INSTRUCTION FOR USE

Reagent kit for Mycobacterium tuberculosis and non-tuberculosis complex DNA detection and their differentiation by multiplex RT-PCR "MTB-test"

IVD

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Content

Introduction

Infectious diseases caused by *Mycobacterium* genus microorganisms (including closely related *Mycolicibacterium* and *Mycobacteroides* genera) especially tuberculosis complex mycobacteria are one of the life quality reduction causes and often lead to death. Diagnostics of these infections promotes adequate diseases treatment, timely treatment selection, choosing an appropriate therapy, monitoring the disease course, evaluating treatment effectiveness and appropriate anti-epidemiological measures implementation.

Target analytes: specific parts of a mycobacteria genomic DNA (*Mycobacterium*, *Mycolicibacterium* and *Mycobacteroides* genera) and Mycobacterium tuberculosis complex.

Target analyte scientific validity lies in the target analyte specificity (DNA sequence unicity) in relation to microbial genomes — tuberculosis and non-tuberculosis complex mycobacteria (including closely related *Mycolicibacterium* and *Mycobacteroides* genera).

According to Tuberculosis in Adults clinical guidelines, A15-A19 (approved by the Ministry of Health of the Russian Federation, 2020)

"Any available for the assay material is used for tuberculosis laboratory testing in accordance with the process localization: sputum, bronchoalveolar lavage, pleural fluid, synovial fluid, ascitic fluid. Urine sediment, ejaculate, prostate secretion, menstrual blood, ear discharge, discharge from fistulas, punctures, biopsy material, spinal fluid, etc. can also be tested in patients with suspected EPTB according to its localization.

It is recommended to conduct at least two sputum or other diagnostic material (molecular biological assay of sputum, bronchoalveolar lavage, bronchial washings, pleural fluid, native sample of tracheal and bronchial tissue or paraffin block, other diagnostic material for Mycobacterium tuberculosis complex) tests with a 2-3 days interval using a set of microbiological and molecular genetic testing methods in patients with suspected tuberculosis to detect Mycobacterium tuberculosis complex."

The reagent kit use area: infectious diseases clinical laboratory diagnostic testing.

Indications and contraindications for use:

Indications for use: reagent kit MTB-test is recommended to use for patients with suspected pulmonary and extrapulmonary tuberculosis, mycobacteriosis.

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-test reagent kit were identified.

Sterility: the device is not sterile.

1. Intended use

MTB-test reagent kit is designed for Mycobacterium tuberculosis complex DNA specific parts detection (*Mycobacterium tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canettii*, *M. caprae*, *M. microti*), 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*) *Mycolicibacterium spp.*, *Mycobacteroides spp*. and their differentiations by multiplex polymerase chain reaction with hybridization-fluorescence detection in a DNA sample 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, washings from environmental objects) in patients with suspected pulmonary and extrapulmonary tuberculosis, mycobacteriosis.

Functional use: obtained results can be used for the diseases caused by the tuberculosis and non-tuberculosis complex mycobacteria (tuberculosis and mycobacteriosis) diagnostics.

Potential consumers of the reagent kit: the kit is designed for research use only.

2. Method principle

Method

Real-time multiplex polymerase chain reaction with hybridization-fluorescence detection.

Test Sample Type

Test 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, washings from environmental objects.

Detection principle

Quantitative detection of the tuberculosis and non-tuberculosis complex mycobacteria DNA and their differentiations (*Mycobacterium tuberculosis* complex, *Mycobacterium* sensu lato, including closely related *Mycolicibacterium* and *Mycobacteroides* genes) by multiplex Real-Time PCR in a DNA

1. PCR Setup;

2. DNA Real-Time PCR amplification with hybridization-fluorescence detection of amplification products;

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, uracil-DNA-glycosylase and an optimized buffer. The enzyme uracil-DNA-glycosidase usage prevents obtaining false-positive results in case of contamination with amplification products while the enzyme is completely inactivated during the first DNA denaturation cycle and does not prevent the amplification of the current reaction products.

The oligonucleotide mixtures contain fluorescent-labeled oligonucleotide probes that hybridize with the complementary amplified target DNA region and get destroyed by a *Taq*-polymerase. The fluorescent dye and quencher separate and fluorescence intensity increases. This allows to register the specific amplification product accumulation by real time measuring the intensity of the fluorescent signal.

The kit contains reagents for multiplex detection of the tuberculosis and non-tuberculosis complex mycobacteria DNA highly specific regions (targets) and their differentiations as well as ICS (Table 1). Tuberculosis complex mycobacteria differentiation is carried out by simultaneous markers IS*6110*, IS*1081* detection while detection of at least one of the markers indicates the Mycobacterium tuberculosis complex presence.

Table 1 – Test targets

Channel corresponding to the fluorophore		
FAM / Green	**HEX / Yellow**	**ROX / Orange**
Mycobacterium tuberculosis complex (IS*6110*, IS*1081*)	ICS	*Mycobacterium* sp. (including *Mycolicibacterium* sp., *Mycobacteroides* sp.)

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

Method limitations

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

The package integrity violation during transportation.

An expired kit usage or the kit storage conditions violation.

Storage conditions violation while samples transportation.

Multiplex PCR reaction takes from 90 to 110 minutes (time for sample preparation is not included) depending on a used cycler.

3. Reagent Kit Components

MTB-test reagent kit comes only in one kit configuration – MTB-test.

Test samples number

The reagent kit comes in one configuration (Table 2) and is intended for 96 reactions that equates to 94 test samples detection, negative and positive samples in a single run of a cycler for 96 wells or 32 single runs of test samples with negative and positive control samples in each run.

Table 2 - "MTB-test" reagent kit components

No.	Reagent	Description	Quantity, volume
1	5x PCR Buffer	Transparent colorless liquid	1 test tube, 480 µl
2	Oligonucleotide mixture	Transparent lilac liquid	1 test tube, 1440 µl
3	Positive control sample (PC)	Transparent colorless liquid	1 test tube, 160 µl
4	Negative control sample (NC)	Transparent colorless liquid	2 test tubes 1600 µl
5	Internal control sample (ICS)	Transparent colorless liquid	1 test tube, 950 µl

Reagent kit components

5x PCR Buffer is ready to use. It contains a thermostable hot-start DNA polymerase, dNTPs (including dUTP), uracil-DNA glycosylase (UDG) 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 reaction in the HEX/Yellow Ct≤32 indicates nucleic acid

extraction efficiency and PCR inhibitors absence. If there is no reaction in the HEX/Yellow channel or if Ct>32 and at the same time there is no reaction in the FAM/Green and ROX/Orange channels, the result should be considered invalid, and a second test starting with DNA extraction should be conducted for the test sample. If the result is invalid again, biomaterial from this patient should be collected for the second time.

Positive control sample (PC) is ready to use and is a plasmid DNA mixture with synthetic inserts of amplified DNA fragments: *Mycobacterium sp.* (including closely related *Mycolicibacterium sp.* and *Mycobacteroides sp. genera*), *M. tuberculosis* complex specific fragments, ICS.

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

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

The kit contains no medicinal products for medical usage and substances of human or animal origin.

4. Reagent Kit Characteristics

4.1. Technical and Performance Characteristics

Table 3

Parameter Name	Characteristics and Standards		Control methods according to TS
1. Technical Characteristics			
1.1.	**Description**	**Reagent volume, µl (±5%)**	
5x PCR Buffer	Transparent colorless liquid	1 test tube, 480 µl	Section 7, clause 7.6
Oligonucleotide mixture	Transparent liquid	1 test tube, 1440 µl	Section 7, clause 7.6
PC	Transparent colorless liquid	1 test tube, 160 µl	Section 7, clause 7.6
NC	Transparent colorless liquid	2 test tubes, 1600 µl	Section 7, clause 7.6
ICS	Transparent colorless liquid	1 test tube, 950 µl	Section 7, clause 7.6
1.2. Completeness	In accordance with clause 1.4 of TS21.20.23-023-97638376-2020		Section 7, clause 7.12
1.3. Labeling	In accordance with clause 4 of TS 21.20.23-023- 97638376-2020		Section 7, clause 7.12
1.4. Packaging	In accordance with clause 4 of TS 21.20.23-023- 97638376-2020		Section 7, clause 7.12
Performance Characteristics			
2.1. Positive result with PC	Fluorescence signal increase registration in test tubes with PC in the FAM, ROX and HEX channels Ct≤30.		Section 7, clause 7.8.1
2.2. Negative result with NC	In the test tubes with NC on the FAM, ROX channels Ct>35 or not specified (no fluorescence accumulation graph), HEX channel Ct>32.		Section 7, clause 7.8.1
2.3 The reaction passage in tubes with a specificity control sample (SC)	In tubes with a SC in the FAM and ROX channels Ct is not specified (no fluorescence accumulation graph), HEX channel Ct≤32.		Section 7, clause 7.8.2
2.4 The reaction passage in tubes with a sensitivity	In tubes with SenC-1 in the FAM and HEX channels in each run (not less than 3 runs) Ct≤35 and the standard deviation value in the SC runs is not more than 5%.		Section 7, clause 7.8.2

control sample (SenC)	In tubes with SenC-2 in the FAM and HEX channels in each run (not less than 3 runs) Ct≤35 and the standard deviation value in the SC runs is not more than 5%.	
	In tubes with SenC-3 in the HEX and ROX channels in each run (not less than 3 runs) Ct≤35 and the standard deviation value in the SenC runs is not more than 5%.	

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

- a sensitivity control sample (SenC-1, SenC-2 and SenC-3) is a mixture of plasmids with synthetic inserts of a mycobacterium genomic DNA fragment and bacteriophage genome fragment in 10% TE buffer (10 mM Tris, 1 mM EDTA).

- a specificity control sample (SC) is a human genomic DNA mixture isolated from Jurkat cell line at 1,000 copies per 5µl (200.000 copies/ml) concentration, in 4.5µl volume with 0.5µl ICS sample addition in accordance with the instruction.

4.2 Analytical efficiency

Table 4- MTB-test reagent kit analytical efficiency

Analytical specificity	**Specific to DNA:** *in the FAM channel: Mycobacterium tuberculosis* complex, including *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canettii*, *M. caprae*, *M. microti*; **in the ROX channel:** *Mycobacterium* sp., and closely related types of *Mycolicibacterium*, *Mycobacteroides* genera. **Absence of non-specific positive amplification results** and DNA presence in the sample**:** **in the FAM channel:** non-tuberculosis complex mycobacteria DNA*: Mycobacterium* sp. (*M. avium*, *M. abscessus*, *M. septicum*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. smegmatis*, *M. xenopi*, *M. ulcerans*, *M. terrae*), *Mycolicibacterium* spp., *Mycobacteroides* spp., as well as heterologous microorganism DNA: *Streptococcus pneumoniae, Staphylococcus aureus, Klebsiella pneumoniae, Haemophilus influenza, Corynebacterium jeikeium, Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila, Pseudomonas aeruginosa, Human herpesvirus 5, Human herpesvirus 1, Human herpesvirus 2,* as well as human genomic DNA from

	Jurkat cells (at least $1x10^6$ GE/ml concentration) **in the ROX channel**: Mycobacterium tuberculosis complex *(M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canettii*, *M. caprae*, *M. microti),* as well as heterologous microorganism DNA *Streptococcus pneumoniae, Staphylococcus aureus, Klebsiella pneumoniae, Haemophilus influenza, Corynebacterium jeikeium, Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila, Pseudomonas aeruginosa, Human herpesvirus 5, Human herpesvirus 1, Human herpesvirus 2,* as well as human genomic DNA from Jurkat cells (at least $1x10^6$ GE/ml concentration)
Analytical sensitivity	**For the tuberculosis complex mycobacteria (FAM channel):** at least 100 genomic DNA copies per 1ml of biomaterial, DNA extraction from a 100µl volume sample and 50µl of eluate. **For mycobacteria (ROX channel):** at least 1000 genomic DNA copies per 1ml of biomaterial, in case of DNA extraction from a 100µl of sample and 50µl of eluate.
Accuracy under repeatability conditions	To precise the accuracy under repeatability control SenC and SC samples were tested for 10 rounds. Repeatability data are obtained within the laboratory for specific equipment and within a specific batch of reagent kit. To precise the accuracy under repeatability the arithmetic mean of the sample, dispersion, standard deviation, and variation index coefficient are calculated based on the data obtained in control samples rounds. Essay results showed that the variation index under repeatability is not higher than 5%.
Accuracy under reproducibility conditions	The assessment of the test system reproducibility is carried out similarly to the accuracy calculation 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 full intra-assay, inter-assay and inter-series reproducibility was observed, the variation coefficient did not exceed 8%.

4.3. Clinical Effectiveness

For clinical essay conduction were used **243 clinical samples** (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) in patients diagnosed with pulmonary and extrapulmonary tuberculosis, mycobacteriosis. The samples were taken from a residual aliquots biological bank accumulated during therapeutic and diagnostic practice at the Samara Regional Clinical Anti-Tuberculosis Dispensary named after N.V. Postnikov and handed over to the Federal Clinical Research Centre of Russia's Federal Medical-Biological Agency.

For the cross-reactivity assessment in clinical trials, the MTB-test reagent kit was also used to test **37 samples** that did not contain the analytes under study, but contained heterologous microorganisms *Streptococcus pneumoniae, Staphylococcus aureus, Klebsiella pneumoniae, Haemophilus influenza, Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila, Pseudomonas aeruginosa, Human herpesvirus 5, Human herpesvirus 1, Human herpesvirus 2* selected from the residual aliquots biological bank based from the test samples pool of Federal Clinical Research Centre of Russia's Federal Medical-Biological Agency.

The clinical samples from patients diagnosed with pulmonary and extrapulmonary tuberculosis, mycobacteriosis were tested during therapeutic and diagnostic practice in Samara Regional Clinical Anti-Tuberculosis Dispensary named after N.V. Postnikov in accordance with the Clinical guidelines. Tuberculosis in adults, A15-A19 (approved by the Ministry of Health of the Russian Federation, 2020) and characterized with the following registered medical devices:

- GenoType Mycobacterium CM Reagents for Mycobacterium tuberculosis complex detection, manufactured by Hain Lifescience GmbH, Germany, registration certificate 2008/02294 dated 15.07.2008 — identifies Mycobacterium tuberculosis complex (but does not differentiate it) and non-tuberculosis complex;

- GenoType MTBC reagents for Mycobacterium tuberculosis complex detection by in vitro PCR testing method, manufactured by Hain Lifescience GmbH, Germany, registration certificate FSZ 2008/02297 dated 15.07.2008 - identifies tuberculosis complex mycobacteria and

differentiates them;

- GenoType Mycobacterium AS reagents for Mycobacterium tuberculosis complex detection by in vitro PCR testing method, manufactured by Hain Lifescience GmbH, Germany, registration certificate FSZ 2008/02296 dated 15.07.2008 — identifies non-tuberculosis complex mycobacteria: M. simiae, M. mucogenicum, M. goodii, M. celatum, M. smegmatis, M. genavense, M. lentiflavum, M. heckeshornense, M. szulgai/M. intermedium, M. phlei, M. haemophilum, M. kansasii, M. ulcerans, M. gastri, M. asiaticum, and M. shimoidei.

There were no failures during the clinical trials. The kits have demonstrated high reliability.

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

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

The results reproducibility is 100%.

DNA isolation from clinical samples was carried out using DNA extraction kits recommended in the instruction for the MTB-test reagent kit usage:

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

- 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 isolation from clinical, produced by the Federal Budget Institute of Epidemiology, Central Research Institute of Epidemiology of Rospotrebnadzor.

Every sample was tested using the MTB-test reagent kit in two rounds and the obtained data were compared with the results obtained by the Samara Regional Clinical Anti-Tuberculosis Dispensary named after N.V. Postnikov during the therapeutic and diagnostic process.

Table 5- Clinical efficiency

Test sample type	Tested analyte	Positive samples observations number	Negative samples observations number	Diagnostic sensitivity with 95% confidence probability	Diagnostic specificity with 95% confidence probability
Sputum	tuberculosis complex mycobacteria	50	46	100% (95%diagnostic interval: 92,89%-100%)	100% (95% diagnostic interval: 92,29%-100%)
	non-tuberculosis complex mycobacteria	16	80	100% (95% diagnostic interval: 79,41%-100%)	100% (95% diagnostic interval: 95,49%-100%)
Bronchoal veolar lavage	tuberculosis complex mycobacteria	28	20	100% (95% diagnostic interval: 87,66%-100%)	100% (95% diagnostic interval: 83,16%-100%)
	non-tuberculosis complex mycobacteria	8	40	100% (95% diagnostic interval: 63,06%-100%)	100% (95% diagnostic interval: 91,19%-100%)
Bronchial washing	tuberculosis complex mycobacteria	38	26	100% (95% diagnostic interval: 90,75%-100%)	100% (95% diagnostic interval: 86,77%-100%)
	non-tuberculosis complex mycobacteria	18	46	100% (95% diagnostic interval: 81,47%-100%)	100% (95% diagnostic interval: 92,29%-100%)
Gastric washing	tuberculosis complex mycobacteria	26	10	100% (95% diagnostic interval: 86,77%-100%)	100% (95% diagnostic interval: 69,15%-100%)
	non-tuberculosis complex mycobacteria	10	26	100% (95% diagnostic interval: 69,15%-100%)	100% (95% diagnostic interval: 86,77%-100%)
Pleural fluid	tuberculosis complex mycobacteria	24	10	100% (95% diagnostic interval: 85,75%-100%)	100% (95% diagnostic interval: 69,15%-100%)
	non-tuberculosis complex mycobacteria	8	26	100% (95% diagnostic interval: 63,06%-100%)	100% (95% diagnostic interval: 86,77%-100%)
Blood	tuberculosis complex mycobacteria	62	16	100% (95% diagnostic interval: 94,22%-100%)	100% (95% diagnostic interval: 79,41%-100%)
	non-tuberculosis complex mycobacteria	16	62	100% (95% diagnostic interval: 79,41%-100%)	100% (95% diagnostic interval: 94,22%-100%)

Urine	tuberculosis complex mycobacteria	30	30	100% (95% diagnostic interval: 88,43%-100%)	100% (95% diagnostic interval: 88,43%-100%)
	non-tuberculosis complex mycobacteria	14	46	100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 92,29%-100%)
Microbiological cultures	tuberculosis complex mycobacteria	12	14	100% (95% diagnostic interval: 73,54%-100%)	100% (95% diagnostic interval: 76,84%-100%)
	non-tuberculosis complex mycobacteria	14	12	100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 73,54%-100%)
Prostate secretion	tuberculosis complex mycobacteria	10	8	100% (95% diagnostic interval: 69,15%-100%)	100% (95% diagnostic interval: 63,06%-100%)
	non-tuberculosis complex mycobacteria	8	10	100% (95% diagnostic interval: 63,06%-100%)	100% (95% diagnostic interval: 69,15%-100%)
Tissue (biopsy and surgical) material	tuberculosis complex mycobacteria	18	10	100% (95% diagnostic interval: 81,47%-100%)	100% (95% diagnostic interval: 69,15%-100%)
	non-tuberculosis complex mycobacteria	10	18	100% (95% diagnostic interval: 69,15%-100%)	100% (95% diagnostic interval: 81,47%-100%)
Synovial fluid	tuberculosis complex mycobacteria	10	4	100% (95% diagnostic interval: 69,15%-100%)	100% (95% diagnostic interval: 39,76 15,8%-100%)
	non-tuberculosis complex mycobacteria	4	10	100% (95% diagnostic interval: 39,76 15,8%-100%)	100% (95% diagnostic interval: 69,15%-100%)
Pericardial fluid	tuberculosis complex mycobacteria	6	4	100% (95% diagnostic interval: 54,07%-100%)	100% (95% diagnostic interval: 39,76 15,8%-100%)
	non-tuberculosis complex mycobacteria	4	6	100% (95% diagnostic interval: 39,76%-100%)	100% (95% diagnostic interval: 54,07%-100%)
Cerebrospinal fluid	tuberculosis complex mycobacteria	10	10	100% (95% diagnostic interval: 69,15%-100%)	100% (95% diagnostic interval: 69,15%-100%)
	non-tuberculosis complex mycobacteria	4	16	100% (95% diagnostic interval: 39,76%-100%)	100% (95% diagnostic interval: 79,41%-100%)

Washings from environmental objects	tuberculosis complex mycobacteria	16	12	100% (95% diagnostic interval: 79,41%-100%)	100% (95% diagnostic interval: 73,54%-100%)
	non-tuberculosis complex mycobacteria	12	16	100% (95% diagnostic interval: 73,54%-100%)	100% (95% diagnostic interval: 79,41%-100%)

5. Risks Associated with the Reagent Kit Use

The risk zone includes the following hazards:

- Functional properties loss of the kit reagents due to transportation, storage or usage under inappropriate conditions;
- Samples cross contamination;
- Clinical material contamination with inhibiting substances in concentrations exceeding the permissible ones;
- Reaction mixtures and tested DNA samples contamination with contents from a PCR tube or PCR products;
- Failure to comply with the requirements for sample preparation, analysis and disposal due to unqualified personnel work;
- An unusable kit usage (after the expiration date or in case of packaging violation).

No risks have been identified in the risk zone area.

Total residual risk of using multiplex RT-PCR MTB-test reagent kit for tuberculosis and non-tuberculosis complex mycobacteria DNA detection and their differentiation is acceptable; the benefit of its usage exceeds the risk.

6. Safety Precautions

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

The reagents included in MTB-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 (test tubes containing PCR products), it is not allowed to open the tubes and spill the contents, as this can lead to contamination of the laboratory area, equipment and reagents with PCR products.

- use the kit strictly for its intended use, 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 expiry date;
- avoid contact with skin, eyes and mucous membranes. In case of contact flush the affected area immediately with water and seek medical assistance.

The 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. A 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[1] with real-time fluorescence detection via channels corresponding to the FAM/Green, HEX/Yellow and ROX/Orange fluorophores: 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 DNA.

1. Disposal tips with an aerosol barrier up to 1,000 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 with optically transparent walls (while using rotary type cyclers): 0.1ml or 0.2ml PCR tubes, or 0.1ml or 0.2ml PCR tubes in strips, or PCR plates with an optically transparent film (e.g., Axygen, USA), compatible with the used cycler;

[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 reagent kit performance.

4. Isolation gown coat and disposable talc-free gloves.

5. Container with disinfectant.

6. Test tube racks for 0.1ml or 0.2ml test 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).

8. For testing sputum and synovial fluid: use mucous material pretreatment reagent MUKOLIZIN, produced by Federal Budget Institute of Science «Central Research Institute of Epidemiology» (registration certificate № FSR 2011/12082 dated 13.03.2019)

8. Test samples

Test sample type

Test 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, washings from environmental objects.

8.1 Clinical material collection

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

The material must be collected before chemotherapy is started.

Sputum. Should be collected in disposable wide-mouth screw cap containers at least 50ml volume. 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 (morning urine middle portion 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 right after prostate massage through rectum. A doctor performs a massage making several vigorous movements from the base to the top. 0.5ml -1ml 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 the lid inner part wrinkling, and labeled.

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. Concentration: 50 000 - 100 000 cells max per 1ml.

Washings from environmental objects. Washings from environmental objects are conducted with transport swabs moistened with sterile saline solution from about a 10cm surface area^2. Main part of the transport swab places into a 1.5ml tube, containing about 500µl of sterile saline solution and the upper part should be broken off and removed.

Initial clinical material transportation, storage and disposal conditions

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, washings from environmental objects: 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. The other clinical material double freezing and thawing 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 places into a 1.5ml Eppendorf type tube. All tubes containing the 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, washings from environmental objects. 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. It is required to leave about 0.2–1.0ml. Thoroughly mix the contents using vortex.

Prostate secretion. Pretreatment of the prostate secretion sample collected after prostate massage through rectum is not required. If it is impossible to obtain the secretion, collect 15-25ml of the first urine portion (which contains prostate secretion) right after the prostate massage (see urine collection rules). 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 a 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, flushes from environmental objects 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 instruction for use for the applied reagent kit.

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

NC sample also undergoes 100µl DNA extraction with ICS 10µl addition. If the reagent kit manufacturer's instructions for DNA extraction allow a larger sample volume usage, adjust the NC volume 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.

8.3. Interfering substances and restrictions on the tested material use

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

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

2) substances found in specific sample types — in that case clinical sample contamination with a biologic agent (hemoglobin, hyaluronic acid) can inhibit a PCR with insufficient purification during the DNA isolation procedure;

3) substances found during the biological material sampling procedure — in that case anticoagulants contained in the blood collection

tube.

The concentrations of interfering substances tested that are expected to be found during normal use of the MTB-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
	Anti-infective drug fluoroquinolones	ofloxacin	0,04 mg/mL
	Anti-infective drug fluoroquinolones	ciprofloxacin	0,05 mg/mL
	Antituberculosis agent	protionamide	0,05 mg/mL
	Aminosalicylic acid derivative. Antituberculosis agent.	capreomicin	0,2 mg/mL
	Antibiotic. Antituberculosis agent	cycloserine	0,05 mg/mL
	Substances encountered during blood collection (anticoagulating agents)	Heparin (anticoagulating agent)	0.15 IU/mL
		Sodium citrate (anticoagulating agent)	0.1 mM/mL

		EDTA-K2 (anticoagulating agent)	0.5 mM/mL

Based on the assay results heparin at 0.15IU/mL concentration and sodium citrate at 0.1IU/mL concentration were classified as PCR inhibitors used as anticoagulant agents for blood sampling. Heparin and sodium citrate usage as anticoagulant agents is prohibited for peripheral blood collection.

Limitations on the test material use:

- test material usage is not allowed if the storage and transportation conditions are violated (temperature, duration, repeated freezing and thawing);
- samples contaminated with extraneous biological material are not allowed to use;
- heparin and sodium citrate cannot be used as anticoagulant in peripheral blood draws.

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 marking "DNase-free" 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 according to the Table 5 right before performing the test.

PCR cabinet, equipment and materials contained in it should be wet cleaned and exposed to UV-radiation for 20-30 minutes before preparing the reactions.

1. Thoroughly mix the test tubes contents with the DNA extracted for the assay, 5x PCR-buffer, oligonucleotide mixture, NC and PC samples, turning each tube up and down 10 times or mixing using vortex at low speed during 3-5 seconds, then remove the drops from the test tube lids by a 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) based on the calculation for each multiplex: the test samples number[2] + 1 PC + 1 NC.

[2] It is recommended to analyze each sample twice to improve the accuracy.

10. Testing procedure

PCR testing includes following steps:

1. PCR Setup;
2. DNA Real-Time PCR amplification with hybridization-fluorescence detection of amplification products;
3. Results Interpretation (fully described in Chapter 11).

A) PCR-test preparation

(carries out in a 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 preparation requires:

1. 5x PCR-buffer - 5µl,
2. Oligonucleotide mixture - 15µl,
3. Sample (test DNA sample, OC and PC samples) - 5µl.

The reaction tubes should be prepared in the following order:

1. Label 0.1 or 0.2ml test tubes or a plate for PCR.
2. In a separate disposable sterile 1.5ml or 2.0ml Eppendorf type test tube prepare the reaction mixture: 5xPCR-buffer (n+3)x5µl and oligonucleotide mixture (n+3)x15µl, where n stands for the tested samples number.
3. Add 20µl of the prepared reaction mixture into each PCR tube.
4. Add 5µl of isolated DNA into corresponding tubes for the test samples. Do not add DNA into the PC and NC tubes.
5. Add 5µl of PC to the corresponding tube.
6. Add 5µl of NC to the corresponding tube.
7. Centrifugate the test tubes during 1-3 seconds to remove the drops from the walls. Use a microcentrifuge-vortex.

B) DNA Real-Time PCR amplification with hybridization fluorescence detection of amplification products;

(performed in the PCR area – a 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 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: quantitative with standards. PCR protocol is specified in the Table 6.

ATTENTION! When using QuantStudio 5 System it is necessary to adjust optical filters before starting the amplification protocol (the VIC signal may be registered by the ROX channel if ΔRn > 100 000, which may lead to false positive results). To do this, click the "Action" button in the "Method" tab, then select "Optical filter settings" in the pop-up menu and in the «PCR Filter» tab choose just the following filter combinations: x1 - m1, x2 - m2, x4 - m4, x5 - m5, x6 - m6.

3. Specify the number and the samples identifiers, mark the tubes layout on the thermoblock matrix according to their layout.
4. Make sure that the FAM/Green, HEX/Yellow, and ROX/Orange detection channels are applied to the amplification program optical measurement parameters.
5. Start PCR with fluorescent signal detection.
6. At the end of the program, proceed to analyze the data.

Table 6 - PCR Protocol

Stage	**Temperature, °C**	**Time, min.: sec.**	**Detection channels**	**Total cycles amount**
1	95	05:00	□	□
2	95	00:15	□	5
	64	00:45	□	
3	95	00:15	□	45
	64	00:45	FAM/Green, HEX/Yellow, ROX/Orange	

11. Results Registry and Interpretation

The results are recorded automatically upon PCR completion via the device software.

Recommendations on setting the threshold line

For all cyclers models, the cycle threshold is set individually for each channel at a level corresponding to 10-20% of the maximum fluorescence level obtained for a PC sample during the last amplification cycle.

The results are interpreted using Ct values of the FAM/Green, HEX/Yellow, and ROX/Orange channels (Table 1). Only Ct values obtained at the PCR with fluorescence detection stage are taken into account (i.e., the corresponding stages 3 – see Table 6).

First, the reaction rate and Ct values in control samples are evaluated. Test samples results interpretation starts only with the correct PC and NC passage.

ATTENTION! If Rotor-Gene Q and similar cyclers are used, activate the functions "Dynamic Tube", "Noise slope correction", set 10% volume in the "Outlier Removal" section.

Results interpretation in control samples

The following results should be obtained for NC and PC (Table 7).

Table 7 - Test results for PC and NC

Control sample	Ct values for detection channels corresponding to fluorophores		
	FAM/Green	HEX/Yellow	ROX/Orange
NC	>35 or absent	≤32	>35 or absent
PC	≤30	≤30	≤30

When obtaining values for the NC that differ from those mentioned in the Table 7, the entire assay results are considered unreliable. In this case, special measures should be taken to eliminate possible contamination.

If PC values differ from those indicated in the Table 7, repeated amplification of the entire sample batch is required. If after repeated amplification PC results differ from those indicated in the Table 7, the reagents must be replaced.

Results interpretation

Results interpretation methods are shown in the Table 8.

The reason for obtaining an invalid result may be the inhibitors presence in the DNA obtained from clinical material, inhibitors presence in the DNA product, incorrect sample protocol implementation, non-compliance with the PCR temperature regime, etc.

Table 9 —Result interpretation method

Ct Values in the detection channels corresponding to fluorophores			Result
FAM / Green	ROX / Orange	HEX / Yellow (ICS)	
–	–	≤ 32	tuberculosis and non-tuberculosis complex mycobacteria DNA are not detected
–	–	> 32	invalid result
≤ 35	not considered	not considered	tuberculosis complex mycobacteria DNA is detected
–	≤ 35	not considered	nontuberculous complex mycobacteria DNA is detected (*Mycobacterium* sp., *Mycolicibacterium* sp., *Mycobacteroides* sp.).
> 35 in one or all channels		not considered	the test result is doubtful for the target corresponding to the channel

NOTE: "not considered" – the result is not taken into account during interpretation; "–" there is no fluorescence signal.

Doubtful results can be caused by a low microorganism concentration in the sample.

In case of an invalid and doubtful result the conclusion is not issued. It is necessary to re-collect biomaterial from a patient and retest it.

If a doubtful result is repeated, repeat the test with a reagents kit from another manufacturer or another method.

12. Storage, Transportation and Usage Conditions

Storage

MTB-test should be stored at -18 °C... -22°C during the entire kit shelf life in the manufacturer's package; it is allowed to store the kit at +2 ...+8 °C up to 30 days.

It is allowed to freeze / thaw MTB-test reagent kit up to 10 times max.

A reagent kit stored in storage conditions violation cannot be used.

Transportation

MTB-test reagent kit can be transported by all types of covered vehicles in accordance with the transportation rules applicable for this transport type.

MTB-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 30 days or at 15°C... 25°C no longer than 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 number in the thermal container and the thermal container size varies according to the transportation duration and conditions.

Reagent kits transported in the temperature regime violation cannot be used.

Shelf Life

MTB-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 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 the components drying 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-test consumer packaging is subject to mechanical destruction with the residues removal as industrial or household waste.

Personnel disposing reagents must comply with the safety rules.

14. Warranty, Contacts

The manufacturer guarantees the MTB-test reagent kit quality and safety during the shelf-life period in compliance with the transportation and storage requirements, as well as in compliance with the rules of use.

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
Phone number: +7 (499) 705-03-75
www.testgene.com

Technical Support Service:

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

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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