IVD For *in Vitro* Diagnostic Use

CE

Escherichioses Screen & Diff Real-TM

Handbook

Real Time PCR Kit for qualitative detection and differentiation of diarrheagenic E.coli (EPEC, ETEC, EIEC, EHEC, and EAgEC)

REF B62-50FRT

REF TB62-50FRT

Σ **50**

NAME

Escherichioses Screen & Diff 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 **Escherichioses Screen & Diff Real - TM** is a Real-Time PCR test for test for qualitative detection and differentiation of diarrheagenic E.coli (EPEC, ETEC, EIEC, EHEC, and EAgEC) DNA in environmental compartments and clinical material.

PRINCIPLE OF ASSAY

Kit **Escherichioses Screen & Diff Real - TM** is based on two major processes: DNA is extracted from samples and amplified by using real time amplification with fluorescent reporter dye probes specific for diarrheagenic E.coli (EPEC, ETEC, EIEC, EHEC, and EAgEC) 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 (B62-50FRT)

Part N° 2– **"Escherichioses Screen & Diff Real - TM":** Real Time amplification kit

- **PCR-mix-1 *EIEC/EHEC***, 0,6 ml;
- **PCR-mix-1 *EPEC/ETEC/EAgEC***, 0,6 ml;
- **PCR-mix-2-Flu**, 2 x 0,3 ml;
- **TaqF Polymerase,** 2 x 0,03 ml;
- **Positive Control DNA *EIEC / EHEC* / STI (C+ $_{EIEC\,/\,EHEC\,/\,STI}$),** 0,1 ml;
- **Positive Control DNA *EPEC / ETEC / EAgEC* (C+ $_{EPEC\,/\,ETEC\,/\,EAgEC}$),** 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 (TB62-50FRT)

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– **"Escherichioses Screen & Diff Real - TM":** Real Time amplification kit

- **PCR-mix-1 *EIEC/EHEC***, 0,6 ml;
- **PCR-mix-1 *EPEC/ETEC/EAgEC***, 0,6 ml;
- **PCR-mix-2-Flu**, 2 x 0,3 ml;
- **TaqF Polymerase,** 2 x 0,03 ml;
- **Positive Control DNA *EIEC / EHEC* / STI (C+ $_{EIEC\,/\,EHEC\,/\,STI}$),** 0,1 ml;
- **Positive Control DNA *EPEC / ETEC / EAgEC* (C+ $_{EPEC\,/\,ETEC\,/\,EAgEC}$),** 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 IC during the DNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- 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

Escherichioses Screen & Diff Real - TM must be stored at -20°C. **DNA-Sorb-B** must be stored at 2-8°C.

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

STABILITY

Escherichioses Screen & Diff 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

Escherichioses Screen & Diff 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;
- *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);
- ⇨ **DNA/RNA-Prep** (Sacace, REF K-2-9);
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006).

Please carry out RNA 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 2 reaction mixes for required number of samples, including controls. First one with **PCR-mix-1 *EIEC* / *EHEC* / STI** plus **PCR-mix-2-Flu** and **polymerase (TaqF)** as second one with **PCR-mix-1 *PEC* / *ETEC* / *EAgEC*** plus **PCR-mix-2-Flu** and **polymerase (TaqF)** (see Table). Vortex the tubes, then centrifuge shortly.

Table. **SCHEME OF REACTION MIXTURE PREPARATION**

	Reagent volume per specified number of reactions (µl)		
Reagent volume per 1 reaction (µl)	10.00	5.00	0.50
Number of reactions	PCR-mix-1	PCR-mix-2-Flu	Polymerase (TaqF)
6	60	30	3.0
8	80	40	4.0
10	100	50	5.0
12	120	60	6.0
14	140	70	7.0
16	160	80	8.0
18	180	90	9.0
20	200	100	10.0
22	220	110	11.0
24	240	120	12.0
26	260	130	13.0
28	280	140	14.0
30	300	150	15.0
32	320	160	16.0

3 Add **15 µl** of prepared reaction mix into each appropriate tube.

4 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

5 Prepare for each panel 3 controls:

- add **10 µl** of **DNA-buffer** to the tube labeled Negative Control Amplification (NCA);
- add **10 µl** of **Positive Control DNA *EIEC* / *EHEC* / STI** to the tube labeled $C+_{EIEC\,/\,EHEC\,/\,STI}$ (Positive Control of Amplification) for **PCR-mix-1 *EIEC* / *EHEC* / STI**;
- add **10 µl** of **Positive Control DNA *EPEC* / *ETEC* / *EAgEC*** to the tube labeled $C+_{EPEC\,/\,ETEC\,/\,EAgEC}$ (Positive Control of Amplification) for **PCR-mix-1 *EPEC* / *ETEC* / *EAgEC*.**

Amplification

Create a temperature profile on your Real-time 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), Rox(Orange)		60	30 s	FAM, JOE/HEX/Cy3, Rox	
	72	10 s	–		72	10 s	–	

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

INSTRUMENT SETTINGS

Escherichioses" test settings for rotor-type instruments (Rotor-Gene 6000, Rotor-Gene Q etc.)

Channel	Threshold	More Settings/Outlier Removal	Slope Correct	Auto gain calibration channel settings
FAM/Green	0.05	10%	On	3-7 FL
JOE/Yellow	0.05	10%	On	3-7 FL
ROX/Orange	0.05	10%	On	5-10 FL

Plate- or modular type instruments

For result analysis, set the threshold line at a level corresponding to 10–20% of the maximum fluorescence signal obtained for Pos C+ sample during the last amplification cycle.

RESULTS ANALYSIS:

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

   PCR-mix-1 *EIEC* / *EHEC* / STI:

   Internal Control is detected on Fam/Green channel,

   EHEC is detected on the Joe/Yellow/HEX/Cy3 channel,

   EIEC on the Rox/Orange/TexasRed channel

   PCR-mix-1 *EPEC* / *ETEC* / *EAgEC:*

   EAgEC is detected on Fam/Green channel,

   EPEC is detected on the Joe/Yellow/HEX/Cy3 channel,

   ETEC on the Rox/Orange/TexasRed channel

Table. Interpretation of results for PCR-analysis

PCR-mix-1	Ct value in channel			Interpretation
	Fam/Green	Joe/Yellow/HEX/Cy3	Rox/Orange/TexasRed	
PCR-mix-1 *EIEC / EHEC* / STI	Pos (< 40)	Neg	Neg	*EIEC* and *EHEC* DNA is not detected
	Neg or Pos (< 40)	Pos (< 40)	Neg	*EHEC* DNA is detected
	Neg or Pos (< 40)	Neg	Pos (< 40)	*EIEC* DNA is detected
	Neg	Neg	Neg	invalid
PCR-mix-1 *EPEC / ETEC /EAg EC*	Pos (< 40)	Neg	Neg	*EAgEC* DNA is detected
	Neg	Pos (< 40)	Neg	*EPEC* DNA is detected
	Neg	Neg	Pos (< 40)	*ETEC* DNA is detected
	Neg	Neg	Neg	*EPEC / ETEC / EAgEC* DNA are not detected

2. The result of the analysis is considered reliable only if the results obtained for both Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct.

Table . Results for controls

PCR-mix-1	Control	Stage for control	Ct value in channel		
			Fam/Green	Joe/Yellow/HEX /Cy3	Rox/Orange /TexasRed
PCR-mix-1-FEP/FRT *EIEC / EHEC* / STI	NCE	DNA extraction	Pos (< 40)	Neg (> 40)	Neg (> 40)
	NCA	Amplification	Neg (> 40)	Neg (> 40)	Neg (> 40)
	C+ *EIEC / EHEC / STI*	Amplification	Pos (< 40)	Pos (< 40)	Pos (< 40)
PCR-mix-1-FEP/FRT *EPEC / ETEC / EAgEC*	NCE	DNA extraction	Neg (> 40)	Neg (> 40)	Neg (> 40)
	NCA	Amplification	Neg (> 40)	Neg (> 40)	Neg (> 40)
	C+*EPEC / ETEC / EAgEC*	Amplification	Pos (< 40)	Pos (< 40)	Pos (< 40)

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of **Escherichioses Screen & Diff Real - TM** PCR kit is ensured by selection of specific primers and probes as well as strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis. Nonspecific responses were absent during examination of human DNA as well as a DNA panel of the following microorganisms:

- *E.coli* strains: O157H7 No. 4, O157H7 No. 23, O157H7 No. 212, O157H7 No. 214, O157H7 No. 1330, O143, O124 No. 227, O144, O86 No. 990, O125 Carioni, O85, O61 No. 10167в/41, O59 No. 9095/41, No. 409 (O34), K12, 3912/41, Krym No. 56, O148H28 B7a, O6 No. 3091, 113/3, 675, O111 No. 153, O62 10524/41, O126 No. 611, M17, Krym No. 1274, 168/59, O57 8198/41, Krym No. 14169, O48, NCTC 9001.
- Strains of other microorganisms: *Salmonella enteritidis* S-6, *S.choleraesuis* 370, *S.typhimurium* 371, *S.dublin* 373, *S.typhi* C1, *S.abortusovis* 372, and *S.gallinarum-pullorum; Shigella flexneri* 851b; *Campylobacter fetus* ssp. *fetus* 25936 and *C.jejuni* ssp. *jejuni* 43435; *Klebsiella K* 65 SW4; *Listeria monocytogenes* USHCH 19 and *L.monocytogenes* USHCH 52; *Proteus vulgaris* 115/98; *Pseudomonas aeruginosa* DH c1; *Staphylococcus aureus* 653 and *S. aureus* 29112; *Morganella morganii* 619 c 01; *Enterococcus faecalis* 356, 12 strains of *Yersinia enterocolitica,* and 6 strains of *Yersinia pseudotuberculosis.*

The specificity of diarrheagenic *E.coli* strains was confirmed by sequence analysis of the studied genome fragments.

Analytical sensitivity

The kit **Escherichioses Screen & Diff Real - TM** allows to detect *E.Coli* DNA in 100% of the tests with a sensitivity of not less than 1000 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

TROUBLESHOOTING

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

- If the signal for C– (except for C– in FAM channel for PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI) and/or for NCA is less than the boundary value, analysis should be repeated starting from the DNA extraction stage.
- If no signal is detected for the positive controls of amplification, it may suggest that the programming of the temperature profile of the used instrument was incorrect, or that the configuration of the PCR reaction was incorrect, or that the storage conditions for kit components did not comply with the manufacturer's instruction, or that the reagent kit expired. Programming of the used instrument, storage conditions, and the expiration date of the reagents should be checked, and then PCR should be repeated.
- If a positive result (the fluorescence curve crosses the threshold line) is detected for a sample that has a fluorescence curve without the typical exponential growth phase (the curve is linear), this may suggest incorrect setting of the threshold line or incorrect calculation of baseline parameters. Such a result should not be considered as positive. Once the threshold line has been set correctly, PCR analysis of the sample should be repeated (if iCycler iQ or iQ5 instruments are used).

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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