DNA-TECHNOLOGY

1542 2026-06-05

IVD

For professional use only

C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit

INSTRUCTION FOR USE

"DNA-Technology Research & Production", LLC,
142281, Russia,
Moscow Region, Serpukhov Urban District,
Protvino, Zheleznodorozhnaya Street, 20
Phone/fax: +7(495) 640.17.71
E-mail: info@dna-technology.com
https://www.dna-technology.com
Customer service department
E-mail: hotline@dna-technology.ru

REF

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

Version: 1542.2026.06.05

TABLE OF CONTENTS

1. INTENDED USE 3
2. METHOD 3
3. CONTENT 4
4. ADDITIONAL REAGENTS AND EQUIPMENT REQUIRED 5
5. TRANSPORT AND STORAGE CONDITIONS 6
6. WARNINGS AND PRECAUTIONS 8
7. SAMPLES 9
8. PROCEDURE 11
9. CONTROLS 14
10. DATA ANALYSIS 15
11. SPECIFICATIONS 16
12. TROUBLESHOOTING 19
13. QUALITY CONTROL 20
14. KEY SYMBOLS 21

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 PCR.

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, DTprime or DTprime II 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	Amount	Volume per tube
Paraffin-sealed PCR mix	Colorless transparent liquid under waxy white fraction	tubes, 6 strips of 8	20 µL in each
Taq polymerase solution	Colorless transparent liquid	1 tube	500 µL
Mineral oil	Colorless transparent viscous oily liquid	1 tube	1.0 mL
Positive control[1]	Colorless transparent liquid	1 tube	130 µL
Strip caps	6 strips of 8		

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

Reagent	Description	Amount	Volume per tube
Paraffin-sealed PCR mix	Colorless transparent liquid under waxy white fraction	48 individual tubes	20 µL in each
Taq polymerase solution	Colorless transparent liquid	1 tube	500 µL
Mineral oil	Colorless transparent viscous oily liquid	1 tube	1.0 mL
Positive control[1]	Colorless transparent liquid	1 tube	130 µL

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

Reagent	Description	Amount	Volume per tube
PCR mix	Colorless or slightly pink transparent liquid	1 tube	600 µL
TechnoTaq MAX polymerase	Colorless transparent viscous liquid	1 tube	30 µL
PCR buffer	Colorless transparent liquid	1 tube	600 µL
Positive control[1]	Colorless transparent liquid	1 tube	130 µL

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

The **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** (package S) is intended for single use and

[1] - marking as C+ is allowed

designed for 48 tests (no more than 12 runs), including analysis of test samples, negative controls and positive controls.

The **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit** (package U) is intended for single use and designed for 96 tests with at least 5 samples per run (3 test samples, negative control and positive control).

4. ADDITIONAL REAGENTS AND EQUIPMENT REQUIRED

The following equipment, reagents and consumables are required:

Equipment, reagents and consumables	Package S		Package U, dosing	
	strips	tubes	manual	automated
UV PCR cabinet	•	•	•	•
Real-time detecting thermal cycler[1]	•	•	•	•
Vortex mixer	•	•	•	•
Vortex rotor for 0.2 mL strips	•	-	-	-
Refrigerator with freezer	•	•	•	•
Tube rack for 0.2 mL tubes	-	•	•	-
Tube rack for 0.2 mL strip tubes	•	-	-	-
Tube rack for 1.5 mL tubes	•	•	•	•
Single channel pipettes (dispensers covering 0.5–10 µL; 2.0–20 µL; 20–200 µL; 200–1,000 µL volume range)	•	•	•	•
RNase and DNase free filtered pipette tips (volume 10 µL, 20 µL, 200 µL, 1,000 µL)	•	•	•	•
Pipette rack	•	•	•	•
RNase and DNase free 1.5 mL microfuge tubes with caps	•	•	•	•
RNase and DNase free 0.2 mL PCR tubes	-	-	•	-
Powder-free surgical gloves	•	•	•	•
Container for used pipette tips, tubes and other consumables	•	•	•	•
DTstream12 M1 or DTstream15 M1 liquid handling station	-	-	-	•
RNase and DNase free filtered pipette tips (volume 200 µL) for DTstream	-	-	-	•
DTpack plate sealing device	-	-	-	•
Centrifuge for microplates (RCF(g) at least 500)	-	-	-	•
Polymer thermal film for microplate sealing	-	-	-	•
384-well PCR microplate	-	-	-	•
Physiological saline solution 0.9% NaCl (sterile)				
Transport medium (if necessary), the following are recommended: – **STOR-F;** – **STOR-M.**				
NA extraction reagent kits[2], the following are recommended: – **PREP-NA;** – **PREP-NA PLUS**; – **PREP-GS;**				

Equipment, reagents and consumables	Package S		Package U, dosing	
	strips	tubes	manual	automated
– **PREP-GS PLUS;** – **PREP-RAPID**; – **PREP-OPTIMA**; – **PREP-MB-RAPID II**.				
Additional equipment for pretreatment of bronchoalveolar lavage; pleural fluid; nasopharyngeal and endotracheal aspirate:				
Biological safety cabinet class II				
RNase and DNase free 1.5 mL microfuge tubes with caps				
Centrifuge for 1.5 mL tubes, RCF(g) at least 12,000				
Disinfectant solution				
Notes: [1] –hereinafter – detecting thermal cycler; the required parameters are indicated below [2] – choice of extraction kit is determined by biomaterial type "•" – the piece of equipment/reagent is required "–" – the piece of equipment/reagent is not required				

The following detecting thermal cyclers are validated for work with the **C. diphtheriae Tox Multiplex 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;
- DTprime in DTprime *X* modification (manufactured by "DNA-Technology R&P", LLC), (only for package U, automated dosing), hereinafter – DTprime *X*;
- DTprime II in DTprime II *X* modification (manufactured by "DNA-Technology R&P", LLC), (only for package U, automated dosing), hereinafter – DTprime II *X*;
- DTlite in DTlite *S* modification (manufactured by "DNA-Technology R&P", LLC), (only for package S, and package U, manual dosing, tubes), 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.

5. TRANSPORT AND STORAGE CONDITIONS

Expiry date – 12 months from the date of production.

5.1. Storage conditions

5.1.1. Package S

- All components of the **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection 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 out of light over the storage period.

5.1.2. Package U

- All components of the **C. diphtheriae Tox Multiplex REAL-TIME PCR Detection Kit,** except for TechnoTaq MAX polymerase, must be stored in a refrigerator or a cooling chamber at

temperatures from 2°C to 8°C over the storage period.

- PCR mix must be stored out of light over the storage period.
- TechnoTaq MAX polymerase must be stored in a freezer at the temperatures from minus 22 °C to minus 18 °C over the storage period.

WARNING! The excessive temperature and light can be detrimental to product performance.

5.2. Transport conditions

Transportation of the reagent kit is carried out in thermoboxes with ice packs by all types of roofed transport at the temperature inside the container corresponding to the storage conditions of the kit components.

5.2.1. Package S

- 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.

5.2.2. Package U

- It is allowed to transport the kit, except for TechnoTaq MAX polymerase, 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.
- It is allowed to transport TechnoTaq MAX polymerase in thermoboxes with ice packs by all types of roofed transport at the temperature inside the thermoboxes up to 25 °C for no longer than 5 days.

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

5.3. Shelf-life of the kit following the first opening of the primary container

5.3.1. Package S

- 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;

5.3.2. Package U

- All components of the kit, except for TechnoTaq MAX polymerase, must be stored in a refrigerator or a cooling chamber at temperatures from 2°C to 8°C over the storage period;
- 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;
- TechnoTaq MAX polymerase must be stored in a freezer at temperatures from minus 22°C to minus 18°C over the storage period.

WARNING! The kits stored under undue regime must not be used.

An expired **C. diphtheriae Tox Multiplex 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.

Conformity of **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.

6. WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use only.
- Molecular biology procedures, such as nucleic acid extraction, PCR-amplification and detection require qualified staff to avoid the risk of erroneous or invalid results, especially due to the degradation of nucleic acids contained in the samples or sample contamination by amplification products.
- Wear powder-free single-use surgical gloves. Wear work clothes and personal protective equipment while working with pathogenic microorganisms. The work clothes and personal protective equipment must be suitable for work to be performed and comply with health and safety requirements.
- Avoid any direct contact with the biological samples, reagents and materials used to carry out the test. Avoid producing spills or generating aerosols. Do not eat/drink components of the kit. Do not inhale gas/fumes/vapor/aerosols produced by the components of the kit. Avoid contact with eyes.
- Samples must be handled under a laminar flow hood.
- Pipettes used to handle samples must only be used for one purpose. The pipettes must be of positive displacement type or be used with aerosol barrier pipette tips.
- The reagents must be handled under a laminar flow hood. The reagents required for amplification must be prepared in such a way to be utilized in a single session.
- Handle and dispose of all biological samples, reagents and materials used to carry out the assay as if infectious[2, 3]. Any material being exposed to biological samples must be treated with disinfecting solution for at least 30 min or autoclaved for 1 hour at 121°C before disposal.
- All of the liquid solutions are designed for single use and cannot be used more than once in amplification reactions.
- Only use the reagents provided in the kit and those recommended by the manufacturer. Do not mix reagents from different batches. Do not use reagents from third party manufacturers' kits.
- All laboratory equipment and tools, including pipettes, test tube racks, laboratory glassware, lab coats, bouffant caps, gloves, etc., as well as reagents must 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 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. Never introduce amplification products in the area designed for extraction/preparation of amplification reactions.
- Do not open the tubes after amplification. Avoid producing accidental spills of the amplification products. Dispose of all PCR waste materials (tubes, tips etc.) only in a closed form in a specialized sealed container with disinfectant solution. Waste materials must be removed in accordance with laboratory internal procedures, and with national and international standards.
- Working surfaces, as well as rooms where NA extraction and PCR are performed, must be disinfected with bactericidal irradiators (UVGI) for 30 min before and after the assay. All surfaces in the laboratory (test tube racks, equipment, tools, etc.) must be treated with disinfecting solution daily.

Emergency actions

Eye Contact: If any component of the kit enters the eyes, flush the eyes gently using potable running water for 15 min or longer, making sure that the eyelids are held open. If pain or irritation occurs, seek medical attention.

[2] - All oligonucleotide components are produced by artificial synthesis in compliance with internal quality control protocol. They do not contain blood or products of blood processing.

[3] - Positive control is produced using artificial DNA synthesis technology, it does not contain parts of infectious agents.

Skin Contact: If any component of this kit comes into contact with the skin and causes discomfort, remove any contaminated clothing. Rinse the affected area with plenty of soap and water. If pain or irritation occurs, seek medical attention.

Ingestion: If any component of this kit is ingested, rinse the mouth with plenty of potable water. If irritation or discomfort occurs, seek medical attention.

Do not use the kit:

- if the transportation and storage conditions have been violated;
- if the appearance of the reagents does not correspond to the product documentation;
- if the packaging of the kit components is breached;
- after the expiry date of the kit.

Adverse health effects are **NOT** anticipated from routine use of this kit in compliance with the current instruction for use.

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,	Isopropyl alcohol	10%

Biomaterial type	Interfering substance (IS)	IS concentration
smears from affected skin areas, bacterial cultures		
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.

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

Sample collection

WARNING! 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 20°C to minus 18°C: for no more than one week
- At temperature minus 70°C: for prolonged period.

WARNING! 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) 16,000 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

8.1 DNA extraction from biological material

NA extraction is performed in accordance with the instructions for use of the corresponding reagent kit.

DNA extraction is carried out according to the extraction kit instructions. **PREP-NA, PREP-NA PLUS, PREP-GS, PREP-GS PLUS, PREP-RAPID, PREP-OPTIMA,** and **PREP-MB-RAPID II** extraction kits are recommended.

WARNING! Run a negative control alongside DNA extraction through all preparation stages. Use physiological saline solution (in volumes per the extraction kit instructions) or the kit provided negative control.

General requirements:

- Follow the DNA preparation recommendations from the NA extraction kit's instruction.
- Use filter tips.
- Open tube caps only when introducing the DNA sample, then close immediately.
- If using strips: close the strip cap after introducing samples before proceeding to the next strip.
- Ensure all tubes are tightly closed after dosing.

For DNA preparations after storage, prepare them as follows before adding to the amplification mixture:

- **PREP-NA, PREP-NA PLUS, PREP-RAPID, PREP-OPTIMA**: vortex (3–5 sec), then centrifuge on vortex (1–3 sec).
- **PREP-GS, PREP-GS PLUS**: vortex (5–10 sec), incubate at 50 °C (5 min) (if the eluate was refrigerated), then centrifuge at 16,000 RCF(g) (1 min).
- **PREP-MB-RAPID II**: centrifuge on vortex carefully (without shaking, 1–3 sec), place into magnetic rack. If supernatant transferred to new tubes — centrifuge (1–3 sec) after shaking.

8.2 Preparing PCR for package S

WARNING!

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

8.2.1 Mark one tube/strip tube with the paraffin-sealed PCR mix for each test sample, C-, and C+.

Example: To test 4 samples, mark 4 tubes for samples, one C- tube and one C+ tube. Total number of tubes is 6.

8.2.2 Shake the tubes with Taq polymerase solution on vortex, then spin down the drops.

8.2.3 Add **10 µL** of Taq polymerase solution to each tube. Avoid paraffin layer break.

8.2.4 Add one **drop** of mineral oil (~20 µL) to each tube. Cover the tubes/strips loosely with caps.

8.2.5 Shake the tube with DNA preparation, C- and C+ on vortex, then spin down the drops.

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 C- which passed whole DNA extraction procedure into the C- tube. Avoid paraffin layer break.

8.2.8 Add **5.0 µL** of C+ into the corresponding tube. Avoid paraffin layer break.

8.2.9 Thoroughly spin down the drops on vortex.

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

Launch the operating software for DT instrument[4]. Add corresponding test[5], specify the number and IDs of the samples, positive and negative controls. Specify position of the tubes/strips in thermal unit (see 8.2.10) and run PCR considering PCR mix volume of 35 µL. See Table 2.

Table 2. 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[6]			Holding		Holding

8.3 Preparing PCR for package U, manual dosing

WARNING!

- The reagents and tubes must be kept away from direct sunlight.
- For amplification use 0.2 mL single-use amplification tubes or 96-well PCR plates[7], sealed hermetically with thermal film. It is not recommended to use strips due to postamplification contamination hazard.

8.3.1 **Mark** the required number of 0.2 mL tubes or a 96-well PCR plate for each test sample, C- and C+.

Note. It is recommended to test at least 5 samples per test (3 test samples, C- and C+).

Example: to test 4 samples, mark 4 tubes/reserve 4 wells for samples, 1 tube/well for C- and 1 tube/well

[4] - Please, apply to Operation Manual for DTprime, DTprime II and DTlite Real-Time PCR instruments PART II.

[5] - The test for DT detecting thermal cyclers is created by entering parameters or is provided by the kit manufacturer.

[6] - holding at 10°C is allowed

[7] - 96-well plates are not used with DTlite and detecting thermal cycler

for C+. The resulting number of tubes/wells is 6.

8.3.2 Shake the tubes with PCR mix on vortex, then spin down the drops.

8.3.3 Add **6.0 µL** of PCR mix to each tube/well (including C- and C+).

8.3.4 Shake the tubes with PCR buffer on vortex, then spin down the drops.

WARNING! Take TechnoTaq MAX polymerase out from the freezer immediately prior to use.

8.3.5 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,
where N is the quantity of samples to be tested taking to account C-, C+.

Example: to test 4 samples, C- and C+ in one PCR run, mark 6 tubes/reserve 6 wells (4 tubes/wells for test samples, 1 tube/well for C- and 1 tube/well for C+). Prepare the mixture of PCR buffer and Taq polymerase for 7 (6+1) tubes/wells. Mix 42 µL of PCR buffer and 2.1 µL of TechnoTaq MAX polymerase.

8.3.6 Vortex the tubes with the mixture of PCR buffer and TechnoTaq MAX and spin down the drops.

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

8.3.7 Add **6.0 µL** of PCR buffer and TechnoTaq MAX polymerase mixture into each tube/well with PCR mix. Cover the tubes loosely.

WARNING! Follow the steps listed in pp. 8.3.8–8.3.14 within two hours after adding PCR buffer and TechnoTaq MAX polymerase mixture to PCR mix.

8.3.8 Shake the tubes with C+ on vortex, then spin down the drops.

8.3.9 Add **6.0 µL** of DNA sample into corresponding tubes/wells. Do not add DNA into the C-, C+ tubes/wells.

8.3.10 Add **6.0 µL** of C- which passed whole DNA extraction procedure into the corresponding tube/well.

8.3.11 Add **6.0 µL** of C+ into the corresponding tube/well.

8.3.12 In case of using **96-well PCR plates**:

8.2.12.1. Place the plate carefully, without shaking into the DTpack sealing device.

8.2.12.2. Seal the PCR plate with polymer thermal film according to the DTpack operation manual.

8.2.12.3. Centrifuge the plate at **RCF(g) 100 for 30 sec**.

8.3.13 In case of using tubes: **Centrifuge** the tubes for 3–5 sec on vortex mixer.

8.3.14 Set the tubes/ PCR plate into the real-time thermal cycler.

8.3.15 Launch the operating software for DT instrument[8]. Add corresponding test[9], specify the number and IDs of the samples, positive and negative controls. Specify position of the tubes in thermal unit (see 8.3.13) and run PCR considering PCR mix volume of 18 µL. See Table 3.

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

[8] - Please, apply to Operation Manual for DTprime, DTprime II and DTlite Real-Time PCR instruments PART II.

[9] - The test for DT detecting thermal cyclers is created by entering parameters or is provided by the kit manufacturer.

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	25[10]			Holding		Holding
√ – optical measurements						

8.4 Preparing PCR for package U, using DTstream

WARNING!

- The reagents and tubes should be kept away from direct sunlight.
- For amplification use 96-well or 384-well[11] PCR plates hermetically sealed with thermal film.

8.4.1 Shake the tube with PCR mix on vortex, then spin down the drops.

8.4.2 Shake the tube with PCR buffer and TechnoTaq MAX polymerase on vortex, then spin down the drops.

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

8.4.3 Prepare the mix of PCR buffer and TechnoTaq MAX polymerase according to the software for DTstream.

8.4.4 Shake the tube with the mix of PCR buffer and TechnoTaq MAX polymerase on vortex, then spin down the drops.

8.4.5 Shake the tubes with DNA preparation, C- and C+ on vortex, then spin down the drops.

8.4.6 Set the tubes with PCR mix, the mix of PCR buffer and TechnoTaq MAX polymerase, DNA samples, positive and negative controls, and PCR microplate 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 microplate without shaking on the working table of DTpack sealing device.

8.4.8 Seal the PCR microplate according to the user manual of DTpack sealing device.

8.4.9 Centrifuge the microplate at **RCF(g) 500 for 30 sec**.

8.4.10 Set the PCR microplate into the real-time thermal cycler.

8.4.11 Launch the operating software for DT instrument[12]. Add corresponding test[13], specify the number and IDs of the samples, positive and negative controls. Specify position of the tubes/plates in thermal unit (see 8.4.10) and run PCR considering PCR mix volume of 18 µL. See Table 3.

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.

[10] - Holding at 10 °C is allowed.

[11] - only for DTprime *X* and DTprime II *X* detecting thermal cyclers

[12] - Please, apply to Operation Manual for DTprime, DTprime II and DTlite Real-Time PCR instruments PART II.

[13] - the test for DT detecting thermal cyclers is created by entering parameters or is provided by the kit manufacturer.

WARNING! 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

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

10.2. Result interpretation is carried out according to Table 4. The results are valid if the conditions for the interpretation of results obtained for control samples are met.

10.3. Invalid 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 recollect biological material from the patient (performed sequentially).

10.4. If a positive result is obtained for C-, the results of the whole batch should be considered invalid. In this case special measures are required for detection and elimination of possible contamination.

10.5. If a negative result is obtained for C+, the results of the whole batch should be considered invalid. In this case it is required to repeat PCR for the whole batch of samples.

Table 4. PCR results interpretation

Detection channel			**Result**		**Interpretation**
Fam	Hex	Cy5			
Test samples					
Cp is specified	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	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	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. 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.
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					
Cp is specified	Is not considered	Cp is specified	+	Tox *C. diphtheriae* gene is detected, *C. diphtheriae* DNA is detected	Positive result. Results of the whole series are valid
Negative control					
Cp is not specified	Cp is specified	Cp is not specified	-	Tox *C. diphtheriae* gene is not detected, *C. diphtheriae* DNA is not detected	Negative result. Results of the whole series are valid

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

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 test 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 (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:

Biomaterial	**Kits for DNA extraction/elution volume, µL**					
	PREP-NA/ 50 µL	**PREP-GS/ 100 µL**	**PREP-NA PLUS, PREP-GS PLUS/ 300 µL**	**PREP-RAPID/ 500 µL**	**PREP-OPTIMA/ 400 µL**	**PREP-MB-RAPID II/ 100 µL**
Smears/scrapes/washings from respiratory tract, mucous membranes, skin in 500 µL of transport medium	50 copies/ sample	100 copies/ sample	300 copies/ sample	500 copies/ sample	400 copies/ sample	100 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 mucous 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)

The performance of the medical device in bacterial cultures assay amounts at 100% (P_{true} = 95.5%).

12. TROUBLESHOOTING

Table 5. 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 any undescribed issues contact our customer service department regarding quality issues with the kit:

Phone: +7(495)640.16.93

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com

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.

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

https://www.dna-technology.com

Manufacturer: "DNA-Technology Research & Production", LLC,

142281, Russia, Moscow Region, Serpukhov Urban District,

Protvino, Zheleznodorozhnaya Street, 20

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

https://www.dna-technology.com

14. KEY 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
	Caution		