Sacace BIOTECHNOLOGIES

EHEC Real-TM

Handbook

Real Time PCR Kit for detection of EHEC (Enterohemorrhagic E. coli)

REF B59-50FRT

REF TB59-50FRT

Σ 50

NAME

EHEC Real - TM

INTRODUCTION

EHEC Real-TM kit is for the qualitative detection of Shiga toxins 1 and 2 (also called Verotoxins) produced by E. coli in cultures derived from clinical stool specimens. EHEC Real-TM kit is used in conjunction with the patient's clinical symptoms and other laboratory tests to aid in the diagnosis of diseases caused by enterohemorrhagic E. coli (EHEC) infections. Shiga toxins can be classified into two main categories: Shiga toxin 1 (ST1) and Shiga toxin 2 (ST2). EHEC strains may produce ST1 or ST2 only or both ST1 and ST2 simultaneously. EHEC are capable of initiating life-threatening illnesses, particularly in young children, the elderly or patients with immune deficiency. The main sources of infection are contaminated, raw or insufficiently heated foods of animal origin, e.g., meat and dairy products. The reservoirs for EHEC are cattle, sheep and goats and it is spread through their feces. These microorganisms can enter food during the processing of meat and dairy products if hygienic conditions are inadequate. The incidence of food infection caused by Shiga toxin-producing E. coli demands reliable and rapid methods of detection. In addition to traditional culture methods, molecular biology techniques are becoming more useful due to their improved specificity and sensitivity.

INTENDED USE

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

PRINCIPLE OF ASSAY

Kit **EHEC 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 *EHEC* 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 (B59-50FRT)

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

- **PCR-mix-1 *EHEC***, 0,6 ml;
- **PCR-mix-2-Flu**, 0,3 ml;
- **TaqF Polymerase,** 0,03 ml;
- **Positive Control *EHEC/IC,*** 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 (TB59-50FRT)

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– **"EHEC Real - TM":** Real Time amplification kit

- **PCR-mix-1 *EHEC***, 0,6 ml;
- **PCR-mix-2-Flu**, 0,3 ml;
- **TaqF Polymerase,** 0,03 ml;
- **Positive Control *EHEC/IC,*** 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 with filter
- Tube racks

STORAGE INSTRUCTIONS

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

Part N° 2 – **EHEC 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

EHEC 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

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

Use of this product should be limited to personnel trained in the techniques of DNA amplification (EN375). 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

EHEC Real - TM can analyze DNA extracted with **DNA-Sorb-B** 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

The following kit is recommended:

⇨ **DNA-Sorb-B** (Sacace, REF K-1-1/B)

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 *EHEC*

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 3 controls:

- add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
- add **10 µl** of ***EHEC/ IC* C+** to the tube labeled. $C+_{EHEC/IC;}$

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),* iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7300/7500 Real Time PCR (Applera), SmartCycler® (Cepheid)

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**
   - ***EHEC*** **is detected on the JOE (Yellow)/HEX/Cy3 channel**

2. The sample is considered to be positive for *EHEC* if in the channel Joe (Yellow)/HEX/Cy3 the value of **Ct** is different from zero (Ct < 35).
3. The sample is considered to be negative if in the channel Joe (Yellow)/HEX/Cy3 for *EHEC* if 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 33.
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 33 a retesting of the sample is required.

Table 1. Results for controls

Control	Stage for control	Ct channel Fam (Green)	Ct channel Joe (Yellow)/HEX/Cy3	Interpretation
NCS	DNA isolation	Pos (< 33)	Neg	Valid result
DNA-buffer	Amplification	Neg	Neg	Valid result
EHEC/*IC* C+	Amplification	Pos (< 33)	Pos (< 33)	Valid result

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 analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *EHEC* primers and probes. The specificity of the kit was 100%. The potential cross-reactivity of the kit was tested against the group control (*Esherichia coli* (*EPEC, ETEC, EAggEC, EIEC*), *Cronobacter sakazakii*; *Enterobacter cloacae*; *Enterobacter aerogenes*; *Pantoea agglomerans*; *Campylobacter spp (C. jejuni, C. coli, C. fetus fetus)*; *Salmomella spp*.; *Yersinia spp*.; *Citrobacter freundii; Clostridium perfringens; Klebsiella pneumoniae; Listeria monocytogenes; Protrus mirabilis; Pseudomonas aeruginosa; Serratia marcessens.*

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

Analytical sensitivity

The kit **EHEC Real - TM** allows to detect *EHEC* 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
	Expiration Date	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
IC	Internal Control	**RUO**	For Research Use Only

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