AmpliSens® *Human enterovirus*-FRT PCR kit
Instruction Manual

RUO

For Professional Use Only

KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Contains sufficient for <n> tests
LOT	Batch code		Use-by Date
RUO	Research Use Only		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limit		Keep dry
	Manufacturer	NCA	Negative control of amplification
	Date of manufacture	C–	Negative control of extraction
	Caution	C+	Positive control of amplification
		IC	Internal control

1. INTENDED USE

AmpliSens® *Human enterovirus*-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of RNA of *Human enterovirus clusters* A, B, C, D (*Human coxsackievirus* A, *Human coxsackievirus* B, *Human echovirus*, *Human poliovirus*, *Human enterovirus* 68-71, 73-78, 89-91) without differentiation between them in the biological material (feces, cerebrospinal fluid, respiratory tract swab) and environmental samples (water sample concentrates) using real-time hybridization-fluorescence detection of amplified products.

NOTE: For research use only. Not for diagnostic procedures

2. PRINCIPLE OF PCR DETECTION

Principle of testing is based on the RNA extraction from the samples of test material with the exogenous internal control sample (Internal Control-FL (IC)) and conducting the RNA reverse transcription reaction and amplification of cDNA fragments of detecting virus and Internal Control-FL (IC) cDNA with hybridization-fluorescence detection. Exogenous internal control (Internal Control-FL (IC)) allows to control all PCR-analysis stages of each individual sample and to identify possible reaction inhibition.

Human enterovirus detection by the polymerase chain reaction (PCR) is based on the amplification of a pathogen genome specific region using special primers. In the 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.

The PCR kit variant FRT-50-0,2 contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and deoxyuridine triphosphate (dUTP). The enzyme UDG recognizes and catalyzes the destruction of the DNA containing deoxyuridine, but has no effect on DNA containing deoxythymidine. Deoxyuridine is absent in the authentic DNA, but is always present in amplicons, because dUTP is a part of dNTP mixture in the reagents for the amplification. Due to the deoxyuridine containing contaminating amplicons are sensitive to the destruction by UDG before the DNA-target amplification. So the amplicons cannot be amplified.

The enzyme UDG is thermolabile. It is inactivated by heating at temperature above 50 °C. Therefore, UDG does not destroy the target amplicons which are accumulated during PCR.

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

Table 1

Channel for fluorophore	FAM	JOE
cDNA-target	Internal Control-FL (IC) cDNA	*Enterovirus* (*Human enterovirus*) cDNA
Target gene	Artificially synthesized sequence	5'UTR

3. CONTENT

AmpliSens® *Human enterovirus*-FRT PCR kit is produced in 5 forms:

variant FRT-50-0,2, REF H-2771-1-2-CE;

variant FRT-50 F, REF H-2773-1-CE;

variant FRT-50 F in bulk[1], REF H-2773-1-CE-B;

variant FRT-L, REF H-2773-1-4-CE;

variant FRT-L in bulk[1], REF H-2773-1-4-CE-B.

Variant FRT-50-0,2 includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *Enterovirus* ready-to-use single-dose test tubes (*under wax)*	clear liquid from colorless to light lilac colour	0.01	55 tubes of 0.2 ml
PCR-buffer-K	red clear liquid	1.1	1 tube
C+ *Enterovirus*	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	1 tube
Internal Control-FL (IC)**	colorless clear liquid	0.5	1 tube

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

** add **10 µl** of **Internal Control-FL (IC)** during the RNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep** protocol).

Variant FRT-50-0,2 is intended for 55 reactions (including controls).

Variant FRT-50 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *Enterovirus*	clear liquid from colorless to light lilac colour	0.6	1 tube
PCR-buffer-C	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
TM-Revertase (MMlv)	colorless clear liquid	0.015	1 tube
RT-G-mix-2	colorless clear liquid	0.015	1 tube
C+ *Enterovirus*	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	1 tube
Internal Control-FL (IC)**	colorless clear liquid	0.5	1 tube

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

** add **10 µl** of **Internal Control-FL (IC)** during the RNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep** protocol).

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

Variant FRT-L includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix *Enterovirus*-Lyo	white powder	—	96 tubes of 0.2 ml
C+ *Enterovirus*	colorless clear liquid	0.5	1 tube
TE-buffer	colorless clear liquid	0.5	1 tube
Internal Control-FL (IC)*	colorless clear liquid	1.0	1 tube
Negative Control (C–)**	colorless clear liquid	1.2	1 tube
Buffer for elution A	colorless clear liquid	1.2	4 tubes

* add **10 µl** of **Internal Control-FL (IC)** during the RNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep** protocol)

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

Variant FRT-L is intended for 96 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- Puncture needles.
- Sterile 50-60-ml plastic container for sampling, storage and transportation of biological material.
- 0.9 % solution of sodium chloride (sterile saline solution) or phosphate-buffered saline (PBS) (Sodium chloride, 137 mM; potassium chloride, 2.7 mM; sodium monophosphate, 10 mM; potassium diphosphate, 2 mM; pH = 7.5 ± 0.2).
- RNA extraction kit.
- Reverse transcription kit for PCR kit variant FRT-50-0,2.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips (up to 1,000 µl) and pipette tips with filters (up to 200 µl, 1000 µl).
- Tube racks.

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

- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Real-time instruments (for example, Rotor-Gene Q (QIAGEN, Germany); CFX 96 (Bio-Rad, USA)).
- Disposable polypropylene tubes:
  a) tightly closed 1.5-ml tubes for sampling.
  b) tightly closed 1.5 and 2-ml tubes for pretreatment
  c) screwed or tightly closed 1.5-ml tubes for reaction mixture preparation for PCR kit variant FRT-50 F.
  d) thin-walled 0.2-ml PCR tubes with optical transparent domed or flat caps or strips of eight 0.2-ml tubes with optical transparent caps if a plate-type instrument is used for PCR kit variant FRT-50 F;
  e) thin-walled 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used for PCR kit variant FRT-50 F.
- 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 the PCR kit if the internal packaging was damaged or its appearance was changed.
- Do not use the PCR kit if the transportation and storage conditions according to the Instruction Manual were not observed.
- 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.
- While observing the conditions of transportation, operation and storage, there are no risks of explosion and ignition.
- Safety Data Sheets (SDS) are available on request.
- The PCR kit is intended for single use for PCR analysis of specified number of samples (see the section “Content”).
- The PCR kit is ready for use in accordance with the Instruction Manual. Use the PCR kit strictly for intended purpose.
- 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.

6. SAMPLING AND HANDLING

AmpliSens® *Human enterovirus*-FRT PCR kit is intended for analysis of the RNA extracted with the use of RNA extraction kits from the biological material (feces, cerebrospinal fluid, respiratory tract swabs) and environmental samples (water sample concentrates).

NOTE: The sampling, transportation and storage of the test material must be carried out in accordance with the requirements of regulatory acts on epidemiological surveillance and prevention of enterovirus (non-polio) infection.

Sampling

Cerebrospinal fluid is obtained in the first days of the disease if clinically indicated in aseptic conditions with the use of disposable puncture needles into disposable dry plastic 1.5-ml tube in an amount of at least 1.0-ml.

The cerebrospinal fluid samples can be stored before the PCR analysis:

- at the temperature from 2 to 8 °C – no more than 1 day;
- at the temperature from minus 24 to minus 16 °C – for 1 week;
- at the temperature not more than minus 68 °C – for a long time.

The cerebrospinal fluid samples can be transported at the temperature from 2 to 8 °C for 1 day.

Feces samples are taken from a disposable reservoir (for example, a petrie dish, disposable plastic bag) placed in a bed-pan or disposable diapers (for younger children). When using a disposable diaper for children with liquid stool, a cotton pad should be placed into diaper before the use for obtaining the sufficient quantity of sample.

NOTE: It is forbidden to take feces samples directly from a bed-pan or another reservoir for multiple use (without distinction of disinfection methods).

Using a separate filter tip or disposable spatula transfer about 1 g of the sample into special disposable plastic container.

The feces samples can be stored before the pretreatment:

- at the temperature from 18 to 25 °C – for 6 hours,
- at the temperature from 2 to 8 °C – for 3 days.

Only one freeze-thawing cycle is required.

The feces samples can be transported at the temperature from 2 to 8 °C for 3 days.

Water sample concentrates are taken according to state and local authorities' requirements.

Water sample concentrates can be stored before the PCR analysis:

- at the temperature from 2 to 8 °C – for 1 day,
- at the temperature from minus 24 to minus 16 °C – for 1 month,
- at the temperature not more than minus 68 °C – for a long time,

Only one freeze-thawing cycle is required.

The water sample concentrates can be transported at the temperature from 2 to 8 °C for 1 day.

Respiratory tract swabs

NOTE: It is recommended to combine nasopharyngeal and oropharyngeal swabs in a single tube. For this purpose, first take the swabs from the mucous membrane of inferior nasal meatus and oropharynx using different swabs and then place the ends of both shafts into one tube containing 500 µl of **Transport Medium for Storage and Transportation of Respiratory Swabs** and analyze them as a single sample.

Nasopharyngeal swabs.

Swabs are obtained using a sterile dry nasopharyngeal flocked swabs with plastic shafts. If the nasal cavity is full of mucus, it is recommended to blow out the nose before the procedure. Gently insert the swab along the external nasal wall to a depth of 2-3 cm towards the inferior nasal concha. Then move the swab slightly lower, insert into the inferior nasal meatus under the inferior nasal concha turbinate to the nasopharynx, rotate and remove along the external nasal wall. The total depth of insertion of the swab should be approximately half of the distance from the nostril to the ear hole (3-4 cm for children and 5-6 cm for adults).

When the material is obtained, insert the working part of the swab into a sterile disposable tube with 500 µl of a **Transport Medium for Storage and Transportation of Respiratory Swabs**, the flexible part of the swab is coiled, then, covering the top of the tube with a lid, the handle of the swab is lowered down, achieving a complete breaking off of the upper part of the swab. Hermetically close and mark the tube with the solution and the working part of the probe.

Nasopharyngeal swabs can be stored before the study:

- at the temperature from 2 to 8 °C - no more than 3 days;
- at the temperature from minus 24 to minus 16 °C - no more than 1 week.

Only one freeze-thaw cycle of test material is allowed.

It is allowed to transport the above-mentioned material at a temperature of 2 to 8 °C for 3 days.

Oropharyngeal swabs

Oropharyngeal swabs are obtained using sterile dry rayon swabs with plastic shafts for oropharyngeal swabs. Rotate the swab over the surface of tonsils, palatine arches, and posterior wall of pharynx.

When the material is obtained, insert the working part of the swab into a sterile disposable tube with 500 µl of a **Transport Medium for Storage and Transportation of Respiratory Swabs**, the flexible part of the swab is coiled, then, covering the top of the tube with a lid, the handle of the swab is lowered down, achieving a complete breaking off of the upper part of the swab. Hermetically close and mark the tube with the solution and the working part of the probe.

Oropharyngeal swabs can be stored before the study:

- at the temperature from 2 to 8 °C - no more than 3 days;
- at the temperature from minus 24 to minus 16 °C - no more than 1 week.

Only one freeze-thaw cycle of the material is allowed.

It is allowed to transport the above-mentioned material at a temperature of 2 to 8 °C for 3 days.

Pretreatment

Pretreatment of w*ater sample concentrates* is not required.

Pretreatment of *cerebrospinal fluid samples* is not required.

Feces samples are to be pretreated.

Preparation of fecal suspension:

1. Take the required number (respectively to the number of samples) of disposable 1.5-ml tubes. Add 1 ml of PBS into each tube (use 15-20 % solution of glycerin in PBS when necessary to store the suspension more than 1 day under refrigeration).
2. Using a new one filter tip or disposable spatula for each sample add 0.1 g (0.1 ml) of feces into each tube and resuspend thoroughly on vortex due to obtain homogenous suspension. Optimal concentration of suspension is ~ 10 % (by the pellet volume after centrifugation). Sediment the drops from the tube caps by short centrifugation on vortex (no more than 10 sec).

Liquid semitransparent feces are used for express filtration without previous obtaining the suspension.

Express filtration of fecal suspension (for viral and bacterial pathogens detection):

1. For express filtration use two tips up to 1 ml (with filter and without filter) and a cut lower part of cotton probe (cotton bud).
2. Put the cut lower part of disposable cotton probe (cotton bud) in the tip without aerosol filter and fix it by pushing into the necked part of the tip.
3. Take 1 ml of fecal suspension by the filter tip, put it in the prepared tip with cotton filter and carry out the pressing-filtration into a new disposable tube. In case of difficult filtration it is recommended to decrease the fecal suspension concentration.
4. 100 µl of filtrate is used for RNA extraction.

The pretreated samples of feces suspensions can be stored before the PCR analysis:

- at the temperature from minus 24 to minus 16 °C – for 1 week,
- at the temperature not more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is required.

The above mentioned material can be transported at the temperature from 2 to 8 °C for 1 day.

Interfering substances and limitations of using test material samples

In order to control the efficiency of RNA extraction, RT and the amplification the Internal Control (Internal Control-FL (IC)) is used in the PCR kit. The Internal Control is added in each biological sample at the extraction stage. The presence of internal control signal after the amplification testifies the effectiveness of nucleic acid extraction and the absence of PCR inhibitors.

Potentially interfering substances

Endogenous and exogenous substances that may be present in biological material (CSF, feces, respiratory tract swabs) used for the study (see Table 2) were selected to assess potential interference.

Model samples of CSF, feces and respiratory tract swabs without adding and with the addition of potentially interfering substances were tested. Model samples of CSF, feces, respiratory tract swabs contained a quality control sample (QCS) with *Enterovirus* RNA at concentrations of $1x10^4$ GE/ml for fecal samples and $5x10^3$ GE/ml for CSF samples, respiratory tract swabs.

Table 2

Type of test material	Potential interference type	Potential interference	Tested sample concentration	Interference
Feces	Endogenous substances	Whole blood	up to 40% volume/volume	Not detected
		Fecal fats	up to 40% volume/volume	Not detected
		Mucin (mucus)	up to 3% weight of the drug to the volume of material	Not detected
	Exogenous substances	‘Enterofuril’ oral suspension	up to 4.25 mg/ml	Not detected
		‘Enterosgel’, oral paste (sweet)	up to 174.75 mg/ml	Not detected
		Dextrin	up to 68.6 mg/ml	Not detected
Cerebrospinal fluid	Endogenous substances	Whole blood	up to 4% volume/volume	Not detected
	Exogenous substances	Ceftriaxone powder for solution for intravenous and intramuscular introduction	up to 8 mg/ml weight of the drug to the volume of material	Not detected
		Iodine, solution for external use alcohol 5%	up to 0,03% volume/volume	Not detected

Type of test material	Potential interference type	Potential interference	Tested sample concentration	Interference
Respiratory tract swabs	Endogenous substances	Whole blood	up to 4% volume/volume	Not detected
		Mucin (mucus)	up to 5% weight of the drug to the volume of material	Not detected
	Endogenous substances	Miramistin	up to 5% volume/volume	Not detected
		Lugol's solution with glycerin	up to 1% volume/volume	Not detected

7. WORKING CONDITIONS

AmpliSens® *Human enterovirus*-FRT PCR kit should be used at the temperature from 20 to 28 °C and relative humidity from 15 to 75 %

8. PROTOCOL

8.1. RNA extraction

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

- **RIBO-prep**.

NOTE: Extract the RNA according to the manufacturer's protocol.

NOTE:
The volumes of reagents and samples when the RNA is extracted by the RIBO-prep reagent kit:

The RNA extraction for each sample is carried out in the presence of **Internal Control-FL (IC).**

Add **10 µl** of **Internal Control-FL (IC)** to each tube.

The volume of the test sample is **100 µl.**

Add **100 µl** of **Negative Control (C–)** reagent into the tube labeled C– (Negative Control of Extraction).

The volume of elution is **50 µl** for PCR kits variant FRT-50-0,2 and variant FRT-50 F or **125 µl** for PCR kit variant FRT-L

NOTE:
It is recommended to carry out the reverse transcription/RT-PCR reaction just after the obtaining RNA samples. It is allowed to store RNA samples at the temperature from 2 to 8 °C for 30 min, at the temperature from minus 24 to minus 16 °C for 1 week and at the temperature not more than minus 68 °C for 1 year.
Only one freeze-thawing cycle for RNA samples is required

8.2. Reverse transcription

In case of PCR kit **variant FRT-50-0,2** it is recommended to use the following kit for the complementary DNA (cDNA) synthesis from RNA:

- **REVERTA-L**.

NOTE: Carry out the reverse transcription according to the manufacturer's protocol.

8.3. Preparing PCR/RT-RCR

8.3.1. Preparing tubes for PCR

Variant FRT-50-0,2

The total reaction volume is **30 µl**, the volume of the cDNA sample is **10 µl.**
Use disposable filter tips for adding reagents, cDNA and control samples into tubes.

1. Prepare the required number of the tubes with **PCR-mix-FL *Enterovirus*** and wax for the amplification of cDNA from test and control samples (for the number of control samples see item 4). Ensure that the wax completely covers the solution on the bottom of the tubes. If this is not the case, do not use these tubes.
2. Add **10 µl** of **PCR-buffer-K** to the surface of the wax layer into each test and control tube ensuring that it does not fall under the wax and mix with **PCR-mix-FL *Enterovirus.***
3. Into the prepared tubes add **10 µl** of the **cDNA samples** obtained by extraction and reverse transcription of the test samples.
4. Carry out the control amplification reactions:

C+ – Add **10 µl** of **C+ *Enterovirus*** to the tube labeled C+ (Positive Control of Amplification)

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

C– – Add **10 µl** of **cDNA** obtained by extraction and reverse transcription of the **Negative Control (C–) reagent** to the tube labeled C– (Negative Control of Extraction).

8.3.2. Preparing tubes for RT-RCR

Variant FRT-50 F

The total reaction volume is **25 µl**, the volume of the **RNA** sample is **10 µl**.
The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, RNA and control samples into tubes.

1. Calculate the required quantity of each reagent for reaction mixture preparation. For one reaction:
   - **10 µl** of **PCR-mix-FL *Enterovirus*,**
   - **5 µl** of **PCR-buffer-C,**
   - **0.5 µl** of **Polymerase (TaqF),**
   - **0.25 µl** of **TM-Revertase (MMlv),**
   - **0.25 µl** of **RT-G-mix-2.**

Prepare the reaction mixture for the total number of test and control samples plus one extra reaction. See numbers of control samples in item 7.
The calculation for the required number of reactions including testing the test and control samples can be performed according to Table 3.

Table 3

Scheme of reaction mixture preparation for variant FRT-50 F

		Reagent volume for specified number of reactions				
Reagent volume per one reaction, µl		**10.0**	**5.0**	**0.25**	**0.5**	**0.25**
Number of test samples	**Number of reactions[2]**	**PCR-mix-FL**	**PCR-buffer-C**	**RT-G-mix-2**	**Polymerase (TaqF)**	**TM-Revertase (MMlv)**
2	**6**	**60**	**30**	**1.5**	**3.0**	**1.5**
4	**8**	**80**	**40**	**2.0**	**4.0**	**2.0**
6	**10**	**100**	**50**	**2.5**	**5.0**	**2.5**
8	**12**	**120**	**60**	**3.0**	**6.0**	**3.0**
10	**14**	**140**	**70**	**3.5**	**7.0**	**3.5**
12	**16**	**160**	**80**	**4.0**	**8.0**	**4.0**
14	**18**	**180**	**90**	**4.5**	**9.0**	**4.5**
16	**20**	**200**	**100**	**5.0**	**10.0**	**5.0**
18	**22**	**220**	**110**	**5.5**	**11.0**	**5.5**
20	**24**	**240**	**120**	**6.0**	**12.0**	**6.0**
22	**26**	**260**	**130**	**6.5**	**13.0**	**6.5**
24	**28**	**280**	**140**	**7.0**	**14.0**	**7.0**
26	**30**	**300**	**150**	**7.5**	**15.0**	**7.5**
28	**32**	**320**	**160**	**8.0**	**16.0**	**8.0**

NOTE: Prepare the reaction mixture just before use.

2. Thaw the tube with **PCR-mix-FL *Enterovirus*.** Thoroughly vortex all the reagents of the PCR kit and sediment the drops by vortex.
3. In a new tube prepare the reaction mixture. Mix the required quantities of **PCR-mix-FL *Enterovirus*, PCR-buffer-C, Polymerase (TaqF), TM-Revertase (MMlv) and RT-G-mix-2.** Sediment the drops by vortex.
4. Take the required number of the tubes or strips for RNA reverse transcription and amplification taking into account the number of test samples and control samples.
5. Transfer **15 µl** of the prepared reaction mixture to each tube. Discard the unused reaction mixture.
6. Add **10 µl** of **RNA samples** extracted from test samples at the RNA extraction stage.

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

7. Carry out the control reactions:

C+ – Add **10 µl** of **C+ *Enterovirus*** to the tube labeled C+ (Positive Control of Amplification)

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

C– – Add **10 µl** of **the sample extracted from the Negative Control (C–) reagent** to the tube labeled C– (Negative control of Extraction).

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

NOTE: Carry out the PCR just after the mix of reaction mixture and RNA-samples and controls. Time of the addition of samples to the reaction mixture and the reaction run on the instrument cannot be more than 10-15 min.

Variant FRT-L

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

1. Take the required number of the tubes with ready-to-use lyophilized reaction mixture **PCR-mix *Enterovirus*-Lyo** for RT-RCR of RNA from test and control samples (see numbers of control samples in point 3).
2. Add **25 µl** of **RNA samples** obtained by extraction.

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

3. Carry out the control reactions:

C+ – Add **25 µl** of **C+ *Enterovirus*** to the tube labeled **C+** (Positive Control of Amplification).

NCA – Add **25 µl** of **TE-buffer** to the tube labeled **NCA** (Negative Control of Amplification).

C– – Add **25 µl** of **the sample extracted from the Negative Control (C–) reagent** to the tube labeled **C–** (Negative Control of Extraction).

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

NOTE: Carry out the RT-PCR just after the mix of reaction mixture and RNA-samples and controls. Time of the addition of samples to the reaction mixture and the reaction run on the instrument cannot be more than 10-15 min.

8.3.3. Amplification

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

Table 4

AmpliSens unified amplification program for rotor-type[3] and plate-type[4] instruments

Step	Temperature, °C	Time	Fluorescent signal detection	Cycles
1	**50**	**15 min**	**–**	**1**
2	**95**	**15 min**	**–**	**1**
3	**95**	**10 s**	**–**	**45**
	60	**20 s**	**FAM, JOE**	

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

NOTE: Any combination of the tests (including tests with reverse transcription and amplification) can be performed in one instrument simultaneously with the use of the unified amplification program. If several tests in "multiprime" format are carried out simultaneously, the detection is enabled in other used channels except for the specified ones. In case of variant FRT-50-0,2 if in one instrument only the tests for the pathogen DNA (cDNA) detection are carried out simultaneously, the first step of reverse transcription (50 °C – 15 min) can be omitted for time saving.

2. Insert tubes into the reaction module of the device.

NOTE: It is recommended to sediment drops from walls of tubes by short centrifugation (1–3 s) before placing them into the instrument.
Insert empty tubes at the edges of reaction module in case of incomplete filling of plate-type instrument.

3. Run the amplification program with fluorescence detection.
4. Analyze results after the amplification program is completed.

[2] number of reactions = number of test samples + controls of extraction stage (C–) and reverse transcription and amplification stage (C+, NCA) + one extra reaction.

[3] For example, Rotor-Gene Q (QIAGEN, Germany).
[4] For example, CFX 96 (Bio-Rad, USA).

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in two channels:

Table 5

Channel for the fluorophore	FAM	JOE
Amplification product	Internal Control-FL (IC) cDNA	*Enterovirus* (*Human enterovirus*) cDNA

Results are interpreted by the crossing (or not-crossing) the S-shaped (sigmoid) fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the sample in the corresponding column of the results grid.
Principle of interpretation is the following:

Table 6

Results interpretation

***Ct* value in the channel for the fluorophore**		**Result**
FAM	**JOE**	
< boundary value	absent or > boundary value	*Enterovirus* RNA **is not detected**
> boundary value or < boundary value	< boundary value	*Enterovirus* RNA **is detected**
absent or > boundary value	absent or > boundary value	**Invalid** result*

* In case of invalid result, the PCR analysis should be repeated for the corresponding test sample starting from the RNA extraction stage.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The result of the analysis is considered reliable only if the results obtained for the controls of amplification and extraction are correct (see Table 7).

Table 7

Results for controls

Control	Stage for control	***Ct* value in the channel for fluorophore**	
		FAM	**JOE**
C–	RNA extraction	< boundary value	absent or > boundary value
NCA	PCR/RT-PCR	absent or > boundary value	absent or > boundary value
C+	PCR/RT-PCR	< boundary value	< boundary value

10. TROUBLESHOOTING

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

1. The *Ct* value determined for the Positive Control of Amplification (C+) in the channel for the JOE fluorophore is greater than the boundary value or absent. The amplification and detection should be repeated for all samples in which the *Human enterovirus* RNA was not detected.
2. The *Ct* value determined for the Negative Control of Extraction (C–) in the channel for the JOE fluorophore is less than the boundary value. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The PCR analysis (beginning with the RNA extraction stage) should be repeated for all samples in which specific RNA was detected.
3. The *Ct* value determined for the Negative Control of Amplification (NCA) in the channel for the JOE fluorophore is less than the boundary value. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The amplification and detection should be repeated for all samples in which specific RNA was detected.
4. The *Ct* value is determined for the test sample, whereas the area of typical exponential growth of fluorescence is absent (the graphic looks like approximate straight line). It is necessary to check the correctness of selected threshold line level or parameters of base line calculation. 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.

11. TRANSPORTATION

AmpliSens® *Human enterovirus*-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® *Human enterovirus*-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-buffer-K included in variant FRT-50-0,2; PCR-mix-FL *Enterovirus*, PCR-buffer-C, Polymerase (TaqF), TM-Revertase (MMlv), RT-G-mix-2 included in variant FRT-50 F). All components of the **AmpliSens® *Human enterovirus*-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-FL *Enterovirus* and PCR-mix *Enterovirus*-Lyo are to be kept away from light.

NOTE: PCR-buffer-K, PCR-mix-FL *Enterovirus*, PCR-buffer-C, Polymerase (TaqF), TM-Revertase (MMlv), RT-G-mix-2 are to be stored at the temperature from minus 24 to minus 16 °C.

NOTE: PCR-mix *Enterovirus*-Lyo is to be kept in packages with a desiccant away from light.

13. SPECIFICATIONS

13.1. Analytical sensitivity (limit of detection)

Table 8

Biological material	Nucleic acid extraction kit	The kit for reverse transcription, amplification and detection		Analytical sensitivity (limit of detection)[5], GE/ml
Cerebrospinal fluid, water sample concentrates	RIBO-prep	REVERTA-L	PCR kit variant FRT-50-0,2	$5x10^3$
		—	PCR kit variant FRT-50 F, variant FRT-L	
Feces	RIBO-prep	REVERTA-L	PCR kit variant FRT-50-0,2	$1x10^4$
		—	PCR kit variant FRT-50 F, variant FRT-L	

The claimed features are achieved while respecting the rules specified in the section *Sampling and Handling*

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *Human enterovirus*-FRT** PCR kit is ensured by the 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.
The PCR kit specificity was tested on the following panels of microorganism strains and RNA/DNA samples:

1. RNA of *Human enteroviruses* (representatives of different genetic clusters – *Human echovirus* 2, 5, 6, 9, 11, 14, 16, 17, 18, 27, 30; *Human coxsackievirus* A4, A5, A6, A9, A16, B3, B4, B5, EV71) *Human poliovirus* 1, 2, 3 (Sabin1, Sabin2, Sabin3)) (the clinical isolates; the specificity was proved by the direct sequencing of the nucleotide sequence), *Human poliovirus* 1, 2, 3 (vaccinal strains) in the concentration no less than $1x10^4$ GE/ml. Positive results were obtained while testing of RNA samples of the strains/clinical isolates listed above;
2. RNA of *Influenza virus* A (H3N2 (NCBI:txid2029290), H1N1 (NCBI:txid1898984)),), *Influenza virus* B, *Rhinoviruses*, *RS viruses*, *Human adenoviruses* –type 3 (GenBank: FJ167580.1), type 5 (GenBank: FJ167596.1), type 7 (GenBank: KU361344.1), type 37 (GenBank: AY048776.1), type 40 (GenBank: FJ167570.1), type 41 (GenBank: FJ167574.1) (in the concentration no less than $1x10^4$ GE/ml) (the clinical isolates; the specificity was proved by the direct sequencing of the nucleotide sequence);
3. Strains ATCC® collection (American Type Culture Collection, USA) in the concentration no less than $1x10^6$ GE/ml: *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™, *Trichophyton mentagrophytes* ATCC® 9533™, *Trichophyton mentagrophytes* ATCC® 9533™, *Vibrio parahaemolyticus* ATCC® 17802™, *Vibrio vulnificus* ATCC® 27562™, *Moraxella catarrhalis* ATCC® 25240™, *Corynebacterium mimutissimum* ATCC® 23348™.

The nonspecific reactions were absent while testing 2[d] and 3[d] panels as well as human DNA (0.2 mg/ml) (Sigma-Aldrich, USA).
The information about interfering substances is specified in the *Interfering substances and limitations of using test material samples*.

13.3. Repeatability, reproducibility

The repeatability and reproducibility of the study were determined by testing positive and negative model samples. Positive samples were a dilution quality control sample (QCS) containing *Enterovirus* RNA with a concentration of $5x10^3$ GE/ml; Negative control (C–) was used as a negative sample.
Repeatability conditions included testing in the same laboratory, by the same operator, using the same equipment within a short period of time. Reproducibility conditions included testing different lots of reagent kit in two independent laboratories, by different operators, on different days, using different equipment.
The results are presented in Table 9.

[5] Number of genome equivalents (GE) of the microorganism per 1 ml of the test material sample.

Table 9

Form	Sample type	Repeatability		Reproducibility	
		Number of samples	Coincidence of results,%	Number of samples	Coincidence of results,%
variant FRT-50-0,2	Positive	10	100	30	100
	Negative	10	100	30	100
variant FRT-50 F	Positive	10	100	30	100
	Negative	10	100	30	100
variant FRT-L	Positive	10	100	30	100
	Negative	10	100	30	100

14. REFERENCES

1. The Picornavirus Pages, [electronic source] - Available from: http://www.picornaviridae.com.
2. Enterovirus surveillance guidelines. Guidelines for enterovirus surveillance in support of the Polio Eradication Initiative//World Health Organization, 2015.

15. QUALITY CONTROL

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

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.07.23 EM	3. Content Footer	REF H-2771-1-2-CE; REF H-2773-1-CE; REF H-2773-1-4-CE were added
28.05.24 HM	Footer	REF H-2773-1-4-CE was added
	Content	Variant FRT-L in bulk was added

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