DNA-TECHNOLOGY

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M.tuberculosis – M.bovis complex
REAL-TIME PCR Detection Kit
INSTRUCTION FOR USE

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R1-P458-23/4INT

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1. INTENDED USE

The **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** is an *in vitro* Nucleic Acid Test (NAT) – pathogen-detection-based product. The **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** is designed to detect *Mycobacterium tuberculosis* and *Mycobacterium bovis* nucleic acids (without differentiation) in human biological samples (cerebrospinal fluid, biopsy material or punctate from lesions of organs and tissues, bronchoalveolar lavage, phlegm, urine, ejaculate) by real-time PCR.

Indications for the use: symptoms of tuberculosis.

The application of the kit does not depend on population and demographic aspects. There are no contradictions for use of the **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit.**

Potential users: qualified personnel trained in molecular diagnostics methods and rules of work in the clinical and diagnostic laboratory.

It is necessary to apply the kit only as directed in this instruction for use.

2. METHOD

Method: polymerase chain reaction (PCR) with real-time results detection; qualitative analysis.

Method principle: The implemented PCR method is based on amplification of a target DNA sequence. The process of amplification includes repeating cycles of thermal DNA denaturation, annealing of primers with complementary sequences and further elongation of polynucleotide chains by Taq-polymerase.

To increase the sensitivity and specificity of the amplification reaction, the use of a hot-start is provided. Hot-start is provided by the PCR-mix preparation consisting of two layers separated by a layer of paraffin. The polymerase chain reaction starts only when paraffin is melted. It excludes non-specific anchoring of primers to DNA target at lower temperatures. Additionally, when the reaction is finished and the tubes cool down, paraffin provides sealing of the mix and additional protection from contamination with amplification products.

DNA probes, each containing a fluorescent label and a fluorescence quencher, are introduced into the PCR-mix. When a specific product is formed, the DNA probe is destroyed and the quenching agent stops affecting the fluorescent label, which leads to an increase in the fluorescence level.

The number of destroyed probes (hence the fluorescence level) increases in proportion to the number of specific amplicons formed, and the fluorescence level is measured at each amplification cycle.

The PCR-mix includes internal control (IC) designed for quality control of polymerase chain reaction.

The DNA probes used to detect the DNA amplification product include the Fam fluorescent tag. The DNA probes used to detect the product of the internal control include the Hex fluorescent tag.

Table 1 shows the detection channels of amplification products.

Table 1. Detection channels of amplification products

Fam	Hex	Rox	Cy5	Cy5.5
Mycobacterium complex *(M.tuberculosis/M.bovis)*	IC	-	-	-

The automatic analysis is available on “DNA-Technology” made instruments: DTlite, DTprime or DTprime II REAL-TIME Thermal Cyclers for **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** (see the catalogue at https://www.dna-technology.com to see available supply options). The current version of the software is available for download at https://www.dna-technology.com/software.

3. CONTENT

The **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** contains PCR-mix, Taq-polymerase solution, mineral oil and positive control sample. The detailed description of content is represented in Table 2.

Table 2. The **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** content, package S for R1-P458-S3/4INT (strips) and R1-P458-23/4INT (tubes)

Reagent	Description	Total volume	Amount
Paraffin sealed PCR-mix	Colorless or pink transparent liquid under waxy white fraction	960 µL (20 µL in each)	Tubes, 6 strips of 8 or 48 individual tubes
Taq-polymerase solution	Colorless transparent liquid	500 µL	1 tube
Mineral oil	Colorless transparent viscous oily liquid	1.0 mL	1 tube
Positive control	Colorless transparent liquid	130 µL	1 tube
Strip caps (for package S, strips)	6 strips of 8		

All components are ready to use and do not require additional preparation for operation.

The **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** is intended for single use and designed for 48 tests (no more than 12 runs), including the analysis of test samples, negative controls and positive controls.

4. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

The following equipment, reagents and consumables are required to work with the **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit**:

- UV PCR cabinet;
- Real-time PCR thermal cycler;
- Vortex mixer;
- Vortex rotor for strips (only for package S, strips);
- Refrigerator with freezer;
- Tube rack for 1.5 mL tubes;
- Tube rack for 0.2 mL tubes;
- Tube rack 0.2 mL stripped tubes (only for package S, strips);
- Single channel pipettes (dispensers covering 2.0-20, 20-200, 200-1,000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL, 200 µL, 1000 µL);
- Pipette rack;
- RNase and DNase free 1.5 mL microfuge tubes with caps;
- Powder-free surgical gloves;
- Container for used pipette tips, tubes and other consumables;
- Physiological sterile saline solution 0.9% NaCl (if necessary);
- Transport medium (if necessary);

– Nucleic acid extraction kit (“DNA-Technology” made **PREP-NA** REF P-034-N/1EU, P-036-N/1EU, P-036-N/2EU and **PREP-GS** REF P-003/1EU, P-003/2EU, P-023/4EU extraction kits are recommended).

Additional equipment for pretreatment of biopsy samples/punctate and DNA extraction using PREP-NA and PREP-GS NA extraction kits:

– Biological safety cabinet class II;

– High speed centrifuge for 1.5 mL tubes (RCF(g) no less than 16,000);

– Solid-state thermostat (supported temperature at least 65 °C);

– Electric laboratory aspirator with trap flask for supernatant removal;

– RNase and DNase free filtered pipette tips for laboratory aspirator;

– Disinfectant solution.

The following detecting thermal cyclers are validated for work with the **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit**:

– DTprime in DTprime *M* modification (manufactured by “DNA-Technology R&P”, LLC), hereinafter **DTprime**;
– DTprime II in DTprime II *M* modification (manufactured by “DNA-Technology R&P”, LLC), hereinafter **DTprime II**;
– DTlite in DTlite *S* modification (manufactured by “DNA-Technology R&P”, LLC), hereinafter **DTlite**;

For the use of detecting thermal cyclers other than those listed above, please consult the reagent kit manufacturer for consultation.

Software:

The most recent version of the DT thermal cyclers software can be downloaded from https://www.dna-technology.com/software.

The OS supported: all versions of Windows starting from 7.

5. TRANSPORT AND STORAGE CONDITIONS

All components of the **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** must be stored at temperatures from 2 °C to 8 °C over the storage period. Paraffin sealed PCR-mix must be stored out of light over the storage period.

The **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** must be transported in thermoboxes with ice packs by all types of roofed transport at temperatures inside the thermoboxes corresponding to storage conditions of the kit components.

It is allowed to transport the kit in thermoboxes with ice packs by all types of roofed transport at the temperature inside the thermoboxes from 2 °C to 25 °C for no longer than 5 days.

Reagent kits transported with violation of temperature conditions must not be used.

Shelf-life of the kit following the first opening of the primary container:

– All components of the kit must be stored in a refrigerator or a cooling chamber at temperatures from 2 °C to 8 °C over the storage period;

– Paraffin sealed PCR-mix must be stored in a refrigerator or a cooling chamber at temperatures from 2 °C to 8 °C and out of light over the storage period.

The kit stored under undue regime should not be used.

An expired **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** must not be used.

We strongly recommend to follow the given instructions in order to obtain accurate and reliable results.

The conformity of the **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** to the prescribed technical requirements is subject to compliance of storage, transportation and handling conditions recommended by manufacturer.

6. WARNINGS AND PRECAUTIONS

Handle and dispose all biological samples, reagents and materials used to carry out the assay as if they were able to transmit infective agents. The samples must be exclusively employed for certain type of analysis. Samples must be handled under a laminar flow hood. Tubes containing different samples must never be opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA. The reagents must be handled under a laminar flow hood. The reagents required for amplification must be prepared in such a way that they can be used in a single session. Pipettes used to handle reagents must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA. Avoid direct contact with the biological samples reagents and materials used to carry out the assay. Wear powder-free surgical gloves. Wear protective clothing (work clothes and personal protective equipment) working with microorganisms classified as particularly pathogenic. The protective clothing and personal protective equipment must comply with the work to be performed and health and safety requirements. Avoid producing spills or aerosol. Any material being exposed to biological samples must be treated for at least 30 minutes with disinfecting solution or autoclaved for 1 hour at 121 °C before disposal.

Molecular biology procedures, such as nucleic acids extraction, PCR-amplification and detection require qualified staff to avoid the risk of erroneous results, especially due to the degradation of nucleic acids contained in the samples or sample contamination by amplification products.

All oligonucleotide components are produced by artificial synthesis technology according to internal quality control protocol and do not contain blood or products of blood processing.

Positive control is produced by artificial DNA synthesis technology. Positive control does not include parts of infectious agents.

All the liquid solutions are designed for single use and can not be used more than once in amplification reactions. Plastic tubes do not contain phthalates. Do not breathe gas/fumes/vapor/spray produced by the components of the kit. Do not eat/drink components of the kit. Avoid contact with eyes. Only use the reagents provided in the kit and those recommended by manufacturer. Do not mix reagents from different batches. Do not use reagents from third party manufacturers' kits. All laboratory equipment, including pipettes, test tube racks, laboratory glassware, lab coats, bouffant caps, etc., as well as reagents should be strictly stationary. It is not allowed to move them from one room to another. Equip separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions. Wear lab coats, gloves and tools, which are exclusively employed for the extraction/preparation of the amplification reaction and for the amplification/detection of the amplification products. Never transfer lab coats, gloves and tools from the area designed for amplification/detection of the amplification products to the area designed for extraction/preparation of amplification reactions. Amplification products must be handled in such a way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be exclusively employed for this specific purpose. Remove PCR waste only in a closed form. Remove waste materials (tubes, tips) only in a special closed container containing a disinfectant solution. Work surfaces, as well as rooms where NA extraction and PCR are performed, must be irradiated with bactericidal irradiators for 30 minutes before and after the work.

Do not open the tubes after amplification. Waste materials are disposed of in accordance with local and national standards. All surfaces in the laboratory (work tables, test tube racks, equipment, etc.) must be treated daily with disinfecting solution.

Emergency actions

Inhalation: Inhalation of the PCR-mix contained within this kit is unlikely, however care should be taken.

Eye Contact: If any component of this kit enters the eyes, wash eyes gently under potable running water for 15 minutes or longer, making sure that the eyelids are held open. If pain or irritation occurs, obtain medical attention.

Skin Contact: If any component of this kit contacts the skin and causes discomfort, remove any contaminated clothing. Wash affected area with plenty of soap and water. If pain or irritation occurs, obtain medical attention.

Ingestion: If any component of this kit is ingested, wash mouth out with water. If irritation or discomfort occurs, obtain medical attention.

Do not use the kit:

- When the transportation and storage conditions are breached;
- When the reagents' appearance does not respond to the kit passport;
- When the kit components packaging is breached;
- After the expiry date provided.

Significant health effects are **NOT** anticipated from routine use of this kit when adhering to the instructions listed in the current manual.

7. SAMPLES

The **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** is designed to detect DNA extracted from cerebrospinal fluid, biopsy material or punctate from lesions of organs and tissues, bronchoalveolar lavage, phlegm, urine, ejaculate.

7.1. General requirements

PCR analysis refers to direct methods of laboratory research; therefore, the collection of biological material must be carried out from the site of infection localization. The decision on studying the localization site shall be taken by the physician according to the collected anamnesis and clinical picture of the disease.

The quality of sampling, sample storage, transport and pretreatment are of great importance for obtaining correct results. Incorrect sampling may lead to unreliable results and, therefore, to the necessity for repeated sampling.

Use RNase and DNase free filtered tips during biomaterial preparation and NA extraction.

To prevent contamination, only open the cap of the tube you are working with and close it before proceeding to the next tube.

7.2. Interfering substances

The presence of PCR inhibitors in a sample may cause controversial (uncertain) results. The sign of PCR inhibition is the simultaneous absence of internal control and specific product of amplification.

PCR inhibitors are the presence of hemoglobin in the DNA sample as a result of incomplete removal during the extraction of DNA from a biomaterial sample containing an impurity of blood, as well as the presence of isopropyl alcohol and methyl acetate in the DNA sample as a result of incomplete removal of washing solutions during sample preparation.

The maximum concentration of interfering substances, which do not affect the amplification of the laboratory control sample and internal control: hemoglobin – 0.35 mg/mL DNA sample, isopropyl alcohol – 100 µL/mL DNA sample, methyl acetate – 100 µL/mL DNA sample.

To reduce the count of PCR inhibitors, it is necessary to follow the principles of taking biological material. Suspecting a large count of PCR inhibitors in the sample, it is recommended to choose DNA extraction methods that allow to remove PCR inhibitors from the sample as much as possible.

7.3. Sample collection

WARNING! Before DNA extraction, preparation of biological material samples is needed.

7.3.1 Cerebrospinal fluid

Cerebrospinal fluid (liquor) is taken with disposable needles into disposable 1.5 mL tubes in an amount of at least 500 µL according to the established procedure.

After sampling, close the tubes tightly and mark them.

7.3.2 Phlegm

Phlegm is collected in disposable graduated sterile wide-mouth vials with screw caps with a volume of at least 50 mL in an amount of at least 1.0 mL.

After sampling, screw the vial tightly and mark it.

7.3.3 Bronchoalveolar lavage

Bronchoalveolar lavage is collected in a sterile container in an amount of about 500 µL according to the established procedure. After sampling, close the container tightly and mark it.

7.3.4 Biopsy/punctate samples

The samples are placed into 1.5 mL tube with transport medium intended by the manufacturer for transporting and storing samples of biological material for PCR studies.

7.3.5 Ejaculate

Before collecting ejaculate (seminal fluid), sexual abstinence is recommended for 3 days before the examination.

Before collecting the ejaculate, the patient urinates in the toilet, completely emptying the bladder.

After urinating, the patient should wash his hands thoroughly with soap and hold the toilet of the external genitals with soap and water. The penis balanus and the foreskin should be dried with a sterile napkin.

The ejaculate is obtained by masturbation and collected in a sterile container with a volume of up to 60 mL.

The container with ejaculate is hermetically closed and marked.

7.3.6 Urine

The first portion of morning urine as a biological material is used in acute inflammatory process of the lower genitourinary tract due to the pronounced painfulness of scraping epithelial cells. The first portion of morning urine is used only for pathogen identification.

The first portion of morning urine in the amount of 10–15 mL is selected for the analysis. It is possible to examine the first portion of urine received 2 or more hours after the previous urination.

The urine is taken into a special dry sterile container with a volume of up to 60 mL, equipped with a hermetically screw-cap.

After the urine collection, container is tightly screwed and marked.

7.4. Transport and storage of the samples

Transport and storage conditions for biological material samples are determined by the instructions for use of the NA extraction kits or transport media used for transport and storage of samples.

Samples may be transported and stored at temperatures from 2 °C to 8 °C no more than 24 hours prior to analysis. When it is impossible to deliver the material in the laboratory during the day, a one-time freezing of the material is allowed. The frozen material is allowed to be stored at temperatures from minus 22 °C to minus 18 °C for one month.

WARNING! Only one freezing-unfreezing of the material is allowed.

7.5. Sample preparation

7.5.1. Cerebrospinal fluid, ejaculate, urine, phlegm

Preparation of biological material (if necessary) is carried out in accordance with the instructions for use of used NA extraction reagent kits/sets.

7.5.2. Bronchoalveolar lavage

Preparation of biological material (if necessary) is carried out in accordance with the instructions for use of used NA extraction reagent kits/sets.

If using PREP-NA and PREP-GS DNA extraction kits:

7.5.2.1. Transfer 500 µL of sample into a 1.5 mL plastic tube.
7.5.2.2. Centrifuge the tube at RCF(g) 12,000 – 16,000 at room temperature (from 18 to 25 °C) for 10 minutes.
7.5.2.3. Remove supernatant leaving approximately 50 µL (precipitate + liquid fraction) in the tube.
7.5.2.4. Add 500 µL of sterile physiological saline solution.
7.5.2.5. Shake the tube on vortex for 3-5 seconds and spin on vortex for 3-5 seconds.
7.5.2.6. Centrifuge the tube at RCF(g) 12,000 – 16,000 for 10 minutes.
7.5.2.7. Remove supernatant leaving approximately 50 µL (precipitate + liquid fraction) in the tube for **PREP-GS** extraction kit or 100 µL (precipitate + liquid fraction) for **PREP-NA** extraction kit.
7.5.2.8. Close the tubes tightly.

The sample is ready for DNA extraction.

7.5.3. Biopsy/punctate samples

Preparation of biological material (if necessary) is carried out in accordance with the instructions for use of used NA extraction reagent kits/sets.

If using PREP-NA and PREP-GS DNA extraction kits:

7.5.3.1. Shake the tubes with biomaterial on vortex for 3-5 seconds and spin on vortex for 3-5 seconds.
7.5.3.2. Remove supernatant.

The sample is ready for DNA extraction.

8. PROCEDURE

DNA extraction from biological material

For NA extraction, we recommend to use reagent kits/sets that are intended for the corresponding types of biomaterial for the purpose of subsequent DNA testing by PCR, for example, **PREP-NA** and **PREP-GS** extraction kits.

Nucleic acids are extracted in accordance with the instructions for use of the reagent kit(s) used.

WARNING!

1. Simultaneously with NA extraction from biological material, it is necessary to prepare a negative control and run it through all stages of sample preparation. For this purpose, it is recommended to use physiological solution or negative control included in the NA extraction reagent kit in the volume specified in the instructions for use of the corresponding kit.
2. DNA extraction from biopsy samples and punctate using **PREP-NA** and **PREP-GS** extraction kits is performed according to the instruction below.

8.1 DNA extraction from biopsy samples and punctate using PREP-NA and PREP-GS extraction kits

8.1.1. General requirements

8.1.3.1. Use RNase and DNase free single use filter tips. Change the tip after each solution removal from the tube. When working with aspirator use RNase and DNase free tips without filter.

8.1.3.2. Do not touch the walls of the tube when adding solution containing biomaterial. If touching occurred, change the tip.

8.1.3.3. To prevent contamination, before introducing DNA only open the caps of the strips you are working with and close them before proceeding to the next one.

8.1.3.4. Treat test samples and negative control (C-) according to the same procedure described in this instruction.

8.1.2. DNA extraction from biopsy samples and punctate using PREP-NA extraction kit

WARNING!

1. Before starting work:
   - heat thermostat to 65 °C;
   - take out the NA extraction reagent kit from the refrigerator and check that there is no precipitate in the lysis solution. In case of precipitation heat the vial with lysis solution on thermostat preheated to 65 °C to dissolve the precipitate completely. Then stir the solution by turning the vial upside down 5-10 times, avoiding foaming. Before use, cool the solution to room temperature (18 °C to 25 °C). The precipitate can also be dissolved at room temperature (18 °C to 25 °C) for approximately 12 hours.
2. If working with aspirator, use RNase and DNase free filter tips.
3. Caps may open during heating! Use tubes with self-lock caps (e.g. Eppendorf Safe-Lock Tubes) or programmable thermostats with a clamp cover (e.g. solid-state programmable small-size thermostat TT-1-“DNA-Technology”).

8.1.2.1. Mark a 1.5 mL plastic tube for each test sample and negative control (C-).

8.1.2.2. Enter 300 µL of lysis solution into each tube with prepared biopsy/punctate samples and into the “C-“ tube. Do not touch the edges of the tube.

8.1.2.3. Add 100 µL of negative control into the “C-“ tube. Close the tubes tightly and shake on vortex for 3-5 seconds.

8.1.2.4. Heat the tubes on thermostat at 65 °C for 30 minutes, spin the tubes on vortex for 3-5 seconds.

8.1.2.5. Transfer supernatant into the corresponding marked tubes for test samples. Do not transfer supernatant into the “C-” tube.

8.1.2.6. Add 400 µL of precipitation buffer into each tube without touching the edges of the tube, close the tubes and shake on vortex for 3-5 seconds.

8.1.2.7. Centrifuge the tubes at RCF(g) 12,000-16,000 for 15 minutes.

8.1.2.8. Remove supernatant fully, using separate tip for each tube. Do not touch the precipitate.

8.1.2.9. Add 500 µL of wash solution No. 1 to the precipitate, close the tubes and mix the contents by carefully turning the tubes upside down 3-5 times.

8.1.2.10. Centrifuge the tubes at RCF(g) 12,000-16,000 for 5 minutes.

8.1.2.11. Remove supernatant fully, using separate tip for each tube. Do not touch the precipitate.

8.1.2.12. Add 300 µL of wash solution No. 2 to the precipitate, close the tubes and mix the contents by carefully turning the tubes upside down 3-5 times.

8.1.2.13. Centrifuge the tubes at RCF(g) 12,000-16,000 for 5 minutes.

8.1.2.14. Remove supernatant fully, using separate tip for each tube. Do not touch the precipitate.

8.1.2.15. Open the tubes and dry the precipitate at 65 °C for 5 minutes.

8.1.2.16. Add 50 µL of dilution buffer to the precipitate, close the tubes.

8.1.2.17. Spin down the drops on vortex for 1-3 seconds.

8.1.2.18. Heat the tubes on thermostat at 65 °C for 10 minutes. Shake the tubes on vortex for 3-5 seconds.

8.1.2.19. Centrifuge the tubes at RCF(g) 12,000-16,000 for 30 seconds.

DNA preparation is ready to be introduced into PCR-mix.

DNA preparation can be stored at temperature from minus 22 °C to minus 18 °C for up to one month or at temperature from minus 72 °C to minus 68 °C for up to one year.

Before using the DNA preparation for PCR thaw the DNA preparation and negative control at room temperature (from 18 °C to 25 °C) or at temperature from 2 °C to 8 °C, then shake the tubes with DNA preparation and negative control on vortex for 3-5 seconds and spin on vortex for 1-3 seconds.

WARNING! It is only allowed to thaw DNA preparation once!

8.1.3. DNA extraction from biopsy samples and punctate using PREP-GS extraction kit

8.1.3.1. Mark a 1.5 mL plastic tube for each test sample and negative control (C-).

8.1.3.2. Enter 150 µL of lysis solution into each tube with prepared biopsy/punctate samples and into the “C-“ tube. Do not touch the edges of the tube.

8.1.3.3. Add 50 µL of sterile physiological saline solution or transport medium into the “C-“ tube. Close the tubes tightly and shake on vortex for 3-5 seconds.

8.1.3.4. Heat the tubes on thermostat at 50 °C for 30 minutes, spin the tubes on vortex for 3-5 seconds.

8.1.3.5. Transfer supernatant into the corresponding marked tubes for test samples. Do not transfer supernatant into the “C-” tube.

8.1.3.6. Thoroughly resuspend the sorbent on vortex. Turn the tube upside down to ensure that the sorbent does not stick to the bottom of the tube. Repeat the mixing of the sorbent if necessary.

8.1.3.7. Add 20 µL of resuspended sorbent to all tubes (including “C-”) without touching the edge of the tube, close the tubes and shake on vortex for 3-5 s.

8.1.3.8. Centrifuge the tubes at RCF(g) 12,000-16,000 for 1 minute.

8.1.3.9. Remove supernatant fully, using separate tip for each tube. Do not touch the precipitate.

8.1.3.10. Add 200 µL of wash solution No. 1 to the precipitate, close the tubes and shake on vortex for 3-5 seconds.

8.1.3.11. Centrifuge the tubes at RCF(g) 12,000-16,000 for 1 minute.

8.1.3.12. Remove supernatant fully, using separate tip for each tube. Do not touch the precipitate.

8.1.3.13. Add 200 µL of wash solution No. 2 to the precipitate, close the tubes and shake on vortex for 3-5 seconds.

8.1.3.14. Centrifuge the tubes at RCF(g) 12,000-16,000 for 1 minute.

8.1.3.15. Add 200 µL of wash solution No. 3 to the precipitate, close the tubes and shake on vortex for 3-5 seconds.

8.1.3.16. Centrifuge the tubes at RCF(g) 12,000-16,000 for 1 minute.

8.1.3.17. Remove supernatant fully, using separate tip for each tube. Do not touch the precipitate.

8.1.3.18. Open the tubes and dry the precipitate at 50 °C for 5 minutes.

8.1.3.19. Without touching the edge of the tube, add 100 µL of elution solution to the precipitate, close the tubes and shake on vortex for 5-10 seconds.

8.1.3.20. Heat the tubes on thermostat at 50 °C for 5 minutes.

8.1.3.21. Centrifuge the tubes at RCF(g) 12,000-16,000 for 1 minute.

The supernatant containing the extracted DNA is ready to be added to the PCR-mix. If the sample is to be stored for more than 7 days, transfer the supernatant to a separate tube.

The obtained DNA preparation can be stored for up to 7 days at temperatures from 2 °C to 8 °C. Before using DNA preparation for PCR repeat 8.1.3.20 – 8.1.3.21.

If you intend to store DNA preparation for more than 7 days, it must be stored at the temperature from minus 22 °C to minus 18 °C.

Storage period of DNA preparation at the temperature from minus 22 °C to minus 18 °C is up to 6 months. In case supernatant containing the extracted DNA was transferred to new tubes after extraction, before using DNA preparation for PCR thaw DNA preparation and negative control at room temperature (from 18 °C to 25 °C) or at a temperature from 2 °C to 8 °C, then shake the tubes with DNA preparation and negative control on vortex for 3-5 seconds and spin on vortex for 1-3 seconds.

WARNING! It is only allowed to thaw DNA preparation once!

8.2 PCR, package S

WARNING!

1. The reagents and tubes should be kept away from direct sun light.
2. When using package S, strips, strictly observe the completeness of the strips and caps for them. Do not use the caps for the strips of the other kits!

8.2.1 Mark one tube/stripped tube with PCR-mix for each test sample, negative control (C-) and positive control (C+).

WARNING! The reagent kit is designed for no more than 12 runs, considering a variable number of test samples, 1 negative control and 1 positive control per run.

Example: to test 4 samples, mark 4 tubes for samples, 1 tube for “C-” and 1 tube for “C+”. The resulting number of tubes is 6.

8.2.2 Vortex the Taq-polymerase solution for 3-5 seconds, then spin for 1-3 seconds.

8.2.3 Add 10 µL of Taq-polymerase solution into each tube. Avoid paraffin layer break.

8.2.4 Add one drop (~20 µL) of mineral oil into each tube. Close the tubes.

8.2.5 Vortex the tubes with samples, “C-” and “C+” for 3-5 seconds and spin down drops for 1-3 seconds.

WARNING!

1. For DNA and negative control, perform the recommendation for the use of DNA preparation from the instruction to the NA extraction reagent kit.
2. If using **PREP-NA**, **PREP-GS** (only if supernatant containing the extracted DNA was transferred into new tubes) extraction kits, shake the tubes with DNA preparation and negative control on vortex for 3-5 seconds and spin on vortex for 1-3 seconds.
3. To prevent contamination, before introducing DNA only open the caps of the strips you are working with and close them before proceeding to the next one. In case of using strips close the strip caps after adding samples before proceeding to the next strip. Close the tubes/strips tightly. Use filter tips.

8.2.6 Add 5.0 µL of DNA sample into corresponding tubes. Do not add DNA into the “C-” and “C+” tubes. Avoid paraffin layer break.

8.2.7 Add 5.0 µL of negative control (C-) which passed whole DNA extraction procedure into “C-” tube and positive control (C+) into corresponding tube. Avoid paraffin layer break.

8.2.8 Spin tubes/strips on vortex for 3-5 seconds.

8.2.9 Set the tubes/strips into the real-time thermal cycler.

8.2.10 Launch the operating software for DT instrument[1]. Add corresponding test[2], specify the number and IDs of the samples, positive and negative controls. Specify the position of the tubes/strips in the thermal unit and run PCR. See Table 3.

Table 3. The PCR program for DTlite, DTprime and DTprime II thermal cyclers

Step	Temperature, °C	Min.	Sec.	Number of cycles	Optical measurement	Type of the step
1	80	0	30	1		Cycle
	94	1	30			
2	94	0	30	5		Cycle
	64	0	15		v	
3	94	0	10	45		Cycle
	64	0	15		v	
4	94	0	5	1		Cycle
5	25[1]	…	…	Holding		Holding
[1] – holding at 10 °C is allowed						

[1] Please, apply to Operation Manual for DTprime, DTprime II and DTlite Real-Time PCR instruments PART II.
[2] Instructions for uploading "files with test parameters" can be found on "DNA-Technology's" website https://www.dna-technology.com/assaylibrary.

9. CONTROLS

The **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** contains positive control. Positive control is a cloned part of the *Mycobacterium* complex *(M.tuberculosis/M.bovis)* genome. It is produced with genetic engineering techniques and characterized by automatic DNA sequencing. The PCR-mix from the kit includes the Internal control (IC). IC is an artificial plasmid intended to assess the quality of PCR performance. To reveal possible contamination a negative control is required.

WARNING! A negative control should go through all stages of DNA extraction. Physiological saline solution can be used as a negative control sample in volumes indicated in supplied instructions.

For **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** the test result is considered valid when:

- the exponential growth of the fluorescence level for the specific product is present, in this case the internal control is not considered;
- the exponential growth of the fluorescence level for the specific product is absent and for internal control is present.

For **M.tuberculosis - M.bovis complex REAL-TIME PCR Detection Kit** the test result is considered invalid when the exponential growth of the fluorescence level for the specific product and for internal control is not observed.

If positive control (C+) does **not** express growing fluorescence of the specific product or positive result, it is required to repeat the whole test. It may be caused by inhibitors, operation error or violation of storage and handling.

If negative control (C-) expresses growing fluorescence of the specific product or positive result, all tests of the current batch are considered false. Decontamination is required.

10. DATA ANALYSIS

10.1. The analysis is performed automatically by the software delivered together with the detecting thermal cycler.

10.2. Result interpretation is performed according to the Table 4. Run results are valid if the interpretation conditions for the results of controls are observed.

Table 4. PCR result interpretation

Detection channel		**Result interpretation**
Fam, Cp	**Hex**, Cp	
Test samples		
Specified	Not considered	***M.tuberculosis* - *M.bovis* complex DNA is detected**
Not specified	**Specified**	*M.tuberculosis* - *M.bovis* complex DNA is not detected
Not specified	Not specified	Unreliable result
Negative control		
Not specified	**Specified**	**Negative result** Run results are valid
Positive control		
Specified	Not considered	**Positive result** Run results are valid

10.3. Unreliable result may be due to the presence of inhibitors in the NA preparation obtained from biological material; incorrect execution of the analysis protocol, noncompliance with the amplification temperature regime, etc. In this case it is necessary to repeat PCR with the available DNA preparation, or to re-extract DNA and perform PCR for this sample, or to re-collect biological material from the patient (performed sequentially).

10.4. If a positive result is obtained for a negative control, the results of the entire run batch are considered unreliable. In this case it is necessary to carry out special measures to identify and eliminate possible contamination.

10.5. If a negative result is obtained for a positive control, the results of the entire run batch are considered unreliable. In this case it is necessary to repeat amplification of the whole batch of samples.

11. SPECIFICATIONS

a. Analytical specificity

For biomaterial samples containing DNA of the detected bacteria, thermal cycler software must register positive amplification result for specific product (*M.tuberculosis* - *M.bovis* genome fragment) on Fam detection channel.

For biomaterial samples not containing DNA of *M.tuberculosis* - *M.bovis*, thermal cycler software must register negative amplification result for specific product (*M.tuberculosis* - *M.bovis* genome fragment) on Fam detection channel and positive amplification result for internal control on Hex detection channel.

There were no nonspecific positive amplification results in the presence of DNA of *Mycobacterium leprae*, *Streptococcus pneumoniae*, *Legionella pneumophila*, *Moraxella catarrhalis*, as well as human DNA in concentration of $1.0x10^8$ copies per mL of sample.

b. Analytical sensitivity (limit of detection)

Limit of detection is 5 copies of *M.tuberculosis* - *M.bovis* DNA per amplification tube.

Limit of detection was established via analysis of laboratory control's serial dilutions.

Limit of detection corresponds to the following DNA concentration values when using the specified NA extraction kits and the end elution (dilution) volumes of the extracted DNA:

Biomaterial	DNA extraction kit	Preparation volume, µL	Limit of detection, copies per sample
- Cerebrospinal fluid, bronchoalveolar lavage, phlegm (extraction from 500 µL of sample); - Biopsy samples/punctate; - Ejaculate (extraction from 100 µL of sample); - Urine (extraction from 1.0 mL of sample)	**PREP-NA**	50	50
	PREP-GS	100	100

c. Diagnostic characteristics

Biomaterial type	Diagnostic sensitivity	Diagnostic specificity
Cerebrospinal fluid	100% (95% CI: 92.89% – 100%)	100% (95% CI: 86.28% – 100%)
Biopsy material from lesions of organs and tissues	100% (95% CI: 92.89% – 100%)	100% (95% CI: 86.28% – 100%)
Punctate from lesions of organs and tissues	100% (95% CI: 92.89% – 100%)	100% (95% CI: 86.28% – 100%)
Bronchoalveolar lavage	100% (95% CI: 92.89% – 100%)	100% (95% CI: 86.28% – 100%)
Phlegm	100% (95% CI: 92.89% – 100%)	100% (95% CI: 86.28% – 100%)
Urine	100% (95% CI: 92.89% – 100%)	100% (95% CI: 86.28% – 100%)
Ejaculate	100% (95% CI: 92.89% – 100%)	100% (95% CI: 86.28% – 100%)
Total	100% (95% CI: 98.95% – 100%)	100% (95% CI: 97.91% – 100%)

d. Reproducibility and repeatability

Reproducibility is 100%.

Repeatability is 100%.

12. TROUBLESHOOTING

Table 8. Troubleshooting

	Result	Possible cause	Solution
C+	-	Operation error PCR inhibition Violation of storage and handling requirements	Repeat whole test Dispose current batch
C-	+	Contamination	Dispose current batch Perform decontamination procedures
IC	Invalid	PCR inhibition	Repeat whole test Resample

If you face to any undescribed issues contact our customer service department:

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com/support

13. QUALITY CONTROL

The quality control procedures performed in accordance with ISO 9001:2015 and ISO 13485:2016:

- observation of quality management in manufacturing of products;
- creation of values for customers;
- maintenance of the best service quality and customer management.

Technical support:

E-mail: hotline@dna-technology.ru

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https://www.dna-technology.com

14. KEY TO SYMBOLS

	Temperature limit		Date of manufacture
	Contains sufficient for <n> tests		Consult instructions for use
	Use-by date		Catalogue number
	Batch code		Manufacturer
	Version		Keep away from sunlight
	Caution		Non-sterile

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