Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

Cronobacter sakazakii Real-TM

Handbook

Real Time PCR Kit for detection of Cronobacter sakazakii

REF B58-50FRT

REF TB58-50FRT

Σ **50**

NAME

Cronobacter sakazakii Real – TM

INTRODUCTION

Acute Intestinal Infection (A.I.I.) are one of the primary causes of hospitalization in infectious disease departments. In accordance with the data provided by the contemporary literature, the most often detectable and generally spread etiological agents of A.I.I. are bacterial microorganisms such as *Shigella spp*. and enteroinvasive *E. coli* (EIEC), *Salmonella spp*., thermophilic group of Campylobacter spp., enteropathogenic E.coli (EPEC) and enteroaggregative E. coli (EAEC) and viral agents such as group A rotaviruses, genotype 2 noroviruses, group F adenoviruses (type 40 and 41) and astroviruses.

INTENDED USE

The **Cronobacter sakazakii Real - TM** is a Real-Time PCR test for the qualitative detection of *Cronobacter sakazakii* in the liquid cultures, water and feces.

PRINCIPLE OF ASSAY

Kit **Cronobacter sakazakii Real - TM** is based on two major processes: isolation of DNA extracted from samples and amplification by real time PCR with fluorescent reporter dye probes specific for *Cronobacter sakazakii* and Internal Control IC. Test contains an (IC) which serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B58-50FRT)

Part N° 2– **"Cronobacter sakazakii Real - TM":** Real Time amplification kit

- **PCR-mix-1.**, 0,6 ml;
- **PCR-mix-2-Flu**, 0,3 ml;
- **TaqF Polymerase,** 0,03 ml;
- **Positive Control** *Cronobacter sakazakii* ***IIC,*** 0,1 ml;
- **Negative Control C-**, 1,2 ml;*
- **Internal Control IC,** 1,0 ml;**
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TB58-50FRT)

Part N° 1 – **"DNA-Sorb-B":** Sample preparation kit

- **Lysis Solution**, 15 ml;
- **Washing Solution 1**, 15 ml;
- **Washing Solution 2,** 50 ml;
- **Sorbent**, 1,25 ml;
- **DNA-eluent**, 5,0 ml.

Contains reagents for 50 extractions

Part N° 2– **"Cronobacter sakazakii Real - TM":** Real Time amplification kit

- **PCR-mix-1.**, 0,6 ml;
- **PCR-mix-2-Flu**, 0,3 ml;
- **TaqF Polymerase,** 0,03 ml;
- **Positive Control** *Cronobacter sakazakii* ***IIC,*** 0,1 ml;
- **Negative Control C-**, 1,2 ml;*
- **Internal Control IC,** 1,0 ml;**
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

**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 (see DNA-Sorb-B REF K-1-1/B protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (module No. 1)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- 60°C ± 5°C dry heat block
- Vortex mixer
- Pipettors (capacity 5-40 µl; 40-200 µl; 200-1000 µl) with aerosol barrier
- 1,5 ml polypropylene sterile tubes
- Disposable gloves, powderless
- Biohazard waste container

Zone 2: RT and amplification:

- Real Time Thermalcycler
- Workstation
- Pipettes
- Tips
- Tube racks

STORAGE INSTRUCTIONS

Part N° 1 – **“DNA-Sorb-B”** must be stored at 2-8°C.

Part N° 2 – **Cronobacter sakazakii Real - TM** must be stored at -20°C.

The complete kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

Cronobacter sakazakii 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:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- 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.

**** Only for Module No.2***

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

Cronobacter sakazakii Real - TM can analyze DNA extracted 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;
- *whole blood* collected in EDTA tubes*;*
- *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 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-Sorb-B** (Sacace, REF K-1-1/B);
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006).

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*

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 **300 µl** of **Lysis Solution** and **10 µl** of **IC**.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control**) to labeled C*neg*.
6. Vortex the tubes, incubate 5 min at 65°C and centrifuge for 5 sec.
7. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
8. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
9. 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.
10. 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.
11. 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.
12. Repeat step 11.
13. Incubate all tubes with open cap for 5 min at 65°C.
16. Resuspend the pellet in **50 µl** of **DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
17. 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.

**** Only for Module No.2***

PROTOCOL (Reaction volume 25 µl):

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

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

2 Prepare the reaction mix for required number of samples.

3 For N reactions mix in a new tube:

10*(N+1) µl of RT-PCR-mix-1

5.0*(N+1) µl of PCR-mix-2

0.5*(N+1) µl of TaqF Polymerase

4 Vortex the tube, then centrifuge shortly. Add **15 µl** of prepared reaction mix into each appropriate tube.

5 Using tips with aerosol filter add **10 µl** of DNA samples obtained at the stage of DNA isolation and mix carefully by pipetting**.**

N.B. If the DNA-Sorb isolation kit is used as a DNA extraction kit, re-centrifuge all the tubes with extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction

6 Prepare for each panel the following controls:

- add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
- add **10 µl** of **Positive Control** *Cronobacter sakazakii* ***IIC*** to the tube labeled *C+;*

Amplification

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

	***Rotor type instruments*[1]**				***Plate type or modular instruments*[2]**			
Stage	***Temp, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***	***Temp,°C***	***Time***	***Fluorescence detection***	***Cycle repeats***
Hold	95	15 min	–	1	95	15 min	–	1
Cycling 2	95	10 s	–	45	95	10 s	–	45
	60	25 s	FAM(Green), JOE(Yellow)		60	30 s	FAM, JOE/HEX/Cy3	
	72	10 s	–		72	10 s	–	

[1] For example Rotor-Gene™ 3000/6000 (Corbett Research, Australia)

[2] For example, *SaCycler-96™ (Sacace),CFX96/* iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7300/7500 Real Time PCR (Applera), SmartCycler® (Cepheid)

INSTRUMENT SETTINGS

Settings for Rotor-type instruments

Channel	Threshold	More Settings/Outlier Removal	Slope Correct	Calibrate/Gain Optimisation…
FAM/Green	*0.05*	*10 %*	*On*	*from 5 Fl to 10 Fl*
JOE/HEX/Cy3/Yellow	*0.05*	*10 %*	*On*	*from 5 Fl to 10 Fl*

Settings for Plate-type instruments

Channel	Threshold
FAM	The threshold line for each channel is set at the level of 10-20 % of maximum fluorescence obtained for the Pos control in the last amplification cycle.
HEX	

RESULTS ANALYSIS:

1. The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line.

   - ***IC DNA*** **is detected on the FAM (Green) channel**
   - ***Cronobacter sakazakii*** **is detected on the JOE (Yellow)/HEX/Cy3 channel**

2. The sample is considered to be positive for *Cronobacter sakazakii* if in the channel Joe (Yellow)/HEX/Cy3 the value of **Ct** is different from zero (Ct < 40).
3. The sample is considered to be negative if in the channel Joe (Yellow)/HEX/Cy3 for *Cronobacter sakazakii* the Ct value is not determined (the fluorescence curve does not cross the threshold line) and in the results table on the channel Fam (Green) value for Internal Control is lower than 40.
4. Occurrence of any value Ct in the table of results for the negative control sample on the Joe (Yellow)/HEX/Cy3 channel and for negative control of amplification (DNA-buffer) (on any of channels) testifies contamination of reagents or samples. In this case results of the analysis for all tests are considered invalid. It is required to repeat the analysis of all tests, and also to take measures to detect and eliminate the source of contamination.
5. No signal with Positive Control indicates incorrect programming of the Real Time instrument: repeat the amplification with correct setting.
6. If the Ct value of the Internal Control is absent or higher than 40 a retesting of the sample is required.

Boundary value of the cycle threshold, Ct

Sample	Rotor-type instruments		Plate-type instruments	
	Green	Yellow	FAM	JOE/HEX/Cy3
EHEC/IC C+	*38*	*38*	*40*	*40*
C-	*38*	*absent*	*40*	*absent*
DNA-buffer	*absent*	*absent*	*absent*	*absent*
Test samples	*38*	*38*	*40*	*40*

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis. Nonspecific reactions were absent while testing human DNA samples and DNA panel of the following microorganisms: 3 strains of *Cronobacter sakazakii*, 4 strains of *Enterobacter cloacae,* 2 strains of *Enterobacter aerogenes*, 2 strains of *Pantoea agglomerans*, 8 strains of *Campylobacter* spp. *(C. jejuni, C. coli* and *C. fetus fetus)*, 31 strains of different serogroups of *Esherichia coli* (including *EHEC, EPEC, ETEC, EAggEC* and *EIEC*), 18 strains of different serogroups *of Salmomella* spp, *12 strains of different species and serogroups* of *Shigella* spp*.,* 22 strains of different species and serogroups of *Yersinia spp*., *Citrobacter freundii, Clostridium perfringens, Klebsiella pneumonia, Listeria monocytogenes, Protrus mirabilis, Pseudomonas aeruginosa, Serratia marcessens.*

Analytical sensitivity

The kit **Cronobacter sakazakii Real - TM** allows to detect *Cronobacter sakazakii* DNA in 100% of the tests with a sensitivity of not less than 1000 copies/ml.

TROUBLESHOOTING

1. Weak or no signal of the IC (FAM (green) channel) for the Negative Control of extraction.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - Improper DNA extraction.
        - ⇒ Repeat analysis starting from the DNA extraction stage
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. JOE (Yellow)/HEX/Cy3 signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

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

* SaCycler™ is a registered trademark of Sacace Biotechnologies
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