $\Delta T_{m(rpoB)}$ calculation is impossible. "not considered" - the Tm values are not taken into account during interpretation.

[13] Different possible contribution of individual mutations to the resistance development and obtaining corresponding rifampicin minimum inhibitory concentrations and its analogues should be taken into account when interpreting drug resistance.

Table 12 - Results interpretation principle for the *katG* gene's **codon 315** (reaction mixtures B and C)

	reaction mixture						Result
	B				C		
	FAM / Green	HEX / Yellow	ROX / Orange	Cy5 / Red	ROX / Orange	Cy5 / Red	
	Standard	S315T1	S315T2	S315N	S315R	S315I	
$\Delta T_{m(katG)}$, °C	**$\Delta T_{m(katG)}$ = 0 ± 2** in all the channels						Polymorphism in the 315th codon is not detected
	$\Delta T_{m(katG)}$ < - 2 in one of the channels: A channel with the lowest $\Delta T_{m(katG)}$ corresponds to the identified polymorphism, the melting peak absence in one of the channels is allowed						The corresponding molecular genetic polymorphism of codon 315 associated with drug resistance to isoniazid is detected. In case of S315T1, T_m in all the channels should correspond to T_m in a PC -2 ± 2°C
	$\Delta T_{m(katG)}$ > 2 or absent						Invalid result

Table 13 - principle of results interpretation for the *inhA* gene (reaction mixture C)

	Reaction mixture C		Result
	FAM / Green	HEX / Yellow	
	Standard	(C-15T)	
$\Delta T_{m(inhA)}$, °C	**0 ± 2**	**0 ± 2**	polymorphysms in the range of -20... +6 are not detected
	not considered	**-5 ± 1**	C- 15T molecular genetic polymorphysm associated with **drug resistance to isoniazid** is detected, while the sample T_m should correspond to T_m in PC-2 ± 2 °C
	> 2	**> 2**	C-15T polymorphysm is not detected (presence of other polymorphysms in the gene region -20... +6 — e.g., A-16G, T-8A, T-8C)
	other $\Delta T_{m(inhA)}$ values or no values		invalid result

The reason for obtaining an invalid result may be the use of clinical material that does not contain mycobacterium tuberculosis complex DNA, low DNA concentration, inhibitors' presence in the DNA sample obtained from clinical material; incorrect analysis protocol execution; non-compliance with the PCR temperature regime; presence of several strains of Mycobacterium tuberculosis complex in the sample, carrying different mutations in the analyzed *rpoB*, *katG* and *inhA gene* regions, et al.

In case of an invalid result 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.

12. Storage, Transportation and Usage Conditions

Storage

MTB-RESIST-I-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-I-Test reagent kit up to 5 times max.

Reagent kit stored in storage conditions violation cannot be used.

Transportation

MTB-RESIST-I-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-I-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 in violation of the temperature conditions cannot be used.

Shelf Life

MTB-RESIST-I-Test validity period 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 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.

13. 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-I-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.

14. Warranty, Contacts

The manufacturer guarantees the MTB-RESIST-I-Test reagent kit quality and safety during the shelf-life period in compliance with the transportation and storage of products 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

Annex A

Symbols used for the reagent kit labeling

Symbol	Description
	Batch code
	Production date
	Use before…
	Number of definitions
	Temperature limits
	Consult the instruction for use