DNA-TECHNOLOGY

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IVD

For professional use only

C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit

INSTRUCTION FOR USE

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REF

R1-P445-S3/4EU
R1-P445-23/4EU
R1-P445-UA/9EU

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1. INTENDED USE

The **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** is an *in vitro* Nucleic Acid Test (NAT) – pathogen-detection-based product. The **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** is intended for detection of DNA and differentiation of toxigenic and nontoxigenic strains of *C. diphtheriae* in human biological material (smears/scrapes from nasopharyngeal, oropharyngeal mucous membrane, smears from affected skin areas) and bacterial cultures from this biomaterial by real-time polymerase chain reaction.

Indications for the assay: symptoms of *C. diphtheriae* infection and epidemiologically related cases; screening for asymptomatic carriage.

The application of the kit does not depend on population and demographic aspects. There are no contradictions for use of the **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit.**

The **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** can be used in clinical and diagnostic laboratories of medical institutions and research practice.

Potential users: personnel qualified in molecular diagnostics methods and working 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 detection of the results in real time; multiplex qualitative analysis.

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 their elongation by DNA-polymerase.

To increase the sensitivity and specificity of amplification reaction, the use of a hot-start is provided. For package S, hot-start is provided by reaction mixture preparation consisting of two layers separated by a layer of paraffin. The polymerase chain reaction starts only when paraffin is melted.

Hot-start for package U is provided by using polymerase whose activity is blocked by antibodies, the activation of the enzyme occurs only after preheating the reaction mixture at 94 °C for 5 minutes.

It excludes non-specific annealing of primers to targets DNA in the initial heating of the tube.

DNA probes, each containing a fluorescent label and a fluorescence quencher, are introduced into the amplification mixture. When a specific product is formed, the DNA probe is destroyed and the effect of the quencher on the fluorescent label stops, which leads to an increase in the fluorescence level recorded by special devices. The number of destroyed probes (and therefore the fluorescence level) increases in proportion to the number of specific amplicons produced. The fluorescence level is measured at each amplification cycle in real time.

The PCR-mix includes the internal control (IC), which is intended to assess the quality of the polymerase chain reaction.

The DNA probes used to detect a specific DNA amplification product include the fluorescent tags Fam and Cy5. The DNA probes used to detect the amplification product of an internal control include the fluorescent dye Hex.

The use of several fluorescent dyes reduces the number of assay tubes, because it makes it possible to simultaneously record the results of different amplification reactions taking place in the same tube. Table 1 shows the detection channels of amplification products.

Table 1. Detection channels of amplification products

Fam	Hex	Rox	Cy5	Cy5.5
C. diphtheriae (tox+)	IC	-	*C. diphtheriae*	-

The automatic analysis is available on "DNA-Technology" made instruments DTlite or DTprime REAL-TIME Thermal Cyclers for **C. diphtheriae Tox Multiplex 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 **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** content is represented in Tables 2-4.

Table 2. The **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** content, package S (standard), strips for R1-P445-S3/4EU

Reagent	Description	Total volume	Amount
Paraffin sealed PCR-mix	Colorless transparent liquid under waxy white fraction	960 µL (20 µL in each tube)	6 8-tube strips
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's caps	6 8-caps		

* - marking as C+ is allowed

Table 3. The **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** content, package S (standard), tubes for R1-P445-23/4EU

Reagent	Description	Total volume	Amount
Paraffin sealed PCR-mix	Colorless transparent liquid under waxy white fraction	960 µL (20 µL in each tube)	48 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

* - marking as C+ is allowed

Table 4. The **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** content, package U, for R1-P445-UA/9EU

Reagent	Description	Total volume	Amount
PCR-mix	Colorless or slightly pink transparent liquid	600 µL	1 tube
TechnoTaq MAX polymerase	Colorless transparent viscous liquid	30 µL	1 tube
PCR-buffer	Colorless transparent liquid	600 µL	1 tube
Positive control*	Colorless transparent liquid	130 µL	1 tube

* - marking as C+ is allowed

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

The kit is intended for single use and designed for 48 tests (package S) including no more than 46 experimental samples, negative control and positive control samples. The kit in the package U is intended for 96 samples and requires no less than 5 samples in a single run (3 experimental samples, positive and negative controls) or on using a dosing device it is possible to run 96 tests simultaneously (94 experimental samples, negative control and positive control samples).

4. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

4.1. Specimen collection

- Sterile single use swabs, single-use sterile flasks and sterile containers to collect clinical material;
- Sterile tubes containing transport medium: "DNA-Technology" made **STOR-F** (REF P-901-1/1EU, P-901-N/1EU, P-901-R/1EU), **STOP-M** (REF P-910-1/1EU) or equivalent, or sterile physiological saline solution for the transportation of the sample.

4.2. DNA extraction and PCR

Preamplification-specimen and control preparation area:

- Biological safety cabinet class II;
- Refrigerator;
- Vortex mixer;
- High speed centrifuge (RCF(g) no less than 16000);
- Solid-state thermostat (temperature range 50-90 °C);
- Tube rack for 1.5 mL tubes;
- 1.5 mL tubes (example, Eppendorf Safe-Lock Tubes);
- Nucleic acid extraction kit ("DNA-Technology" made **PREP-NA** (REF P-002/1EU), **PREP-NA PLUS** (REF P-002/2EU), **PREP-GS** (REF P-003/1EU), **PREP-GS PLUS** (REF P-003/2EU), **PREP-RAPID** (REF P-001/1EU), **PREP-MB RAPID** (REF P-116-N/4EU, REF P-116-A/8EU) and **PREP-OPTIMA** (REF P-016-N/2EU, REF P-016-1/2EU, REF P-015-N/2EU) extraction kits are recommended);
- Physiological saline solution 0.9% NaCl (Sterile) (if needed);
- Electric laboratory aspirator with trap flask for the removal of supernatant;
- RNase and DNase free pipette tips for aspirator with trap flask;
- Single channel pipettes (dispensers covering 0.2-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL, 200 µL, 1000 µL);
- Pipette stand;
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Preamplification-reagent preparation area:

- UV PCR cabinet;
- Refrigerator;
- Freezing chamber (in case of using package U REF R1-P445-UA/9EU);

- Vortex mixer;
- Vortex rotor for strips (in case of using package S, strips REF R1-P445-S3/4EU);
- Tube rack for 1.5 mL tubes;
- PCR tube rack for 0.2 mL tubes or strips;
- 0.2 mL PCR tubes (in case of using package U REF R1-P445-UA/9EU);
- Single channel pipettes (dispensers covering 0.5-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 10 µL, 20 µL, 200 µL, 1000 µL);
- Pipette stand;
- DTstream M1 dosage instrument (only for automated dosing in case of using package U REF R1-P445-UA/9EU);
- Device for tray sealing DTpack ("DNA-Technology", LLC) (only for automated dosing in case of using package U REF R1-P445-UA/9EU);
- Centrifuge for microtrays (RCF(g) no less than 500) (only for automated dosing in case of using package U REF R1-P445-UA/9EU);
- Polymer thermal seal for microtray sealing (only for automated dosing in case of using package U REF R1-P445-UA/9EU);
- PCR microtray (only for automated dosing in case of using package U REF R1-P445-UA/9EU);
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Post-Amplification – Amplification detection area:

- Real-time PCR thermal cycler.

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. STORAGE AND HANDLING REQUIREMENTS

Expiry date – 12 months from the date of production.

All components of the **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** except TechnoTaq MAX polymerase (package U) must be stored at temperatures from 2 °C to 8 °C during the storage period.

TechnoTaq MAX polymerase (package U) must be stored at temperatures from minus 18 °C to minus 22 °C during the storage period.

PCR-mix must be stored at temperatures from 2 °C to 8 °C and away from light during the storage period. The excessive temperature and light can be detrimental to product performance.

The kit must be transported in thermoboxes with ice packs by all types of roofed transport at temperatures corresponding to storage conditions of the kit components.

Transportation of the kit, except the TechnoTaq MAX polymerase, is allowed in thermoboxes with ice packs by all types of roofed transport at temperatures from 2 °C to 25 °C but for no more than 5 days and should be stored at temperatures from 2 °C to 8 °C immediately on receipt.

It is allowed to transport the TechnoTaq MAX polymerase in thermoboxes with ice packs by all types of roofed transport at temperatures up to 25 °C but for no more than 5 days and should be stored at temperatures from minus 18 °C to minus 22 °C immediately on receipt.

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

- components of the kit except TechnoTaq MAX polymerase should be stored at temperatures from 2 °C to 8 °C during the storage period;
- TechnoTaq MAX polymerase should be stored at temperatures from minus 18 °C to minus 22 °C during the storage period;
- PCR-mix for amplification should be stored at temperatures from 2 °C to 8 °C and away from light during the storage period.

The kit stored under undue regime should not be used.

An expired the **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** should not be used.

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

The conformity of the **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** to the prescribed technical requirements is subject to compliance of storage, transportation and handling conditions recommended by manufacturer.

Contact our official representative in EU by quality issues of the **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit**.

6. WARNINGS AND PRECAUTIONS

Only personnel trained in the methods of molecular diagnostics and the rules of work in the clinical and diagnostic laboratory are allowed to work with the kit.

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 cannot 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

Smears/scrapes from nasopharyngeal, oropharyngeal mucous membrane, smears from affected skin areas and bacterial cultures from this biomaterial are used for the assay.

Sampling, sample processing procedures and storage are carried out in accordance with the instructions to the DNA extraction kit from biological material.

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.

The following endogenous and exogenous interfering substances are considered to be PCR inhibitors that may be present in the DNA sample: Hemoglobin, mucus (mucin), and pharmaceuticals (Pinosol, chlorhexidine bigluconate, Rhinofluimucil, Octenisept) present in the DNA sample as a result of incomplete removal during DNA extraction from the biomaterial sample, and isopropyl alcohol and methyl acetate remaining in the DNA sample as a result of incomplete removal of wash solutions during sample preparation.
Maximum concentrations of interfering substances at which PCR inhibition was not observed are shown in the table below.

Biomaterial type	Interfering substance (IS)	IS concentration
Endogenous substances		
nasopharyngeal and oropharyngeal mucous membrane smears/scrapes, smears from affected skin areas	Hemoglobin	0.35 mg/mL
nasopharyngeal and oropharyngeal mucosa swabs/scrapes	Mucus (mucin)	20 %
Exogenous substances		
nasopharyngeal and oropharyngeal mucous membrane smears/scrapes	Pinosol	2%
nasopharyngeal and oropharyngeal mucous membrane smears/scrapes, smears from affected skin areas	Chlorhexidine bigluconate	5%
nasopharyngeal and oropharyngeal mucous membrane smears/scrapes	Rhinofluimucil	5%
nasopharyngeal and oropharyngeal mucous membrane smears/scrapes, smears from affected skin areas	Octenicept	2%
nasopharyngeal and oropharyngeal mucous membrane smears/scrapes, smears from affected skin areas, bacterial cultures	Isopropyl alcohol	10%
nasopharyngeal and oropharyngeal mucous membrane smears/scrapes, smears from affected skin areas, bacterial cultures	Methyl acetate	10%

General requirements

PCR analysis is a direct method, so biological material sampling must be carried out from the location of the infectious process. The decision about analyzing the sampling location is done by a physician according to anamnesis and clinical picture.

The quality of taking a sample of biomaterial, its storage, transportation and pre-processing have a great importance for obtaining correct results.

Incorrect sample taking can lead to invalid results and the need for resampling.

Sampling, sample processing procedures and storage are carried out in accordance with the instructions to the DNA extraction kit from biological material.

ATTENTION! Before DNA extraction pre-processing of samples is needed.

Sample collection

ATTENTION! Pretreatment, sampling and storage of the material is carried out in accordance with the user manual for DNA extraction kit.

Smears/scrapes from nasopharyngeal and oropharyngeal mucous membranes; smears from affected skin areas

Sample taking is made with special sterile single-use tools – probes, cytobrushes, and swabs depending on the source of biological material according to established procedure.

Order of taking:

1 Open the tube with a transport medium.

2 After sample taking put the swab into the tube with transport medium and rinse it thoroughly for 10-15 seconds. Avoid spraying of solution.

3 Remove swab from solution, press it to the wall of tube and squeeze the rest of the liquid. Throw out the swab.

4 Close the tube tightly and mark it.

Bacterial cultures

Material is taken from liquid and solid media using a disposable microbiological loop, spatula or pipette.

Place the recommended amount of liquid bacterial culture or colonies from solid medium into a 1.5 mL disposable plastic tube containing the manufacturer's transport medium for transporting and storing bacterial culture samples for PCR assays.

NOTE – If necessary, the material (a single colony of cells or 100 µL of liquid medium) is taken into a 1.5 mL disposable plastic tube with 500 µL of sterile physiological solution previously added.

Close the tube tightly and mark it.

Transportation and storage of samples

Periods for transport and storage of smears/samples from nasopharyngeal and oropharyngeal mucous membranes; smears from affected skin areas and bacterial cultures are determined by the instructions for the recommended reagent kits for DNA extraction or for the transport medium.

Bacterial cultures taken in saline are allowed to be transported and stored:

- At temperature from 2 °C to 8 °C: for no more than 1 day
- At temperature from minus 18 °C to minus 20 °C: for no more than one week
- At temperature minus 70 °C: for prolonged period.

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

Sample preparation

Smears/scrapes from nasopharyngeal and oropharyngeal mucous membranes; smears from affected skin areas

1. Centrifuge the tube at RCF(g) 16000 for 10 minutes.
2. Remove the supernatant, leaving the volume of precipitate + liquid fraction in the tube that is recommended in the instruction for the DNA extraction kit.

8. PROCEDURE

DNA extracting from biological material

DNA extraction is carried out in accordance with the instruction to the extraction kit. DNA extraction kits for subsequent usage of DNA in PCR and intended for corresponding types of biomaterial are recommended.

ATTENTION! Independently of DNA extraction kit used, a negative control sample should go through all stages of DNA extraction. Physiological saline solution or negative control sample from an extraction kit can be used as a negative control in volumes as indicated.

Assay procedure

8.1 Preparing PCR for package S

ATTENTION! The reagents and tubes should be kept away from direct sun light.

ATTENTION! When using package S (R1-P445-S3/4EU), strips, strictly observe the completeness of the strips and caps for them. Do not use the caps for the strips from the other kits!

8.1.1 Mark the required number of tubes with paraffin sealed PCR-mix for each test sample, positive control (C+) and negative control (C-).

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.

8.1.2 Vortex the Taq-polymerase solution for 3-5 seconds, then spin for 1-3 seconds to collect the drops.

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

8.1.4 Add one drop (~20 µL) of mineral oil into each tube.

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

ATTENTION! In case of using **PREP-GS DNA Extraction Kit**. After vortexing centrifuge the tubes with the DNA preparation at RCF(g) 16000 for one minute to precipitate the sorbent. If, after isolation, the supernatant containing the isolated DNA was transferred to new tubes, centrifugation is carried out for 1-3 seconds in a vortex mixer.

In case of using **PREP-MB RAPID DNA Extraction Kit**, after vortexing put the tubes with the DNA preparation in magnetic rack. If, after isolation, the supernatant containing the isolated DNA was transferred to new tubes, centrifugation is carried out for 3-5 seconds in a vortex mixer.

ATTENTION! Open the cap of the tube, add DNA sample (or control sample), then close the tube before proceeding to the next DNA sample to prevent contamination. In case of using tubes in strips, close the strip before proceeding to the next strip to prevent contamination. Close the tubes/strips tightly. Use filter tips.

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

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

8.1.8 Spin tubes/strips for 3-5 seconds.

8.1.9 Set the tubes/strips into the Real-time Thermal Cycler.

8.1.10 Launch the operating software for DT instrument[1]. Add corresponding test[2], specify the number and IDs of the samples, positive and negative control samples. Specify the position of the tubes/strips in the thermal unit (see 8.1.9) and run PCR. See Table 5.

Table 5. The PCR program for DTlite and DTprime 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	10[1]			Holding		Holding
[1] – holding at 25°C is allowed						

8.2 Preparing PCR for package U, manual dosing

ATTENTION! The reagents and tubes should be kept away from direct sunlight.

8.2.1 Mark the required number of 0.2 mL tubes for each test sample, positive control (C+) and negative control (C-).

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 tube with PCR-mix for 3-5 seconds, then spin in vortex for 1-3 seconds to collect the drops.

8.2.3 Add to each tube 6.0 µL of PCR-mix.

8.2.4 Vortex the TechnoTaq MAX polymerase and PCR-buffer for 3-5 seconds, then spin for 1-3 seconds to collect the drops.

ATTENTION! TechnoTaq MAX polymerase should be got out from the freezer immediately prior to use.

8.2.5 Prepare the mixture of PCR-buffer and TechnoTaq MAX polymerase. Add into the one tube:

6.0 x (N+1) µL of PCR-buffer,
0.3 x (N+1) µL of TechnoTaq MAX polymerase,
N is a quantity of the samples to be tested taking to account “C-”, “C+”.

Example: for simultaneous testing of 4 samples, “C-” and “C+” in one PCR run, mark 6 tubes (4 tubes for samples to be tested, 1 tube for “C-” and 1 tube for “C+”). Prepare the mixture of PCR-buffer and Taq-polymerase for 7 (6+1) tubes. Mix 42 µL of PCR-buffer and 2.1 µL of TechnoTaq MAX polymerase.

8.2.6 Vortex the tube with the mixture of PCR-buffer and TechnoTaq MAX polymerase for 3-5 seconds, then spin in vortex for 1-3 seconds to collect the drops.

ATTENTION! Mixture of PCR-buffer and TechnoTaq MAX polymerase must be prepared immediately prior to use.

[1] Please, apply to Operation Manual for DTprime 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.

8.2.7 Add 6.0 µL of PCR-buffer and TechnoTaq MAX polymerase mixture into each tube with PCR-mix.

ATTENTION! Follow the steps listed in pp. 8.2.8 – 8.2.13 within two hours after addition of PCR-buffer and TechnoTaq MAX polymerase mixture to PCR-mix.

8.2.8 Vortex the tubes with samples, "C-" and "C+" for 3-5 seconds and spin down drops for 1-3 seconds.

ATTENTION! In case of using **PREP-GS DNA Extraction Kit**. After vortexing centrifuge the tubes with the DNA preparation at RCF(g) 16000 for one minute to precipitate the sorbent. If, after isolation, the supernatant containing the isolated DNA was transferred to new tubes, centrifugation is carried out for 1-3 seconds in a vortex mixer.

In case of using **PREP-MB RAPID DNA Extraction Kit**, after vortexing put the tubes with the DNA preparation in magnetic rack. If, after isolation, the supernatant containing the isolated DNA was transferred to new tubes, centrifugation is carried out for 3-5 seconds in a vortex mixer.

ATTENTION! Open the cap of the tube, add DNA sample (or control sample), then close the tube before proceeding to the next tube to prevent contamination. Close the tubes tightly. Use filter tips.

8.2.9 Add 6.0 µL of DNA sample into the corresponding tubes. Do not add DNA into the "C-", "C+" tubes.

8.2.10 Add 6.0 µL of negative control (C-) which passed whole DNA extraction procedure into the corresponding tube. Add 6.0 µL of positive control sample (C+) into the corresponding tube.

8.2.11 Spin the tubes for 3-5 seconds.

8.2.12 Set the tubes into the Real-time Thermal Cycler.

8.2.13 Launch the operating software for DT instrument[3]. Add corresponding test[4], specify the number and ID's of the samples, positive and negative control samples. Specify the position of the tubes in the thermal unit (see 8.2.12) and run PCR. See Table 6.

Table 6. The PCR program for DTlite and DTprime Thermal Cyclers for package U

Step	Temperature, °C	Min.	Sec.	Number of cycles	Optical measurement	Type of the step
1	80	0	5	15		Cycle
	94	0	5			
2	94	5	00	1		Cycle
3	94	0	30	5		Cycle
	64	0	15		√	
4	94	0	10	45		Cycle
	64	0	15		√	
5	94	0	5	1		Cycle
6	10[1]			Holding		Holding
[1] – holding at 25°C is allowed						

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

8.4 Preparing PCR using DTStream (only for package U)

ATTENTION! The reagents and tubes should be kept away from direct sunlight.

8.4.1 Vortex the tube with PCR-mix for 3-5 seconds, then spin for 1-3 seconds to collect the drops.

8.4.2 Vortex the TechnoTaq MAX polymerase and PCR buffer for 3-5 seconds, then spin in vortex for 1-3 seconds to collect the drops.

ATTENTION! TechnoTaq MAX polymerase should be taken out from the freezer immediately prior to use.

8.4.3 Prepare the mixture of PCR buffer with TechnoTaq MAX polymerase according to the user manual for dosing device DTstream.

8.4.4 Vortex the tube with the mixture for 3-5 seconds, the spin in vortex for 1-3 seconds to collect the drops.

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

ATTENTION! In case of using **PREP-GS DNA Extraction Kit**. After vortexing centrifuge the tubes with the DNA preparation at RCF(g) 16000 for one minute to precipitate the sorbent. If, after isolation, the supernatant containing the isolated DNA was transferred to new tubes, centrifugation is carried out for 1-3 seconds in a vortex mixer.

ATTENTION! In case of using **PREP-MB RAPID DNA Extraction Kit**, vortex the tubes for 3-5 seconds on a vortex mixer, put the tubes with the DNA preparation in magnetic rack and transfer the supernatant containing the isolated DNA to new tubes. If, after DNA extraction, the supernatant containing the isolated DNA was already transferred to new tubes, centrifugation is carried out for 3-5 seconds in a vortex mixer.

8.4.6 Set the tubes with PCR-mix, the mixture of PCR-buffer and TechnoTaq MAX polymerase, DNA samples, positive and negative controls and PCR microtray on the DTstream working table and conduct dosage of the components according to DTstream user manual.

8.4.7 After the end of dosing program on DTstream put the PCR microtray without shaking on the working table of DTpack sealing device.

8.4.8 Run the process of sealing of PCR microtray according to the user manual of DTpack sealing device.

8.4.9 Centrifuge the microtray at RCF(g) 500 for 30 seconds.

8.4.10 Set the PCR microtray into the Real-time Thermal Cycler.

8.4.11 Launch the operating software for DT instrument[5]. Add corresponding test[6], specify the number and ID’s of the samples, positive and negative control samples. Specify the position of the tubes/strips in the thermal unit (see 8.3.10) and run PCR. See Table 6.

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

9. CONTROLS

The **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** contains positive control sample. 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.

ATTENTION! A negative control sample should go through all stages of DNA extraction. Physiological saline solution or negative control sample from an extraction kit can be used as a negative control in volumes as indicated.

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 is absent for the specific product and is present for internal control.

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 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

1. Registration of the results is carried out automatically during amplification by the software provided with detecting thermocycler.

When the amplification program is complete, an information message will appear on the screen and you will be prompted to proceed to the analysis of the results. A graph will show the fluorescence vs. cycle number for all channels used for each assay in the thermoblock.

After the end of the run the software registers “+” or “-” in the table with results. In this case a conclusion can be formed.

2. The results are interpreted according to Table 7. The run results are valid if the conditions for interpreting the results obtained for the control samples are fulfilled.

Table 7. PCR results interpretation

Detection channel			Result		Interpretation
Fam	**Hex**	**Cy5**			
Analyzed samples					
Cp is specified	Is not considered	Cp is specified	+	Tox *C. diphtheriae* gene is detected, *C. diphtheriae* DNA is detected	*C. diphtheriae* toxigenic strains (*tox+* gene)* DNA is detected
Cp is not specified	Is not considered	Cp≤35	+	Tox *C. diphtheriae* gene is not detected, *C. diphtheriae* DNA is detected	*C. diphtheriae* nontoxigenic strains (*tox-* gene) DNA is detected
Cp is not specified	Is not considered	Cp>35	+	*C. diphtheriae* DNA is detected. Insufficient DNA amount to determine presence or absence of tox *C. diphtheriae* gene	The obtained result may be due to low content of *C. diphtheriae* in test sample, cross-contamination by high copy samples, or PCR inhibition**
Cp is not specified	Cp is specified	Cp is not specified	-	Tox *C. diphtheriae* gene is not detected, *C. diphtheriae* DNA is not detected	*C. diphtheriae* DNA is not detected
Cp is specified	Is not considered	Cp is not specified	Invalid	Tox *C. diphtheriae* gene is detected, *C. diphtheriae* DNA is not detected	Invalid result
Cp is not specified	Cp is not specified	Cp is not specified	Invalid	Invalid result	Invalid result
Positive control sample					
Cp is specified	Is not considered	Cp is specified	+		Positive result. Results of the whole series are valid
Negative control sample					
Cp is not specified	Cp is specified	Cp is not specified	-		Negative result. Results of the whole series are valid

* - true toxigenicity must be confirmed by phenotypic tests in all cases.

** - You should perform a one-time repeated sampling and/or repeated DNA extraction and PCR. If the result reoccurs, the final result shall be as follows: "*C. diphtheriae* DNA detected. Insufficient DNA amount to determine presence or absence of tox *C. diphtheriae* gene".

3. For samples with negative results on the three detection channels, the "Result" column will show "invalid" (invalid result). An unreliable result can be due to the presence of inhibitors in the DNA preparation obtained from biological material; incorrect performance of the analysis protocol; non-compliance with the amplification temperature regime, etc. In this case, PCR with the available DNA preparation needs to be repeated, or the DNA must be isolated and PCR performed again, or the biological material must be taken again (performed sequentially).

4. In case the results for negative control sample are different from those in Table 7, the results of all series are considered invalid. In this case decontamination procedures are required.
5. In case the results for positive control sample are different from those in Table 7, it is required to repeat amplification for all series.

11. SPECIFICATIONS

a. Analytical specificity

In samples of human biological material containing *C. diphtheriae* DNA, the detection thermal cycler software records positive amplification results for the specific product (*C. diphtheriae* DNA fragment) during amplification.

In samples of human biological material containing toxigenic *C. diphtheriae* DNA, the detection thermal cycler software records positive amplification results for specific products (*C. diphtheriae* DNA fragments and the *C. diphtheriae tox gene*) during amplification.

In samples of biological material not containing C. diphtheriae DNA, the detection thermal cycler software records negative amplification results for the specific product and positive amplification results for the internal control (IC).

The absence of nonspecific positive amplification results has been shown during the examination in a high concentration of DNA of microorganisms causing infectious diseases, and / or normally present in the loci of biomaterial collection: *Streptococcus bovis, Streptococcus sanguinis, Escherichia coli, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus anginosus, Staphylococcus aureus, Burkholderia spp., Klebsiella pneumoniae, Proteus spp., Morganella morganii, Pseudomonas aeruginosa, Enterobacter cloacae, Acinetobacter spp., Enterococcus spp*., and human DNA at a concentration higher than 750 ng per sample.

An analytical specificity assay shows specific and no nonspecific positive amplification results in high concentration DNA preparations isolated from 33 cultures: seven *Corynebacterium ulcerans (tox+)*, four *Corynebacterium ulcerans* (NTTB, *tox+*), four Corynebacterium ulcerans (tox-), five *Corynebacterium pseudodiphtheriticum (tox-)*, five *Corynebacterium diphtheriae* (*mitis* biotype, *tox*-), five *Corynebacterium diphtheriae* (*gravis* biotype, *tox*-), three *Corynebacterium diphtheriae* (NTTB, *tox+*).

b. Analytical sensitivity

The limit of detection is 5 DNA copies per amplification tube, which corresponds to 10^3 DNA copies/mL. The limit of detection is set by analyzing serial dilutions of a laboratory control sample (LC).

The limit of detection of the test sample depends on the reagent kit(s) used for DNA extraction and the final elution volume of the extracted DNA, e.g., swabs/scrapes from respiratory tract in 500 µL of transport medium:

Kits for DNA extraction/elution volume, µL				
PREP-NA/ 50	**PREP-GS/ 100**	**PREP-NA PLUS PREP-GS PLUS, PREP-MB RAPID/ 300**	**PREP-RAPID/ 500**	**PREP-OPTIMA/ 400**
50 copies/sample	100 copies/sample	300 copies/sample	500 copies/sample	400 copies/sample

c. Diagnostic characteristics

The evaluation of diagnostic characteristics (diagnostic sensitivity and diagnostic specificity) was performed during the count of correctly determined *Corynebacterium diphtheriae* with differentiation of toxigenic and nontoxigenic strains detected with the tested "*C. diphtheriae* Tox" kit of reagents.

Biomaterial type	*C. diphtheriae* of nontoxigenic strains (tox- gene)		*C. diphtheriae* of toxigenic strains (tox+ genes)	
	Diagnostic sensitivity	Diagnostic specificity	Diagnostic sensitivity	Diagnostic specificity
Nasopharyngeal and oropharyngeal mucus membrane smears/scrapes	100% (92.9 - 100)	100% (97.6 - 100)	100% (92.9 - 100)	100% (97.6 - 100)
Smears from affected skin areas	100% (92.9 - 100)	100% (97.6 - 100)	100% (92.9 - 100)	100% (97.6 - 100)

d. Effectiveness characteristics

The effectiveness of the medical device in bacterial cultures assay amounts at 100% (Ptrue=95.5%).

12. TROUBLESHOOTING

Table 8. Troubleshooting

	Result	**Possible cause**	**Solution**
C+	-	Operation error PCR inhibition Violation of storage and handlingrequirements	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 regarding quality issueswith the kit:

Phone: +7(495)640.16.93

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com

13. QUALITY CONTROL

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

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

Contact our customer service by quality issues of **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit**.

Technical support:

E-mail: hotline@dna-technology.ru

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14. KEY TO SYMBOLS

IVD	*In vitro* diagnostic medical device		Date of manufacture
	Temperature limit		Consult instructions for use
	Contains sufficient for <n> tests	REF	Catalogue number
	Use-by date		Manufacturer
LOT	Batch code		Keep away from sunlight
VER	Version		Caution

REF R1-P445-S3/4EU
R1-P445-23/4EU
R1-P445-UA/9EU

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