IVD For *in Vitro* Diagnostic Use

CE

Corynebacterium diphtheriae / tox genes Real-TM

Handbook

Real Time PCR Kit for qualitative detection of Corynebacterium diphtheriae and gene encoding toxins differentiation of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans*

REF B2842-100FRT

Σ 100

NAME

Corynebacterium diphtheriae / tox-genes Real-TM

INTRODUCTION

Corynebacterium diphtheriae is the pathogenic bacterium that causes diphtheria. *C. diphtheriae* produces diphtheria toxin which alters protein function in the host by inactivating the elongation factor EF-2. This causes pharyngitis and 'pseudomembrane' in the throat. The diphtheria toxin gene is encoded by a bacteriophage found in toxigenic strains, integrated into the bacterial chromosome.

INTENDED USE

The **Corynebacterium diphtheriae / tox-genes Real-TM** is a Real-Time PCR test for the qualitative detection and differentiation of *Corynebacterium diphtheriae* and genes encoding toxins of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* in the biological material (nasopharyngeal swabs, oropharyngeal swabs, swabs from disease sites, germ culture).

PRINCIPLE OF ASSAY

Kit **Corynebacterium diphtheriae / tox-genes Real-TM** is based on two major processes: DNA is extracted from samples and amplified using real time amplification with fluorescent reporter dye probes specific for rpoB gene site of Corynebacterium diphtheria, gene encoding Corynebacterium diphtheria toxin, gene encoding Corynebacterium ulcerans toxin as well as amplification of Internal Control IC. The test contains an exogenous Internal Control (IC) which serves as an extraction and amplification control for each individually processed specimen and to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B2842-100FRT)

Part N° 1– **"Corynebacterium diphtheriae / tox-genes Real-TM":** Real Time amplification kit

- **PCR-mix-1 *C.diphtheriae* / tox genes**, 1,2 ml;
- **PCR-mix-2-FRT**, 0,6 ml;
- **TaqF Polymerase,** 0,06 ml;
- **Pos Control C+ *C.diphtheriae* / tox genes*,** 0,2 ml;
- **Negative Control C-****, 1,2 ml;
- **Internal Control IC***,** 1,0 ml;
- **DNA-buffer******, 0,2 ml;

Contains reagents for 110 tests.

** must be used as Positive Amplification Control during the amplification procedure;*
*** must be used in the isolation procedure as Negative Control of Extraction;*
**** add 10 µl of Internal Control during the DNA isolation directly to the sample/lysis mixture;*
***** DNA Buffer reagent must be used as Negative Amplification Control during the amplification procedure.*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Desktop microcentrifuge for "eppendorf" type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator, Freezer

Zone 2: Real Time amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

Corynebacterium diphtheriae / **tox-genes Real-TM** must be stored at **2-8°C** except the reagents **PCR-mix-1** ***C.diphtheriae*** / **tox genes**, **PCR-mix-2-FRT** and **TaqF Polymerase** that must be stored at **-20°C**. The kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

Corynebacterium diphtheriae / **tox-genes Real-TM** test is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

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

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Corynebacterium diphtheriae / **tox-genes Real-TM** can analyze extracted DNA from:

- Nasopharyngeal swabs;
- Oropharyngeal samples*;*
- Swabs from disease sites (eye, ear, wound, skin lesions, etc.);
- Germ culture.

It is recommended to process samples immediately after collection. Store samples at 2–8 °C for no longer than 24 hours, or freeze at –20/80°C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

- ⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006) for bacterial pellet/colony from culture and liquid transport media.

Please carry out DNA extraction according to the manufacture’s instruction. Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION

DNA/RNA Prep (Sacace, REF K-2-9) extraction protocol (reagent provided separately):

1. Prepare required number of 1.5 ml disposable polypropylene micro centrifuge tubes including one tube for Negative Control of Extraction (**Negative Control**, **C-**).
2. Add to each tube **300 µl** of **Lysis Sol** and **10 µl** of **Internal Control.**
3. Add **100 µl** of samples to the appropriate tubes using pipette tips with aerosol barriers.
4. Prepare Controls as follows:
    - add **100 µl** of **Negative Control C–** to the tube labeled C*neg*
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 7-10 sec.
6. Add **400 µl** of **Prec Sol** and mix by vortex. Centrifuge all tubes at 13,000 r/min for 5 min and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
7. Add **500 µl of Wash Sol 3** into each tube. Vortex vigorously to ensure pellet washing. Centrifuge all tubes at 13,000 r/min for 60 sec and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
8. Add **200 µl of Wash Sol 4** into each tube. Vortex vigorously to ensure pellet washing. Centrifuge all tubes at 13,000 r/min for 60 sec and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
9. Incubate all tubes with open caps at **65 °C for 5 min**.
10. Resuspend the pellet in **50 µl of RE-buffer** (elution volume can be increased up to 90 µl). Incubate for 5 min at 65°C and vortex periodically.
11. Centrifuge the tubes at 13000g for 60 sec.

The supernatant contains RNA/DNA ready for amplification. If amplification is not performed the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at - 20°/-80°C.

PCR PROTOCOL (Reaction volume 25 µl):

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

1 Prepare required quantity of PCR reaction tubes for samples and controls (N).

2 Prepare in the new sterile tube for each sample **10*(N+1) µl** of **PCR-mix-1 *C.diphtheriae* / tox genes**, **5,0*(N+1) µl** of **PCR-mix-2-FRT** and **0.5*(N+1) µl** of **TaqF Polymerase.** Prepare the Reaction Mix just before its use. Vortex and centrifuge for 2-3 sec.

3 Add to each tube **15 µl** of **Reaction Mix.**

4 Add **10 µl** of **extracted DNA** sample to the appropriate PCR tube containing the Reaction Mix. Mix by pipetting.

5 Prepare for each session 3 controls:

- add **10 µl** of extracted **Negative Control C-** to the PCR tube labeled Negative Control of Extraction;
- add **10 µl** of **DNA-buffer** to the PCR tube labeled Negative Amplification Control;
- add **10 µl** of **Pos Control C+ *C.diphtheriae* / tox genes** to the PCR tube labeled Positive Amplification Control.

6. Insert the tubes in the thermalcycler.

AMPLIFICATION

1. Create a temperature profile on your rotor-type[1] or plate-type[2] instrument as follows:

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of cycles*
Hold	50	15 min	–	1
Hold	95	15 min	–	1
Cycling	95	10 s	–	45
	60	20 s	FAM, JOE, ROX, Cy5	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)
[2] For example, SaCycler-96™ (Sacace), iQ5™, CFX™ (BioRad); Mx3000P™/3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied), SmartCycler® (Cepheid)

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 3000/6000, RotorGene Q)

Channel	Calibrate/Gain Optimisation…	Threshold	Dynamic tube	Slope Correct	More Settings/ Outlier Removal
FAM/Green	from 5Fl to 10Fl	0,1	on	on	5 %
JOE/Yellow	from 5Fl to 10Fl	0,1	on	on	5 %
ROX/Orange	from 5Fl to 10Fl	0,1	on	on	5 %
Cy5/Red	from 5Fl to 10Fl	0,1	on	on	5 %

Plate-type instruments (SaCycler-96, iQ, CF, Mx3000/3005, ABI 7500

Channel	Threshold
FAM	Set the threshold line at the level corresponding to 10-20 % of maximum fluorescence level obtained for C+ sample at the last amplification cycle
JOE/HEX	Set the threshold line at the level corresponding to 10 % of maximum fluorescence level obtained for C+ sample at the last amplification cycle
ROX	
Cy5	

RESULTS ANALYSIS:

The targets are detected by four different fluorescence channels as specified in the table below:

Fluorescence channel	FAM	JOE	ROX	Cy5
Target	Internal Control	*Corynebacterium diphtheria tox* gene	*Corynebacterium diphtheria rpo B* gene	*Corynebacterium ulcerans tox* gene

The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line and interpreted as follow:

- ***Corynebacterium diphtheriae*** **DNA is detected (*C. diphtheriae* specie is identified)**, if the *Ct* value detected in FAM and ROX channel is less than the boundary *Ct* value. Moreover, the fluorescence curves of the sample should cross the threshold line with a typical exponential growth of fluorescence.
- ***Corynebacterium diphtheriae*** **DNA containing *C. diphtheriae* tox gene is detected**, **(identification of potentially toxicogenic *C. diphtheriae*)** if the *Ct* value in FAM, ROX and JOE channels is less than the boundary *Ct* value. Moreover, the fluorescence curve of the sample should cross the threshold line with a typical exponential growth of fluorescence.
- ***Corynebacterium diphtheriae*** **DNA is not detected** in a sample if the *Ct* value is not detected (absent) in ROX channel (the fluorescence curve does not cross the threshold line), whereas the *Ct* value in FAM channel is less than the boundary *Ct* value.
- ***Corynebacterium diphtheriae*** **tox gene is not detected** in a sample if the *Ct* value is not detected (absent) in JOE channel (the fluorescence curve does not cross the threshold line), whereas the *Ct* value in FAM channel is less than the boundary *Ct* value.
- ***Corynebacterium ulcerans*** **tox gene is detected** in a sample if the *Ct* value in Fam and Cy5 channels is less than the boundary *Ct* value, whereas the *Ct* value in ROX channel is not detected (absent). Moreover, the fluorescence curve of the sample should cross the threshold line with a typical exponential growth of fluorescence.
- ***Corynebacterium ulcerans*** **tox gene is not detected** in a sample if the *Ct* value is not detected (absent) in Cy5 channel (the fluorescence curve does not cross the threshold line), whereas the *Ct* value in FAM channel is less than the boundary *Ct* value.

- If the *Ct* value detected in FAM, ROX and Cy5 channels is less than the boundary *Ct* value, is detected ***Corynebacterium diphtheriae*** **DNA containing pseudogene which is similar to the *Corynebacterium ulcerans* tox gene**.
- If the *Ct* value detected in JOE channel is less than the boundary *Ct* value, and the *Ct* value is not detected (absent) in ROX channel, whereas the *Ct* value detected in FAM channel is less than the boundary *Ct* value, the PCR analysis should be repeated for the specific clinical sample starting from the DNA extraction stage. If it is obtained the same result, the conclusion is that is supposedly detected ***Corynebacterium pseudotuberculosis*** **DNA containing *Corynebacterium diphtheriae* tox gene.**
- The result is **invalid** if the *Ct* value in ROX and FAM channels is not detected (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated for the specific clinical sample starting from the DNA extraction stage. If it is obtained the same result, re-sampling of material is recommended.
- The result is **equivocal** if for a specific clinical sample**:**
  a) the *Ct* value detected in JOE channel is greater than the boundary *Ct* value, whereas the *Ct* value is absent in ROX and Cy5 channels, and the *Ct* value detected in FAM channel is less than the boundary *Ct* value. In such cases, re-sampling of material is recommended. If it is obtained the same result or the *Ct* value detected in JOE channel is less than the boundary value, the sample is positive.
  b) the *Ct* values detected in JOE and Cy5 channels are greater than the boundary *Ct* value, whereas the *Ct* value is absent in ROX channel, and the *Ct* value detected in FAM channel is less than the boundary *Ct* value. In such cases, re-sampling of material is recommended. If it is obtained the same result or the *Ct* values detected in JOE and/or Cy5 channels are less than the boundary value, the sample is considered positive for the respective channels.
  c) the *Ct* values detected in ROX and/or JOE and/or Cy5 channel are greater than the boundary *Ct* value, whereas the *Ct* value detected in FAM channel is less than the boundary *Ct* value. In such cases, the PCR analysis should be repeated for the specific clinical sample starting from the DNA extraction stage. If it is obtained the same result or the *Ct* values detected in ROX and/or JOE and/or Cy5 channels are less than the boundary value, the sample is considered positive for the respective channels.

Table for boundary values of control samples:

Sample	Rotor-type instruments				Plate-type instruments			
	Channel for fluorophore							
	FAM	JOE	ROX	Cy5	FAM	JOE	ROX	Cy5
NCA	-	-	-	-	-	-	-	-
C-	< 35	-	-	-	< 38	-	-	-
C+	< 33	< 33	< 33	< 33	< 35	< 35	< 35	< 35

Table for results interpretation and boundary values of clinical samples:

***Ct* value in the channel for the fluorophore**					**Result**
FAM		**JOE**	**ROX**	**Cy5**	
Internal Control		*Corynebacterium diphtheriae* (*tox C. Diphtheria* gene)	*Corynebacterium diphtheria (rpo B* gene*)*	*Corynebacterium ulcerans* (*tox C. Ulcerans gene*)	
Rotor-type instruments	**Plate-type instruments**	**Rotor- and plate-type instruments**			
Determined or absent		Absent	≤ 42	Absent or > 42	*C.diphtheriae* **DNA is detected**
Determined or absent		≤ 42	≤ 42	Absent or > 42	*C.diphtheriae* **DNA** containing *C.diphtheriae* tox gene **is detected**
Determined or absent		≤ 42	≤ 42	≤ 42	*C.diphtheriae* **DNA** containing *C.diphtheriae* tox gene **is detected**. *C.ulcerans* tox gene **DNA is detected**
Determined or absent		Absent	≤ 42	≤ 42	*C.diphtheriae* **DNA** containing gene similar to the *C.ulcerans* tox gene **is detected**
Determined or absent		Absent	Absent	≤ 42	*C.diphtheriae* **DNA is NOT detected**. *C.ulceransgene* tox gene **DNA is detected**
≤ 35	≤ 38	Absent	Absent	Absent or > 42	*C.diphtheriae* DNA, *C.diphtheriae* tox gene DNA, *tox C.ulcerans* tox gene DNA **are NOT detected**
Absent or > 35	Absent or > 38	Absent or > 42	Absent or > 42	Absent or > 42	**Invalid***
Determined or absent		> 42	≤ 42	Absent or > 42	**Equivocal***
Determined or absent		> 42	> 42	≤ 42	**Equivocal***
Determined or absent		Absent	> 42	≤ 42	**Equivocal***
Determined or absent		> 42	Absent	≤ 42	**Equivocal***
≤ 35	≤ 38	≤ 42	Absent	Absent	**Equivocal***
Determined or absent		> 42	≤ 42	≤ 42	**Equivocal***
Determined or absent		≤ 42	Absent	≤ 42	**Equivocal***
Determined or absent		≤ 42	> 42	≤ 42	**Equivocal***
Determined or absent		≤ 42	Absent	Absent or > 42	**Equivocal***
Determined or absent		≤ 42	> 42	Absent or > 42	**Equivocal***
≤ 35	≤ 38	Absent	> 42	Absent or > 42	**Equivocal***
≤ 35	≤ 38	> 42	> 42	Absent or > 42	**Equivocal***

***** In case of **invalid/equivocal result** see the procedure as described in the above section **RESULTS ANALYSIS**

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific pathogen's primers and probes. The specificity of the kit was 100%. The potential cross-reactivity of the kit was tested against the group control of the following microrganisms: *Corynebacterium pseudodiphtheriticum, Corynebacterium pseudotuberculosis, Corynebacterium ulcerans (*when identifying *C.diphtheriae), Corynebacterium xerosis, Corynebacterium urealiticum, Corynebacterium amycolatum, Corynebacterium jeikeium, Streptococcus spp., Moraxella catarrhalis, Staphilococcus aureus, Staphilococcus saprophiticus, Haemophilus influenzae, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Mycobacteria tuberculosis 27294 105, Neisseria flava, Neisseria sicca, Neisseria mucosa, E. coli ATCC, NCTC, 01577 27u7, Enterococcus faecalis, Mycoplasma pneumoniae, Chlamydophila pneumoniae, Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Legionella pneumophila, Shigella flexneri, Shigella sonnei, Salmonella Enteritidis, Yersinia enterocolitica* and also human genomic DNA

It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **Corynebacterium diphtheriae** / **tox-genes Real-TM** allows to detect *Corynebacterium diphteriae* and tox genes DNA in 100% of the tests with a sensitivity of not less than 1000 GE/ml*.

Test material	Pathogen	Nucleic acid extraction kit	Analytical sensitivity, (limit of detection), GE/ml[1]
Nasopharyngeal swabs, oropharyngeal swabs, swabs from disease sites	*Corynebacterium diphtheriae*	DNA/RNA Prep	1000
	Corynebacterium diphtheria tox gene		
	Corynebacterium ulcerans tox gene		

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

Target gene:

Fluorescence channel	FAM	JOE	ROX	Cy5
DNA-target	Internal Control	*Corynebacterium diphtheriae* toxin DNA	*Corynebacterium diphtheriae* DNA	*Corynebacterium ulcerans* toxin DNA
Target gene	DNA synthetic sequence	*gene tox C. diphtheriae*	*gene rpo B*	*gene tox C. ulcerans*

TROUBLESHOOTING

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

1. The *Ct* value detected for the Positive Control of Extraction (PCE) in any of the channels 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 detected for the Negative Control of Extraction (C–) in ROX and/or JOE and/or Cy5 channels. Probably contamination of laboratory with amplification fragments or contamination of reagents. Take appropriate measures for detecting and elimination the contamination source. 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 detected for the Negative Control of amplification (NCA) in FAM and/or JOE and/or ROX and/or Cy5 channels. Probably contamination of laboratory with amplification fragments or contamination of reagents. Take appropriate measures for detecting and elimination of contamination source. The amplification and detection should be repeated for all samples in which specific DNA was detected.
4. The *Ct* value is detected for the clinical sample, whereas the typical exponential growth of fluorescence is absent (the graphic looks like approximate straight line). It is necessary to check the setup of the threshold line level or other parameters. 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.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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