AmpliSens® Escherichioses–FRT
PCR kit

RUO

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code	Σ	Sufficient for
RUO	Research Use Only		Use-by Date
VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer	**NCA**	Negative control of amplification
	Date of manufacture	**C–**	Negative control of extraction
ETEC	Enterotoxigenic *E.coli*	**C+** *EIEC / EHEC / STI*, **C+** *EPEC / ETEC / EAgEC*	Positive control of amplification
EHEC	Enterohemorrhagic *E.coli*	**IC**	Internal control
EAgEC	Enteroaggregative *E.coli*	***EPEC***	Enteropathogenic *E.coli*
		EIEC	Enteroinvasive *E.coli*

1. INTENDED USE

AmpliSens® Escherichioses-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection and differentiation of diarrheagenic *E.coli* (*EPEC*, *ETEC*, *EIEC* (in conjunction with *Shigella* spp.), *EHEC*, and *EAgEC*) DNA in environmental samples and biological material by real-time hybridization-fluorescence detection of amplified products.

NOTE: For research use only. Not for diagnostic procedures

2. PRINCIPLE OF PCR DETECTION

Detection of different groups of diarrheagenic *E.coli* (*EPEC*, *ETEC*, *EIEC* (in conjunction with *Shigella* spp.), *EHEC*, *EAgEC*) by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific primers. In real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

AmpliSens® Escherichioses-FRT PCR kit is a qualitative test that contains the Internal Control (IC). It must be used in the isolation procedure in order to control the isolation process of each individual sample and to identify possible reaction inhibition.

AmpliSens® Escherichioses-FRT PCR kit uses "hot-start," which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE	ROX
PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI			
DNA-target	Internal Control-FL (IC) DNA	*EHEC* DNA	*EIEC* DNA
Target gene	Artificially synthesized sequence	Stx1/2 (Shiga Toxin type 1 and 2 genes)	ipaH (Invasion plasmid antigen)
PCR-mix-1-FEP/FRT *EPEC* / *ETEC* / *EAgEC*			
DNA-target	*EAgEC* DNA	*EPEC* DNA	*ETEC* DNA
Target gene	aggR transcriptional activator of fimbria gene	eae intimin protein gene	LT (Heat-labile enterotoxin gene)

3. CONTENT

AmpliSens® Escherichioses-FRT PCR kit is produced in 2 forms:

variant FRT-50 F, REF R-B62(RG,iQ)-CE;

variant FRT-50 F in bulk[1], REF R-B62(RG,iQ)-CE-B.

Variant FRT-50 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI	clear liquid from colorless to light lilac colour	0.6	1 tube
PCR-mix-1-FEP/FRT *EPEC* / *ETEC* / *EAgEC*	clear liquid from colorless to light lilac colour	0.6	1 tube
PCR-mix-2-FRT	colorless clear liquid	0.3	2 tubes
Polymerase (TaqF)	colorless clear liquid	0.03	2 tubes
Positive Control DNA *EIEC* / *EHEC* / STI (C+ *EIEC / EHEC / STI*)	colorless clear liquid	0.1	1 tube
Positive Control DNA *EPEC* / *ETEC* / *EAgEC* (C+ *EPEC / ETEC / EAgEC*)	colorless clear liquid	0.1	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-)*	colorless clear liquid	1.2	1 tube
Internal Control-FL (IC)**	colorless clear liquid	1.0	1 tube

* must be used in the extraction procedure as Negative Control of Extraction.

** add **10 µl** of **Internal Control-FL (IC)** during the DNA extraction procedure directly to the sample/lysis mixture (see **DNA-sorb-B**, **RIBO-sorb**, or **RIBO-prep** protocols).

Variant FRT-50 F is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2-ml reaction tubes.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia), iCycler iQ or iQ5 (Bio-Rad, USA), or equivalent).
- Disposable polypropylene microtubes for PCR (0.5- or 0.2-ml; for example, Axygen, USA).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. 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 regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid inhalation of vapors, samples and reagents contact with the skin, eyes, and mucous membranes. Harmful if swallowed. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice if necessary.
- Safety Data Sheets (SDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where the previous step was performed.

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

[1] In bulk form contains unlabeled tubes. Tubes with identical reagent are packed in one bag with label.

6. SAMPLING AND HANDLING

NOTE: Obtaining samples of biological materials for PCR-analysis, transportation and storage are described in the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® Escherichioses-FRT PCR kit is intended for the analysis of DNA extracted with DNA extraction kits from the environmental samples and biological material:

- *water samples* (pretreatment is not required);
- *feces* (pretreatment should be carried out as described in manufacturer's handbook [1]).

7. WORKING CONDITIONS

AmpliSens® Escherichioses-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. DNA extraction

It is recommended to use the following nucleic acid extraction kits:

- **DNA-sorb-B**;
- **RIBO-sorb**;
- **RIBO-prep**.

NOTE: Extract DNA according to the manufacturer's instructions.

8.2. Preparing PCR

The total reaction volume is 25 µl, the volume of DNA sample is 10 µl.

NOTE: The reaction mixture components should be mixed just before the analysis with respect to the required number of reaction tubes, including test and control samples according to the Table 2.
Note that even for the analysis of one test or control DNA sample it is necessary to run all controls of the PCR amplification stage (Positive Control of Amplification (C+), Negative Control of Amplification (NCA) for each type of mixture. It is recommended to mix the reagents for an even number of reactions for the purpose of a more exact dosage.

8.2.1. Preparing tubes for PCR

1. Thaw the reagents and vortex the tubes thoroughly. Make sure that there are no drops on the tube caps.
2. Prepare the required number of tubes (including controls).
   Mix **PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI** with **PCR-mix-2-FRT** and **polymerase (TaqF)** as well as **PCR-mix-1-FEP/FRT *EPEC* / *ETEC* / *EAgEC*** with **PCR-mix-2-FRT** and **polymerase (TaqF)** (see Table 2). Thoroughly vortex the tubes. Make sure that there are no drops on the tube caps.

Table 2

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[2]	**PCR-mix-1-FRT**	**PCR-mix-2-FRT**	**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. Transfer **15 µl** of the resultant mixture to the prepared tubes.
4. Add **10 µl** of **DNA** obtained from test or control samples using tips with aerosol barrier. Dispose the unused reaction mixture.

NOTE: Avoid transferring sorbent together with the DNA sample in case of extraction by RIBO-sorb or DNA-sorb-B kits.

5. Carry out the control amplification reactions:

NCA – Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

C– – Add **10 µl** of **the sample extracted from the Negative Control of Extraction** to the tube labelled **C–.**

C+ *EIEC / EHEC / STI* – 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.**

C+*EPEC / ETEC / EAgEC* – 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*.**

[2] The number of samples and IC (N), controls of amplification, and one extra sample (N+3+1)

8.2.2. Amplification

1. Program the real-time amplification instrument according to manufacturer's manual. Create a temperature profile on your instrument as follows:

Table 3

Amplification program

	Rotor-type Instruments[3]			Plate-type Instruments[4]		
Step	**Temperature, °C**	**Time**	**Cycles**	**Temperature, °C**	**Time**	**Cycles**
Hold	**95**	15 min	1	**95**	15 min	1
Cycling 1	**95**	10 s	45	**95**	10 s	45
	60	25 s *fluorescent signal detection*		**60**	25 s *fluorescent signal detection*	
	72	10 s		**72**	10 s	

Fluorescent signal is detected in the channels for the FAM, JOE and ROX fluorophores.

2. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin*.
3. Insert tubes into the reaction module of the device.
4. Run the amplification program with fluorescence detection.
5. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

9.1 Results interpretation

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in three channels: for FAM, JOE and ROX fluorophores.
The results are interpreted by the presence (or absence) of an intercept between the fluorescence curve and the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the DNA sample in the corresponding column of the results grid.
The principle of interpretation is presented in Table 4.

Table 4

Results interpretation

PCR-mix-1	*Ct* value in the channel for fluorophore			Interpretation
	FAM	**JOE**	**ROX**	
PCR-mix-1-FEP/FRT *EIEC / EHEC* / STI	< boundary value	> boundary value	> boundary value	*EIEC/ Shigella* spp. and *EHEC* DNA is not detected
	> or < boundary value	< boundary value	> or < boundary value	*EHEC* DNA is detected
	> or < boundary value	> or < boundary value	< boundary value	*EIEC/ Shigella* spp. DNA is detected
	> boundary value	> boundary value	> boundary value	invalid
PCR-mix-1-FEP/FRT *EPEC / ETEC /EAgEC*	< boundary value	> or < boundary value	> or < boundary value	*EAgEC* DNA is detected
	> or < boundary value	< boundary value	> or < boundary value	*EPEC* DNA is detected*
	> or < boundary value	> or < boundary value	< boundary value	*ETEC* DNA is detected
	> boundary value*	> boundary value	> boundary value	*EPEC / ETEC / EAgEC*[5] DNA are not detected

* If *Ct* value of a sample obtained in the channel for JOE fluorophore is less than the boundary value in case PCR-mix-1-FEP/FRT *EIEC / EHEC* / STI was used, the ***"DNA EHEC* is detected"** result will be displayed.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit. See also Guidelines [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 (see Table 5).

Table 5

Results for controls

PCR-mix-1	Control	Stage for control	*Ct* value in the channel for fluorophore		
			FAM	**HEX**	**ROX**
PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI	C-	DNA extraction	< boundary value	> boundary value or absent	> boundary value or absent
	NCA	PCR	> boundary value or absent	> boundary value or absent	> boundary value or absent
	C+ *EIEC / EHEC / STI*	PCR	< boundary value	< boundary value	< boundary value
PCR-mix-1-FEP/FRT *EPEC / ETEC / EAgEC*	C-	DNA extraction	> boundary value or absent	> boundary value or absent	> boundary value or absent
	NCA	PCR	> boundary value or absent	> boundary value or absent	> boundary value or absent
	C+ *EPEC / ETEC / EAgEC*	PCR	< boundary value	< boundary value	< boundary value

10. TROUBLESHOOTING

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

1. If the signal determined for the Positive Control of Amplification (C+) in the channels for JOE, FAM or ROX is greater than the boundary *Ct* value, the amplification and detection should be repeated for all samples in which DNA of different groups of diarrheagenic *E.coli* was not detected in the respective channel with the corresponding PCR-mix-1-FEP/FRT.
2. If the signal determined for the Negative Control of Extraction (C–) (except for PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI in the channel for FAM fluorophore) and/or the Negative Control of Amplification (NCA) in the all channels less than the boundary *Ct* value, the PCR analysis should be repeated starting from the DNA extraction stage for all samples in which DNA of pathogens was detected in the corresponding channel.

[3] For example, Rotor-Gene 3000, Rotor-Gene 6000, Rotor-Gene Q, or equivalent.
[4] For example, iCycler iQ, iQ5, Mx3000P, Mx3000, or equivalent.
[5] If the result is positive for PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI in the FAM channel.

11. TRANSPORTATION

AmpliSens® Escherichioses-FRT PCR kit should be transported at 2–8 °C for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® Escherichioses-FRT** PCR kit (except for PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI, PCR-mix-1-FEP/FRT *EPEC* / *ETEC* / *EAgEC*, PCR-mix-2-FRT, and polymerase (TaqF)) are to be stored at 2–8 °C when not in use. All components of the **AmpliSens® Escherichioses-FRT** PCR kit are stable until the expiration date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI, PCR-mix-1-FEP/FRT *EPEC* / *ETEC* / *EAgEC*, PCR-mix-2-FRT and polymerase (TaqF) are to be stored at the temperature from minus 24 to minus 16 °C

NOTE: PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI and PCR-mix-1-FEP/FRT *EPEC* / *ETEC* / *EAgEC* are to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

Pathogen	Biological material	Nucleic acid extraction kit	PCR kit	Sensitivity, GE/ml [6]
EPEC	Feces	RIBO-prep	Variant FRT-50 F	$1x10^3$
ETEC				
EIEC / *Shigella* spp				
EHEC				
EAgEC				

NOTE: The claimed analytical performance characteristics of **AmpliSens® Escherichioses-FRT** PCR kit are guaranteed only when additional reagent kits DNA-sorb-B, RIBO-sorb, or RIBO-prep (manufactured by FBIS CRIE) are used.

13.2. Specificity

The analytical specificity of **AmpliSens® Escherichioses-FRT** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published 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 (American Type Culture Collection): *Acinetobacter baumanii* ATCC® 19606™, *Bacteroides fragilis* ATCC® 25285™, *Bordetella bronchiseptica* ATCC® 10580™, *Bordetella bronchiseptica* ATCC® 4617™, *Bordetella pertussis* ATCC® 9340™, *Candida albicans* ATCC® 14053™, *Candida guilliermondii* ATCC® 6260™, *Candida krusei* ATCC® 14243™, *Clostridium difficile* ATCC® 9689™, *Clostridium septicum* ATCC® 12464™, *Corynebacterium jeikeium* ATCC® 43734™, *Corynebacterium xerosis* ATCC® 373™, *Eggerthella lenta* (*Eubacterium lentum*) ATCC® 43055™, *Enterobacter aerogenes* ATCC® 13048™, *Enterobacter cloacae* ATCC® 13047™, *Enterococcus faecalis* ATCC® 29212™, *Enterococcus faecalis* (vancomycin resistant) ATCC® 51299™, *Enterococcus faecium* ATCC® 35667™, *Erysipelothrix rhusiopathiae* ATCC® 19414™, *Escherichia coli* ATCC® 25922™, *Escherichia coli* ATCC® 35218™, *Fluoribacter* (*Legionella*) *dumoffii* ATCC® 33279™, *Haemophilus infuenzae* ATCC® 33930™, *Haemophilus influenzae* ATCC® 9006™, *Haemophilus influenzae* ATCC® 10211™, *Haemophilus parainfluenzae* ATCC® 7901™, *Klebsiella oxytoca* ATCC® 49131™, *Klebsiella pneumoniae* ATCC® 27736™, *Legionella pneumophila* ATCC® 33152™, *Listeria grayi* (*murrayi*) ATCC® 25401™, *Listeria innocua* ATCC® 33090™, *Listeria monocytogenes* ATCC® 7644™, *Moraxella* (*Branhamella*) *catarrhalis* ATCC® 25238™, *Moraxella* (*Branhamella*) *catarrhalis* ATCC® 8176™, *Neisseria meningitidis* ATCC® 13102™, *Neisseria meningitidis* ATCC® 13090™, *Neisseria lactamica* ATCC® 23970™, *Neisseria gonorrhoeae* ATCC® 19424™, *Neisseria gonorrhoeae* ATCC® 49926™, *Peptoniphilus* (*Peptostreptococcus*) *anaerobius* ATCC® 27337™, *Proteus mirabilis* ATCC® 12453™, *Proteus vulgaris* ATCC® 6380™, *Propionibacterium acnes* ATCC® 11827™, *Pseudomonas aeruginosa* ATCC® 15442™, *Rhodococcus equi* ATCC® 6939™, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* ATCC® 14028™, *Serratia marcescens* ATCC® 14756™, *Staphylococcus aureus* ATCC® 6538P™, *Staphylococcus aureus* (*MRSA*) ATCC® 43300™, Staphylococcus aureus ATCC® 29213™, *Staphylococcus aureus* ATCC® 25923™, *Staphylococcus aureus* ATCC® 33862™, *Staphylococcus aureus* (*MRSA*) ATCC® 33591™, *Staphylococcus aureus* subsp. *aureus* ATCC® 12600™, *Staphylococcus epidermidis* ATCC® 12228™, *Staphylococcus haemolyticus* ATCC® 29970™, *Staphylococcus saprophyticus* ATCC® 49907™, *Stenotrophomonas maltophilia* ATCC® 13637™, *Stenotrophomonas maltophilia* ATCC® 13637™, *Streptococcus agalactiae* ATCC® 12386™, *Streptococcus agalactiae* ATCC® 13813™, *Streptococcus equisimilis* ATCC® 12388™, *Streptococcus equi* subsp. *equi* ATCC® 9528™, *Streptococcus bovis* (Group D) ATCC® 9809™, *Streptococcus mutans* ATCC® 35668™, *Streptococcus pneumoniae* ATCC® 49619™, *Streptococcus pneumoniae* ATCC® 6303™, *Streptococcus pneumoniae* ATCC® 27336™, *Streptococcus pneumoniae* ATCC® 6305™, *Streptococcus pyogenes* ATCC® 19615™, *Streptococcus salivarius* ATCC® 13419™, *Streptococcus uberis* ATCC® 700407™, *Vibrio parahaemolyticus* ATCC® 17802™, *Vibrio vulnificus* ATCC® 27562™, *Moraxella catarrhalis* ATCC® 25240™.

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

14. REFERENCES

1. Handbook “Sampling, Transportation, and Storage of Clinical Material for PCR Diagnostics”, developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology” of Federal Service for Surveillance on Consumers’ Rights Protection and Human Well-Being.
2. Guidelines to the **AmpliSens® Escherichioses-FRT** PCR kit for qualitative detection and differentiation of diarrheagenic *E.coli* DNA in environmental samples and biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection, developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology”.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science “Central Research Institute for Epidemiology” ISO 13485-Certified Quality Management System, each lot of **AmpliSens® Escherichioses-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

AmpliSens®

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[6] Genome equivalents (GE) of the pathogen agent per 1 ml of a sample.