AmpliSens® *Corynebacterium diphtheriae* / **tox-genes-FRT** PCR kit

CE IVD

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Contains sufficient for <n> tests
LOT	Batch code		Use-by Date
IVD	*In vitro* diagnostic medical device		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limit		Keep dry
	Manufacturer	NCA	Negative control of amplification
	Date of manufacture	C–	Negative control of extraction
EC REP	Authorized representative in the European Community	C+	Positive control of amplification
	Caution	IC	Internal control

1. INTENDED USE

AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Corynebacterium diphtheriae* DNA and genes encoding toxins of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* in the biological material (naso- and oropharyngeal swabs, swabs from affected areas of skin) using real-time hybridization-fluorescence detection of amplified products. The material for PCR is DNA-samples extracted from test material.

Indications and contra-indications for use of the reagent kit

The reagent kit is used to study the biological material taken from patients suspected of diphtheria etiology of the disease (laryngotracheitis, laryngitis, croup); from patients with tonsillitis with a pathological effusion on the tonsils, with suspicion of the pharyngeal (paratonsillar) abscess; infectious mononucleosis; stenosing laryngotracheitis; from persons who were in contact with the source of the infection (for epidemiological reasons); from persons newly admitted to specialized long-stay institutions for children and adults (for prophylactic purposes).

There are no contra-indications with the exception of cases when the material cannot be taken for medical reasons.

The results of PCR analysis are taken into account in complex diagnostics of disease.

NOTE: The reagent kit can be used for the purpose of screening *C.diphtheriae* carriage, as well as for the purpose of excluding Diphtheria diagnosis or for making a preliminary diagnosis of diphtheria. It should be noted that some *C.diphtheriae* with a toxin gene are not capable of producing the toxin (not toxic). Therefore, after positive results of PCR studies (detection of *C.diphtheriae* DNA and *C.diphtheriae* toxin gene), the toxicity of *C.diphtheriae* should be additionally confirmed using other laboratory methods, in accordance with the current regulatory acts.

2. PRINCIPLE OF PCR DETECTION

Principle of testing is based on the DNA extraction from the samples of test material with the exogenous internal control sample (Internal Control-FL (IC)) and simultaneous amplification of DNA fragments of the detected microorganism and DNA of the internal control with hybridization-fluorescence detection. Exogenous internal control (Internal Control-FL (IC)) allows to control all PCR-analysis stages of each individual sample and to identify possible reaction inhibition.

Amplification of DNA fragments with the use of specific primers and Taq-polymerase enzyme are performed with the DNA samples obtained at the extraction stage. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

The PCR kit variant FRT-100 F contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and deoxyuridine triphosphate (dUTP). The enzyme UDG recognizes and catalyzes the destruction of the DNA containing deoxyuridine, but has no effect on DNA containing deoxythymidine. Deoxyuridine is absent in the authentic DNA, but is always present in amplicons, because dUTP is a part of dNTP mixture in the reagents for the amplification. Due to the deoxyuridine containing contaminating amplicons are sensitive to the destruction by UDG before the DNA-target amplification. So the amplicons cannot be amplified.

The enzyme UDG is thermolabile. It is inactivated by heating at temperature above 50 °C. Therefore, UDG does not destroy the target amplicons which are accumulated during PCR.

The results of amplification are registered in the following fluorescence channels.

Table 1

Channel for fluorophore	FAM	JOE	ROX	Cy5
DNA-target	Internal Control-FL (IC) DNA	*Corynebacterium diphtheriae* toxin DNA	*Corynebacterium diphtheriae* DNA	*Corynebacterium ulcerans* toxin DNA
Target gene	Artificially synthesized sequence	*C. diphtheriae* tox gene	*rpo B* gene	*C. ulcerans* tox gene

3. CONTENT

AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT PCR kit is produced in 2 forms:

variant FRT-100 F REF H-2842-1-CE.

variant FRT-L REF H-2843-1-4-CE.

Variant FRT-100 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *C.diphtheriae* / tox genes	clear liquid from colorless to light lilac colour	1.2	1 tube
PCR-buffer-B	colorless clear liquid	0.6	1 tube
Polymerase (TaqF)	colorless clear liquid	0.06	1 tube
C+ *C.diphtheriae* / tox genes	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Internal Control-FL (IC)*	colorless clear liquid	1.0	1 tube
Negative Control (C-)**	colorless clear liquid	1.2	1 tube

* add **10 µl** of **Internal Control-FL (IC)** during the RNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep**, REF K2-9-Et-100-CE protocol).

** must be used in the extraction procedure as Negative Control of Extraction.

Variant FRT-100 F is intended for 110 reactions (including controls).

Variant FRT-L includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix *C.diphtheriae* / tox genes-Lyo	white powder	-	96 tubes of 0.2 ml
C+ *C.diphtheriae* / tox genes	colorless clear liquid	0.5	1 tube
TE-buffer	colorless clear liquid	0.5	1 tube
Internal Control-FL (IC)*	colorless clear liquid	1.0	1 tube
Negative Control (C-)**	colorless clear liquid	1.2	1 tube
Buffer for elution A	colorless clear liquid	1.2	1 tube

* add **10 µl** of **Internal Control-FL (IC)** during the RNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep**, REF K2-9-Et-100-CE protocol).

** must be used in the extraction procedure as Negative Control of Extraction.

Variant FRT-L is intended for 96 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- Transport medium for storage and transportation of respiratory swabs.
- 0.9 % saline solution or 0.01 M potassium-phosphate buffer (pH 7.0).
- Pediatric nasopharengeal flocked swab with plastic applicator – for sampling from mucous membrane of inferior nasal meatus of children.
- Flocked or fiber swabs for collecting nasopharyngeal specimens from kids and adults.
- Vortex mixer.
- Vacuum aspirator with flask for removing supernatant.
- DNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free and pipette tips with filters (up to 10 µl, 100 µl, 200 µl).
- Tube racks.
- PCR box.
- Real-time instruments with 4 (or more) independent detection channels (for example, Rotor-Gene Q (QIAGEN, Germany), CFX96 (Bio-Rad, USA)).
- Disposable polypropylene PCR tubes:
  a) screwed or tightly closed 1.5-ml tubes for reaction mixture preparation for PCR kit variant FRT-100 F;
  b) thin-walled 0.2-ml PCR tubes with optical transparent domed or flat caps or strips of eight 0.2-ml tubes with optical transparent caps if a plate-type instrument is used for PCR kit variant FRT-100 F;
  c) thin-walled 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used for PCR kit variant FRT-100 F.
- Refrigerator with the range from 2 to 8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store all-positive material (samples, controls) away from all other reagents and add it to the reaction mix in a distantly separated facility. Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterward.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use the PCR kit if the internal packaging was damaged or its appearance was changed.
- Do not use the PCR kit if the transportation and storage conditions according to the Instruction Manual were not observed.
- Do not use a kit after its expiration date.
- Dispose of all samples and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid inhalation of vapors, samples and reagents contact with the skin, eyes and mucous membranes. Harmful if swallowed. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice if necessary.
- While observing the conditions of transportation, operation and storage, there are no risks of explosion and ignition.
- Safety Data Sheets (SDS) are available on request.
- The PCR kit is intended for single use for PCR analysis of specified number of samples (see the section “Content”).
- The PCR kit is ready for use in accordance with the Instruction Manual. Use the PCR kit strictly for intended purpose.
- Use of this product should be limited to personnel trained in the DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment, and reagents to the area where the previous step was performed.

⚠ Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING

AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT PCR kit is intended for the analysis of DNA extracted with DNA extraction kits from the biological material (naso- and oropharyngeal swabs, swabs from the affected areas of skin).

Sampling

Naso- and oropharyngeal swabs are obtained using different swabs from the mucosa of inferior nasal meatus and then from the oropharynx, with the working ends of swabs are placed in one tube with 500 µl of **Transport Medium for Storage and Transportation of Respiratory Swabs** (REF 959-CE, REF 957-CE, REF 958-CE) and tested as one sample.

Nasopharyngeal swabs are obtained through the inferior nasal meatus using sterile dry flocked swabs with plastic shafts for nasopharyngeal swabs. If the nasal cavity is full of mucus it is recommended to blow the nose before the procedure. Gently insert the swab along the external nasal wall to a depth of 2–3 cm towards the inferior nasal concha. Then move the swab slightly lower, insert it in the inferior nasal meatus under the inferior nasal concha, rotate, and remove along the external nasal wall. The total depth of insertion of the swab should be approximately half of the distance from the nostril to the ear hole (3-4 cm for children and 5-6 cm for adults).

When the material is obtained, insert the swab till the place of break into a sterile disposable tube with 500 µl of **Transport Medium for Storage and Transportation of Respiratory Swabs** (REF 959-CE, REF 957-CE, REF 958-CE). Furthermore the flexible part of the swab is winded in a spiral. Then covering the tube with a cap, lift down the handle of the swab achieving complete break of the upper part of the swab. Tightly close the tube with the solution and the working part of swab and mark it.

Oropharyngeal samples are obtained using sterile dry rayon swabs with plastic shafts for oropharyngeal swabs. Rotate the swab over the surface of tonsils, palatine arches, and posterior wall of pharynx.

When material is obtained, insert the working part of the swab into a sterile disposable tube with 500 µl of **Transport Medium for Storage and Transportation of Respiratory Swabs** (REF 959-CE, REF 957-CE, REF 958-CE). Break off the end of shaft to allow tight closing of tube cap. Close and mark the tube with the solution and the swab.

The test material can be stored before PCR analysis:

- at the temperature from 2 to 8 °C – no more than 3 days;
- at the temperature from minus 24 to minus 16 °C – for 1 week.

Only one freeze-thawing cycle is acceptable.

Swabs from affected areas of skin are taken with a sterile dry probe. The swabs from affected skin surface are taken after preliminary removing of crusts. When the material is obtained, place the working part of the probe into the sterile disposable tube with 500 µl of **Transport Medium for Storage and Transportation of Respiratory Swabs** (REF 959-CE, REF 957-CE, REF 958-CE) or 0.9 % saline solution or 0.01 M potassium-phosphate buffer (pH 7.0). Break off the end of shaft to allow tight closing of tube cap. Close and mark the tube with the solution and the swab.

The swabs from affected areas of skin can be stored before PCR analysis:

- at the temperature from 2 to 8 °C – no more than 3 days;
- at the temperature from minus 24 to minus 16 °C – for 1 week.

Only one freeze-thawing cycle is acceptable.

It is allowed to transport the above-mentioned material at the temperature from 2 to 8 °C for 3 days.

Pretreatment

Pretreatment of nasopharyngeal swabs, oropharyngeal swabs, swabs from affected areas of skin is not required.

Interfering substances and limitations of using test material samples

In order to control the DNA extraction efficiency and possible reaction inhibition the Internal Control (Internal Control-FL (IC)) is used in the PCR kit. The Internal Control is added in each biological sample at the extraction stage. The presence of internal control signal after the amplification testifies the effectiveness of nucleic acid extraction and the absence of PCR inhibitors.

Potential interfering substances

Endogenous and exogenous substances that may be present in the biological material (nasopharyngeal swabs, oropharyngeal swabs, swabs from the affected areas of skin) used for the study were selected to assess potential interference

Nasopharyngeal and oropharyngeal swabs

Samples of nasopharyngeal and oropharyngeal swabs without adding and with the addition of potentially endogenous (mucin) and exogenous (aqueous solution of hexidine-chlorine for local and outdoor use, 5 %, and Lugol's solution with glycerin, 1 %) potential interfering substances were tested (Table. 2).

Samples of nasopharyngeal and oropharyngeal swabs with added quality control samples (QCS) containing *C.diphtheriae* DNA, *C.diphtheriae tox* gene and *C. ulcerans tox* gene were tested. Final concentration of each QCS was $1x10^4$ GE/ml.

Swabs from the affected areas of skin

Swabs from the affected areas of skin from patients with allergic non-infectious dermatoses without adding and with the addition of potentially endogenous (hemoglobinum) and exogenous (aqueous solution of hexidine-chlorine for local and outdoor use, 5 %) potential interfering substances were tested.

Swabs from the affected areas of skin with added quality control samples (QCS) containing *C.diphtheriae* DNA, *C.diphtheriae tox* gene and *C. ulcerans tox* gene were tested. Final concentration of each QCS was $1x10^4$ GE/ml.

Table 2

Type of tested material	Type of potential interferent	Potential interferent	Tested concentration in a sample	Interference presence
Nasopharyngeal swabs, oropharyngeal swabs	Endogenous substances	Mucin	5 %	Not detected
	Exogenous substances	Aqueous solution of hexidine-chlorine diegluconate	2,5 %	Not detected
		Lugol's solution with glycerin	0,5 %	Not detected
Swabs from the affected areas of skin	Endogenous substances	Haemoglobin	0,21 g/ml	Not detected
	Exogenous substances	Aqueous solution of hexidine-chlorine diegluconate	2,5 %	Not detected

7. WORKING CONDITIONS

AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT PCR kit should be used at the temperature from 20 to 28 °C and relative humidity from 15 to 75 %.

8. PROTOCOL

8.1. DNA Extraction

It is recommended to use the following nucleic acid extraction kits:

— **RIBO-prep**, REF K2-9-Et-100-CE.

If using the RIBO-prep kit extract the DNA according to the manufacturer's protocol.

The volumes of reagents and samples when the DNA is extracted by the RIBO-prep reagent kit:

The DNA extraction for each sample is carried out in the presence of **Internal Control-FL (IC).**

NOTE: Add **10 µl** of **Internal Control-FL (IC)** to each tube.

The volume of the test sample is **100 µl.**

Add **100 µl of Negative Control (C–)** into the tube labeled C– (Negative Control of Extraction).

The volume of elution is **50 µl** for PCR kit variant FRT-100 F or **100 µl** for PCR kit variant FRT-L

NOTE: The buffer included in the RIBO-prep kit of reagents, as well as the buffer for elution included in PCR kit variant FRT-L is used for elution during DNA extraction from test samples

8.2. Preparing PCR

8.2.1 Preparing tubes for PCR

Variant FRT-100 F

The total reaction volume is **25 µl**, the volume of the DNA sample is **10 µl**.

1. Calculate the required quantity of each reagent for reaction mixture preparation. For one reaction:
   - **10 µl** of **PCR-mix-FL *C.diphtheriae* / tox genes,**
   - **5 µl** of **PCR-buffer-B,**
   - **0.5 µl** of **polymerase (TaqF)**.
2. Prepare the reaction mixture for the total number of test and control samples plus several extra reactions. See numbers control samples in item 4.

NOTE: Prepare the reaction mixture just before use.

3. Thaw the tubes with **PCR-mix-FL *C.diphtheriae* / tox genes.** Thoroughly vortex all the reagents of the PCR kit and sediment the drops by vortex.
4. In a new tube prepare the reaction mixture. Mix the required quantities of **PCR-mix-FL *C.diphtheriae* / tox genes, PCR-buffer-B** and **polymerase (TaqF)**. Sediment the drops by vortex.
5. Take the required number of the tubes or strips taking into account the number of test samples and control samples
6. Transfer **15 µl** of the prepared reaction mixture to each tube. Discard the unused reaction mixture.

Table 3

Scheme of reaction mixture preparation for variant FRT-100 F

		Reagent volume for specified number of reactions		
Reagent volume per one reaction, µl		**10.0**	**5.0**	**0.5**
Number of test samples	**Number of reactions[1]**	**PCR-mix-FL**	**PCR-buffer-B**	**Polymerase (TaqF)**
2	**6**	**60**	**30**	**3.0**
4	**8**	**80**	**40**	**4.0**
6	**10**	**100**	**50**	**5.0**
8	**12**	**120**	**60**	**6.0**
10	**14**	**140**	**70**	**7.0**
12	**16**	**160**	**80**	**8.0**
14	**18**	**180**	**90**	**9.0**
16	**20**	**200**	**100**	**10.0**
18	**22**	**220**	**110**	**11.0**
20	**24**	**240**	**120**	**12.0**
22	**26**	**260**	**130**	**13.0**

[1] Number of test samples including the control of extraction stage (N), controls of amplification, and one extra reaction (N+3+1).

		Reagent volume for specified number of reactions		
Reagent volume per one reaction, µl		**10.0**	**5.0**	**0.5**
Number of test samples	**Number of reactions[1]**	**PCR-mix-FL**	**PCR-buffer-B**	**Polymerase (TaqF)**
24	**28**	**280**	**140**	**14.0**
26	**30**	**300**	**150**	**15.0**
28	**32**	**320**	**160**	**16.0**
30	**34**	**340**	**170**	**17.0**
32	**36**	**360**	**180**	**18.0**
68	**72**	**720**	**360**	**36.0**
92	**96**	**960**	**480**	**48.0**

7. Add **10 µl** of **DNA samples** extracted from test samples at the DNA extraction stage using tips with filter.
8. Carry out the control amplification reactions:

C- – Add **10 µl** of **the sample extracted from the Negative Control (C-) reagent** to the tube with reaction mixture
C+ – Add **10 µl** of **C+ *C.diphtheriae* / tox genes** to the tube with reaction mixture
NCA – Add **10 µl** of **TE-buffer** to the tube with reaction mixture

Variant FRT-L

The total reaction volume is **25 µl**, the volume of the **DNA** sample is **25 µl**.
Use disposable filter tips for adding reagents, DNA and control samples into tubes.

1. Take the required number of the tubes with ready-to-use lyophilized reaction mixture **PCR-mix *C.diphtheriae* / tox genes-Lyo** for amplification of DNA from test and control samples (see the number of control samples in point 3).

2 Add **25 µl** of **DNA samples** extracted from test samples into the prepared tubes.

3 Carry out the control reactions:

C+ – Add **25 µl** of **C+ *C.diphtheriae* / tox genes** to the tube labeled **C+** (Positive Control of Amplification).
NCA – Add **25 µl** of **TE-buffer** to the tube labeled **NCA** (Negative Control of Amplification).
C- – Add **25 µl** of **the sample extracted from the Negative Control (C-) reagent** to the tube labeled **C–** (Negative Control of Extraction).

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

8.2.2. Amplification

1. Create a temperature profile on your instrument as follows:

Table 3

AmpliSens unified amplification and fluorescence detection program for rotor-[2] and plate-type instruments[3]

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	50	15 min	–	1
2	95	15 min	–	1
3	95	10 s	–	45
	60	20 s	**FAM, JOE, ROX, Cy5**	

NOTE: Any combination of the tests (including tests with reverse transcription and amplification) can be performed in one instrument simultaneously with the use of the unified amplification program. If several tests in "multiprime" format are carried out simultaneously, the detection is enabled in other used channels except for the specified ones. If only the tests for DNA detection are performed in one instrument then the first step of reverse transcription (50 °C – 15 minutes) can be omitted for time saving.

2. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin*.
3. Insert tubes into the reaction module of the instrument.

NOTE: Insert empty tubes at the edges of reaction module in case of incomplete filling of plate-type instrument.

4. Run the amplification program with fluorescence detection.
5. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in four channels:

Table 5

Channel for the fluorophore	FAM	JOE	ROX	Cy5
Signal registration, indicating the amplification product accumulation	Internal Control-FL (IC) DNA	*Corynebacterium diphtheria* toxin DNA, *tox* gene	*Corynebacterium diphtheria* DNA, *rpo B* gene	*Corynebacterium ulcerans* toxin DNA, *tox* gene

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the cDNA sample in the corresponding column of the results grid.
Principle of interpretation is the following

Table 6

Results interpretation

***Ct* value in the channel for the fluorophore**				**Result**
FAM	**JOE**	**ROX**	**Cy5**	
Internal Control (IC)	***C.diphtheriae C.diphtheriae tox* gene)**	***C.diphtheriae* (*rpoB* gene)**	***C.ulcerans* (*C.ulcerans tox* gene)**	
determined or absent	absent	≤ boundary value	absent or > boundary value	*C.diphtheriae* DNA **is detected**
determined or absent	≤ boundary value	≤ boundary value	absent or > boundary value	*C.diphtheriae* DNA containing *C.diphtheriae tox* gene **is detected**
determined or absent	≤ boundary value	≤ boundary value	≤ boundary value	*C.diphtheriae* DNA containing *C.diphtheriae tox* gene **is detected**. *Corynebacterium ulcerans* tox gene **is detected**
determined or absent	absent	≤ boundary value	≤ boundary value	*C.diphtheriae* DNA containing gene which is similar to *C.ulcerans tox* gene **is detected**.
determined or absent	absent	absent	≤ boundary value	*C.diphtheriae* **DNA is NOT detected**. *C.ulcerans tox* gene **is detected**.
≤ boundary value	absent	absent	absent or > boundary value	*C.diphtheriae* DNA, *C.diphtheriae tox* gene, *C.ulcerans tox* gene **are NOT detected**
absent or > boundary value	absent or > boundary value	absent or > boundary value	absent or > boundary value	**Invalid***
determined or absent	≤ boundary value	≤ boundary value	absent or > boundary value	**Equivocal*** If the result is repeated, it is concluded that *C.diphtheriae* DNA and low content of *C.diphtheriae tox* gene are detected. It is recommended to repeat the material sampling
determined or absent	> boundary value	> boundary value	≤ boundary value	**Equivocal*** If the result is repeated, it is concluded that *C.ulcerans tox* gene and low content of *C.diphtheriae* DNA and *C.diphtheriae tox* gene are detected. It is recommended to repeat the material sampling
determined or absent	absent	> boundary value	≤ boundary value	**Equivocal*** If the result is repeated, it is concluded that low content of *C.diphtheriae* DNA, containing gene which is similar to *C.ulcerans tox* gene are detected. It is recommended to repeat the material sampling
determined or absent	> boundary value	absent	≤ boundary value	**Equivocal*** If the result is repeated, it is concluded that low content of *C.pseudotuberculosis* DNA containing *C.diphtheria tox* gene is detected. *C.ulcerans tox* gene is detected. It is recommended to repeat the material sampling
≤ boundary value	≤ boundary value	absent	absent	**Equivocal*** If the result is repeated, it is concluded that *C.pseudotuberculosis* DNA containing *C.diphtheriae tox* gene or low content of *C.diphtheriae* DNA containing *C.diphtheriae tox* gene is detected. It is recommended to repeat the material sampling
determined or absent	> boundary value	≤ boundary value	≤ boundary value	**Equivocal*** If the result is repeated, it is concluded that *C.ulcerans tox* gene, *C.diphtheriae* DNA and low content of *C.diphtheriae tox* gene are detetcted. It is recommended to repeat the material sampling
determined or absent	≤ boundary value	absent	≤ boundary value	**Equivocal*** If the result is repeated, it is concluded that *C.pseudotuberculosis* DNA containing *C.diphtheriae tox* gene is detected. *C.ulcerans tox* gene is detected
determined or absent	≤ boundary value	> boundary value	≤ boundary value	**Equivocal*** If the result is repeated, it is concluded that *C.ulcerans* tox gene and low content of *C.diphtheriae* DNA containing *C.diphtheriae tox* gene are detected. It is recommended to repeat the material sampling
determined or absent	≤ boundary value	absent	absent or > boundary value	**Equivocal*** If the result is repeated, it is concluded that *C.pseudotuberculosis* DNA containing *C.diphtheriae tox* gene
determined or absent	≤ boundary value	> boundary value	absent or > boundary value	**Equivocal*** If the result is repeated, it is concluded that low content of *C.diphtheriae* DNA containing *C.diphtheriae tox* gene is detected. It is recommended to repeat the material sampling
≤ boundary value	absent	> boundary value	absent or > boundary value	**Equivocal*** If the result is repeated, it is concluded that *C.diphtheriae* DNA is detected
≤ boundary value	> boundary value	> boundary value	absent or > boundary value	**Equivocal*** If the result is repeated, it is concluded that low content of *C.diphtheriae* DNA and *C.diphtheriae tox* gene is detected

[2] For example, Rotor-Gene Q (QIAGEN, Germany).
[3] For example, CFX 96 (Bio-Rad).

***** In case of **invalid/equivocal result** it is necessary to repeat PCR-study of the corresponding test sample, starting from the DNA extraction stage.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The results of the analysis is considered reliable only if the results obtained for controls of amplification and extraction stages are correct (according to Table 7 and the *Important Product Information Bulletin* enclosed to the PCR kit).

Table 7

Results for controls

Control	Stage for control	*Ct* value in the channel for the fluorophore			
		FAM	JOE	ROX	Cy5
C-	DNA extraction	< boundary value	absent	absent	absent
NCA	PCR	absent	absent	absent	absent
C+	PCR	< boundary value	< boundary value	< boundary value	< boundary value

10. TROUBLESHOOTING

Results of analysis are not taken into account in the following cases:

1. The *Ct* value determined for the Positive Control of Extraction (PCE) in any of the channels (see Table 5) is greater than the boundary *Ct* value or absent. Amplification and detection should be repeated for all the samples in which the specific DNA was not detected.
2. The *Ct* value is determined for the Negative Control of Extraction (C–) in the channels for ROX and/or JOE and/or Cy5 fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected.
3. The *Ct* value is determined for the Negative Control of amplification (NCA) in the channels for the FAM and/or JOE and/or ROX and/or Cy5 fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The amplification and detection should be repeated for all samples in which specific DNA was detected.
4. The *Ct* value is determined for the test sample, whereas the area of typical exponential growth of fluorescence is absent (the graphic looks like approximate straight line). It is necessary to check the correctness of selected threshold line level or parameters of base line calculation. If the result has been obtained with the correct level of threshold line (base line), the amplification and detection should be repeated for this sample.

If you have any further questions or if you encounter problems, please contact our Authorized representative in the European Community.

11. TRANSPORTATION

AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT PCR kit should be transported at 2–8 ºC for no longer than 5 days. PCR kit can be transported at 2–25 °C for no longer than 3 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-buffer-B, polymerase (TaqF) and PCR-mix-FL *C.diphtheriae* / tox genes from PCR kit variant FRT-100 F). All components of the **AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT** PCR kit are stable until the expiry date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-buffer-B, polymerase (TaqF) and PCR-mix-FL *C.diphtheriae* / tox genes are to be stored at the temperature from minus 24 to minus 16 °C

NOTE: PCR-mix-FL *C.diphtheriae* / tox genes is to be kept away from light

NOTE: PCR-mix *C.diphtheriae* / tox genes-Lyo is to be kept in packages with a desiccant away from light

13. SPECIFICATIONS

13.1. Analytical sensitivity (limit of detection)

Table 8

Test material	Pathogen	Nucleic acid extraction kit	PCR kit	Analytical sensitivity, (limit of detection), GE/ml[4]
Naso- and oropharyngeal swabs, swabs from the affected areas of skin	*Corynebacterium diphtheriae*	RIBO-prep	variant FRT-100 F, variant FRT-L	1000
	Corynebacterium diphtheria tox gene			
	Corynebacterium ulcerans tox gene			

The claimed features are achieved while respecting the rules specified in the section *Sampling and Handling*

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes have been checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The PCR kit detects DNA fragments of claimed test microorganisms of the following strains from NCTC® collection (National Collection of Type Cultures Diphtheriae Reference Laboratory, Central Health Laboratory (CPHL), UK) with concentration no more than $1x10^3$ GE/ml:

- *Corynebacterium diphtheriae* NCTC® №10356 (tox-) – *C.diphtheriae* DNA is detected, genes encoding *C.diphtheriae* and *C.ulcerans* toxins are not detected;
- *Corynebacterium diphtheriae* NCTC® №10648 (tox+) – *C.diphtheriae* DNA and gene encoding *C.diphtheriae* toxin are detected, gene encoding *C.ulcerans* toxin is not detected;
- *Corynebacterium ulcerans* NCTC® №7910 (tox+) – gene encoding *C.ulcerans* toxin is detected, *C.diphtheriae* DNA and gene encoding *C.diphtheriae* toxin are not detected.

The analytical specificity was proved on the following microorganisms as well as on human DNA:

— Strains from ATCC® collection (American Type Culture Collection, USA) and GCPM collection (State collection of pathogenic microorganisms Ministry of Health of Russia) with concentration no less than $1x10^6$ GE/ml: *Corynebacterium xerosis* ATCC® 7711D-5™, *Corynebacterium urealyticum* ATCC® 43044™, *Corynebacterium amycolatum* ATCC® 49368™, *Corynebacterium jeikeium* ATCC® 43734™, *Corynebacterium pseudodiphtheriticum* 25;

— Strains from ATCC® (American Type Culture Collection, USA), NCTC® (National Collection of Type Cultures, UK) and GCPM (State collection of pathogenic microorganisms of the Ministry of Health of Russia) with concentration no less than $1x10^6$ GE/ml: *Bordetella pertussis* 703 L 6, *Streptococcus pneumoniae* ATCC® 49619™, *Streptococcus agalactiae* ATCC® 13813™, *Streptococcus pyogenes* Dick – I, *Staphylococcus saprophyticus* ATCC® 15305™, *Haemophilus influenzae* 423, *Proteus mirabilis* 3177, *Klebsiella pneumoniae* 418, *Pseudomonas aeruginosa* ATCC® 9027™, *Neisseria flava* ATCC® 14221™, *Neisseria sicca* 5, *Neisseria mucosa* ATCC® 19693™, *Escherichia coli* M 17, *Salmonella enteritidis* 5765, *Salmonella typhimurium* 79, *Moraxella catarrhalis* ATCC® 8193™, *Yersinia enterocolitica* 134, *Enterococcus faecalis* NCTC® 775, *Staphylococcus aureus* ATCC® 6538-P, *Mycobacterium bovis* Ravenel 700204.

— Human DNA with concentration 1 mg/ml.

The nonspecific responses were not observed while testing the DNA samples of the above mentioned microorganisms, as well as human DNA.

The clinical specificity of **AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT** PCR kit was confirmed in laboratory clinical trials.

The information about known interfering substances is specified in the *Interfering substances and limitations of using test material samples*.

13.3. Reproducibility

Reproducibility was determined in two independent laboratories by different operators in different days using different instruments by testing samples containing *C.diphtheriae* DNA, *C.diphtheriae tox* gene and *C.ulcerans tox* gene. The final concentration was $1x10^3$ GE/ml. Results of claimed DNA fragments detection coincided for all repeaters of test samples.

13.3. Diagnostic characteristics

Table 8

The results of testing AmpliSens®*Corynebacterium diphtheriae* / tox-genes-FRT PCR kit in comparison with the reference assay

Samples type	The results of application of AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT PCR kit		Results of using the reference assay[5]	
			Positive	Negative
Naso- and oropharyngeal swabs	300 samples were tested	**Positive**	150	0
		Negative	0	150
Swabs from the affected areas of skin	300 samples were tested	**Positive**	150	0
		Negative	0	150

Table 9

The results of testing AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT PCR kit

Samples type	Sample description	Number of samples, pcs	Result	
			Positive	Negative
Naso- and oropharyngeal swabs	Biological material containing *Corynebacterium diphtheriae* DNA and *Corynebacterium diphtheriae* toxin gene	50	50	0
	Biological material containing *C.diphtheriae* DNA and not *C.diphtheriae* toxin gene	50	50	0
	Biological material containing DNA encoding *Corynebacterium ulcerans* toxin gene	50	50	0
	Biological material not containing *Corynebacterium diphtheria* DNA, *Corynebacterium diphtheriae* toxin gene and Corynebacterium *ulcerans* toxin gene	150	0	150
Swabs from the affected areas of skin	Biological material containing *Corynebacterium diphtheriae* DNA and *Corynebacterium diphtheriae* toxin gene	50	50	0
	Biological material containing *C.diphtheriae* DNA and not *C.diphtheriae* toxin gene	50	50	0
	Biological material containing DNA encoding *Corynebacterium ulcerans* toxin gene	50	50	0
	Biological material not containing *Corynebacterium diphtheria* DNA, *Corynebacterium diphtheriae* toxin gene and Corynebacterium *ulcerans* toxin gene	150	0	150

The test material was naso- and oropharyngeal swabs, swabs from the affected areas of skin negative in the study with the comparison kit, and model samples of clinical material contaminates with *Corynebacterium diphtheriae* NCTC® №10356™ (tox-), *Corynebacterium diphtheriae* NCTC® №10648™ (tox+) and *Corynebacterium ulcerans* NCTC® №7910™ (tox+) microorganism strains with concentration $1×10^3$ GE/ml, simulating biological material from diphtheria patients and pathogen carriers.

Table 10

Diagnostic characteristics of AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT PCR kit

Sample type	Diagnostic sensitivity, (with a confidence level of 95 %) in the interval (%)	Diagnostic specificity, (with a confidence level of 95 %) in the interval (%)
Naso- and oropharyngeal swabs	92.9 – 100	97.6 – 100
Swabs from the affected areas of skin	92.9 – 100	97.6 – 100

[4] Number of genome equivalents (GE) of the microorganism per 1 ml of the test material sample.

[5] DS-DIF-CORYNE (RCP «Diagnostic systems», Ltd) biochemical test-system for differentiation of bacteria from *Corynebacterium* genus (as well as diphtheria pathogen) and determination of its toxigenic properties was used as reference method.

14. REFERENCES

1. Berger A., Hogardt M., Konrad R., Sing A. Detection methods for laboratory diagnosis of diphtheria//Corynebacterium diphtheriae and related toxigenic species. – Springer Netherlands, 2014. – P. 171-205.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of the **AmpliSens® *Corynebacterium diphtheriae* / tox-genes-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

AmpliSens®

EC REP

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List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
14.05.18 PM	3. Content	The volume of the reagent was specified
30.05.18 PM	3. Content	The name of the reagent was specified
18.09.19 PM	Through the text	Text formatting was changed. Corrections according to the template. Test material was specified – "nasopharyngeal swabs, oropharyngeal swabs" were changed to "naso- and oropharyngeal swabs", "swabs from disease sites" were changed to "swabs from affected areas of skin", "germ culture" was deleted
	6. Sampling and handling	The information about sampling and transportation of nasopharyngeal and oropharyngeal swabs was specified. The information about germ culture was deleted. The "Interfering substances and limitations of using test material samples" subsection was rewritten
	8. Protocol	The information about buffer from RIBO-prep kit and PCR kit variant FRT-L was specified
	9. Data analysis	Results interpretation was presented in table form
	13. Specifications	The information in Analytical specificity subsection was changed. The Reproducibility subsection was added. Values for diagnostic sensitivity and specificity with a confidence level were changed
	14. References	The section was actualized
26.05.20 MA	Page Footer	Phrase "Not for use in the Russian Federation" was added
18.03.21 VA	—	The name, address and contact information for Authorized representative in the European Community was changed