For Professional Use Only

Clostridium difficile + ToxA + ToxB Real-TM

Handbook

Real Time PCR Kit for qualitative detection of Clostridium difficile and gene encoding toxins

REF B1972-100FRT

100

NAME

Clostridium difficile + ToxA + ToxB Real-TM

INTRODUCTION

C difficile is a ubiquitous bacterium found in soil, hospital environments, child care facilities and nursing homes. It is a spore-forming, Gram-positive bacillus, which can spread via the fecal-oral route; patient-to-patient transmission has been well documented within hospitals.

Important pathophysiological features of *C difficile* include heat-resistance of the spore (allowing environmental persistence) and toxin production. Two toxins (A and B) can be produced causing as a final result fluid secretion in the intestine, mucosal damage and interstitial inflammation.

INTENDED USE

The **Clostridium difficile + ToxA + ToxB Real-TM** is a Real-Time PCR test for the qualitative detection of *Clostridium difficile* and differentiation of genes encoding toxins in the biological material (feces) and water.

PRINCIPLE OF ASSAY

Kit **Clostridium difficile + ToxA + ToxB 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 *Clostridium difficile*, genes encoding toxin A and B as well as the Internal Control IC. The test detects an endogenous human sequence as Internal Control (IC) which serves as an sampling, extraction and amplification control for each individually processed specimen and to identify any possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B1972-100FRT)

Part N° 1– **"Clostridium difficile + ToxA + ToxB Real-TM":** Real Time amplification kit

- **PCR-mix-1 *Clostridium diff* + ToxA + ToxB**, 0,960 ml;
- **Diluent**, 2,0 ml x 2;
- **TaqF Polymerase,** 0,05 ml;
- **Pos Control C+ *Clostridium diff* + ToxA + ToxB *,** 0,2 ml;
- **Exogenous Internal Control,** 1 ml

Contains reagents for 110 tests.

** must be used as Positive Amplification Control during the amplification procedure;*
*** add 10 µl of the exogenous internal control to the sample/lysis mixture at the beginning of the extraction process*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit
- 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

Clostridium difficile + ToxA + ToxB Real-TM must be stored at **-20°C**. The kit can be shipped at 2-8°C but should be stored at -20°C immediately on receipt.

STABILITY

Clostridium difficile + ToxA + ToxB 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

For Professional 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

Clostridium difficile + ToxA + ToxB Real-TM can analyze extracted DNA from:

- *Liquid cultures;*
- *water:* centrifuge 10-20 ml for 10 min at maximum speed. Discard the supernatant and leave about 100 µl of solution for DNA extraction;
- *feces:*
  - Prepare 20% feces suspension by adding in 5 ml tube of 4ml of Saline Solution and 1,0 gr (approx. 1,0 ml) of feces. Vortex to get the homogeneous suspension and centrifuge for 5 min to 7000-12000g and using a micropipette with a plugged aerosol barrier tip transfer in a new sterile 1,5 ml tube 100 µl of the bacterial fraction (white-yellowish line between the sediment and the supernatant)
  - Add 800 µl of PBS or Saline Solution. Vortex to get the homogeneous suspension and centrifuge for 5 min to 7000-12000g. Remove and discard the supernatant
  - Resuspend the pellet in 0,3 ml of PBS or Saline Solution.

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 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-Sorb-B** (Sacace, REF K-1-1/B);
- ⇨ **DNA/RNA-Prep** (Sacace, REF K-2-9);
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006).

Please carry out DNA extraction according to the manufacture’s instruction. Add 10 µl of Exogenous Internal Control to the sample/lysis mixture at the beginning of the extraction process.

SPECIMEN AND REAGENT PREPARATION

DNA-Sorb-B (Sacace, REF K-1-1/B) extraction protocol (reagent provided separately):

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60°C until disappearance of ice crystals.
2. Prepare required quantity of 1.5 ml polypropylene tubes.
3. Add to each tube **10 µl** of **Exogenous Internal Control** and **300 µl** of **Lysis Solution**.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Vortex the tubes, incubate 5 min at 65°C and centrifuge for 5 sec.
6. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically
8. Centrifuge all tubes for 1 min at 5000g 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 tubes.
9. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and centrifuge for 1 min at 5000g 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 tubes.
10. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 1 min at 10000g 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 tubes.
11. Repeat step 10.
12. Incubate all tubes with open cap for 5 min at 65°C.
13. Resuspend the pellet in **50 µl** of **DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
14. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. The amplification can be performed on the same day of extraction.

PROTOCOL (Reaction volume 25 µl):

Total reaction volume is **35 µl**, the volume of DNA sample is **7 µl**.

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

2 Prepare in the new sterile tube for each sample **7*(N+1) µl** of **PCR-mix-1 *Clostridium diff* + ToxA + ToxB**, **21*(N+1) µl** of **Diluent** and **0.3*(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 **28 µl** of **Reaction Mix.**

4 Add **7 µl** of **extracted DNA** sample to the appropriate PCR tube containing the Reaction Mix. Mix by pipetting.

5 Prepare for each session 2 controls:

- add **7 µl** of **Diluent** to the PCR tube labeled Negative Amplification Control;
- add **7 µl** of **Pos Control C+ *Clostridium diff* + ToxA + ToxB** to the PCR tube labeled Positive Amplification Control.

6. Insert the tubes in the thermalcycler.

AMPLIFICATION

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

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
Hold	95	90 sec	–	1
Cycling	95	15 sec		40
	60	30 sec	FAM(Green), JOE(Yellow), ROX(Orange), Cy5(Red)	
	72	40 sec		

For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
Hold	94	90 sec	–	1
Cycling	94	10 sec		40
	64	25 sec	FAM(Green), JOE(Yellow), ROX(Orange), Cy5(Red)	
	72	20 sec		

For example, SaCycler-96™ (Sacace), iQ5™, CFX™ (BioRad); Mx3000P™/3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied).

INSTRUMENT SETTINGS

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

	Dynamic Tube				**on**
	Slope correct				**on**
	Ignore First				**5**
	Eliminate Cycles before				**10**
	Channel	**Outlier Removal**		**Threshold**	**Finishing cycle (FC)**
		NTC Threshold	**Reaction Efficiency threshold**		
Toxin A	Green	10%	off	0,01	35
Toxin B	Yellow	20%	off	0,01	35
Clostridium difficile	Orange	10%	off	0,01	35
Internal control	Red	15%	off	0,02	35

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	Toxin A	Toxin B	*Clostridium difficile*	*Internal Control*

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

	Ct FAM (Green) Toxin A	**Ct HEX (Yellow) Toxin B**	**Ct ROX (Orange) *C. difficile***	**Ct Cy5 (Red) IC**	**Result**
PC +	Ct defined	Ct defined	Ct defined	Ct defined	**Valid**
	Ct absent	**Ct absent**	**Ct absent**	**Ct absent**	**Invalid**. It is required to repeat the analysis.
NC-	**Ct absent**	**Ct absent**	**Ct absent**	**Ct absent**	**Valid**
	Ct defined			**Ct absent**	**Invalid**. Contamination by *Clostridium difficile DNA*. It is required to repeat the analysis.
	Ct absent	**Ct absent**	**Ct absent**	**Ct defined**	**Invalid**. Contamination by IC. It is required to repeat the analysis.
Clinical samples	**Ct absent**	**Ct absent**	**Ct defined**	**Ct defined**	**PRESENCE** of *Clostridium difficile* DNA. **ABSENCE** of Toxin A and Toxin B genes.
	Ct defined	**Ct defined**	**Ct defined**	**Ct defined**	**PRESENCE** of *Clostridium difficile* DNA. **PRESENCE** of Toxin A and Toxin B genes.
	Ct absent	**Ct absent**	**Ct absent**	**Ct defined**	**ABSENCE** of *Clostridium difficile* DNA. **ABSENCE** of Toxin A and Toxin B genes.
	Ct absent	**Ct absent**	**Ct absent**	**Ct absent**	Inhibition of PCR reaction or improper DNA isolation. It is required to repeat the analysis for the specific clinical sample.

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 **Clostridium difficile + ToxA + ToxB Real-TM** allows to detect *Clostridium difficile* and tox genes DNA in 100% of the tests with a sensitivity not less than 1000 copies/ml.

Target regions

Clostridium difficile: 23S rRNA
Toxin A: toxA gene
Toxin B: toxB gene

TROUBLESHOOTING

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

1. The *Ct* value for the Positive Control of Amplification (PCA) is absent in any of the channels. 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 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.
3. 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
RUO	For Research Use Only	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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