AmpliSens® *Enterovirus / Parechovirus*-FRT PCR kit

IVD

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code		Contains sufficient for <n> tests
IVD	*In vitro* diagnostic medical device		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
EC REP	Authorized representative in the European Community	C+	Positive control of amplification
		IC	Internal control

1. INTENDED USE

AmpliSens® *Enterovirus / Parechovirus*-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of RNA of *Human enterovirus* clusters A, B, C, D without differentiation, and RNA of *Human parechovirus* clusters A, B without differentiation in the biological material (feces, cerebrospinal fluid, swabs from respiratory tract, sputum, tissue (biopsy, autopsy) material from human), and environmental samples (water sample concentrates) using real-time hybridization-fluorescence detection of amplified products. The material for PCR is cDNA samples obtained by reverse transcription of RNA extracted from test material.

Indications and contra-indications for use of the reagent kit

The reagent kit can be used for investigation of biological material in clinical laboratory medicine (diagnostics), taken from the persons suspected of enteroviral and/or parechoviral infection without distinction of form and presence of the manifestation, and for investigating of environmental samples and autopsy material in order to determine post-mortem diagnosis.

There are no contra-indications with the exception of cases when the material cannot be taken for medical reasons.

NOTE: The results of PCR analysis are taken into account in complex diagnostics of disease.

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)), the RNA reverse transcription reaction and amplification of cDNA fragments of detecting viruses and cDNA of the internal control (Internal Control-FL (IC)) with hybridization-fluorescence detection. Exogenous internal control (Internal Control-FL (IC)) allows to control all PCR-analysis stages for each individual sample and to identify possible reaction inhibition.

Amplification of cDNA fragments with the use of specific primers and Taq-polymerase enzyme are performed with the RNA samples obtained at the extraction stage. 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 contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and deoxyuridine triphosphate (dUTP).

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

Table 1

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

3. CONTENT

AmpliSens® *Enterovirus / Parechovirus*-FRT PCR kit is produced in 1 form:
variant FRT-50-0,2, REF H-3751-1-2-CE
Variant FRT-50-0,2 includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *Enterovirus / Parechovirus* 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	colorless clear liquid	1.1	1 tube
C+ *Enterovirus / Parechovirus*	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).

4. ADDITIONAL REQUIREMENTS

- Transport medium for storage and transportation of respiratory swabs.
- Puncture needles.
- Flocked or fiber swabs for collecting nasopharyngeal specimens from kids and adults.
- Sterile swab with viscose tip in individual package – for sampling from oropharynx of children and adults (it is acceptable to use it for sampling from mucous membrane of inferior nasal meatus of adults).
- Sterile plastic container (50-60 ml) for sampling, storage and transportation of biological samples.
- Reagent for pretreatment of viscous fluids (sputum, aspirates).
- 0.9 % of sodium chloride (sterile saline solution) or phosphate buffered saline (PBS) (137 mM sodium chloride; 2,7 mM potassium chloride; 10 mM sodium monophosphate; 2 mM potassium diphosphate; pH=7,5±0,2).
- Glycerin for long-term storage of biological material (feces) under low-temperature freezing conditions.
- Microcentrifuge for Eppendorf tubes (RCF max. 12,000 x g).
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and mallet) or homogenizer for pretreatment of viscera material.
- RNA extraction kit.
- Reverse transcription kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips (up to 1,000 µl) and pipette tips with aerosol filters (up to 100 µl, 200 µl, 1000 µl).
- Tube racks.
- Vortex mixer.
- 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 and 2-ml tubes for sampling.
  b) screwed or tightly closed 1.5 and 2-ml tubes for pretreatment.
- 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® *Enterovirus / Parechovirus*-FRT PCR kit is intended for analysis of the RNA extracted with RNA extraction kits from the biological material (feces, cerebrospinal fluid, swabs from respiratory tract, sputum, tissue material from human), 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 tubes 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 – no more than 1 week;
- at the temperature not more than minus 68 °C – for a long time.

The cerebrospinal fluid samples can be transported at 2–8 °C for 1 day.

Feces. Sampling feces are taken from a disposable reservoir (for example, Petrie dish, disposable plastic bag) placed in a bed-pan or disposable diapers (for children). When using a disposable diaper for children with watery consistency of feces, a cotton swab 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 (or 1ml) of the sample into special sterile plastic container.

The feces samples can be stored before the pretreatment:
- at the temperature from 18 to 25 °C – no more than 6 hours;
- 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-thawing cycle is required.

The feces samples can be transported at 2–8 °C for 3 days.

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

The water sample concentrates 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 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 material can be transported at 2–8 °C for 1 day.

Sputum or *tracheal aspirate* is collected into sterile hermetic disposable plastic containers after gargling the oral cavity with water.

The test material 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 – no more than 1 week.

Only one freeze-thawing cycle is required.

The material can be transported at 2–8 °C for 1 day.

Swabs from respiratory tract

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 are obtained through the inferior nasal meatus using sterile dry flocked swabs with plastic shafts. If the nasal cavity is full of mucus it is recommended to blow 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 it in the inferior nasal meatus under the inferior nasal concha, 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 **Transport Medium for Storage and Transportation of Respiratory Swabs**, the flexible part of the swab is minimized by a spiral, then, covering the top of the tube with a lid, the handle of the swab is lowered down, achieving complete breaking off the end of shaft. Close and mark the tube with the solution and the swab.

The test material can be stored before the analysis:
- 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-thawing cycle is acceptable.

The material can be transported at 2–8 °C for 3 days.

Oropharyngeal swabs are obtained using sterile dry rayon swabs by rotating 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 **Transport Medium for Storage and Transportation of Respiratory Swabs**. Break off the end of shaft to allow tight closing of tube cap. Close and mark the tube with the solution and the swab.

The test material can be stored before PCR analysis:
- at the temperature from 2 to 8 °C – for 3 days;
- at the temperature from minus 24 to minus 16 °C – for 1 week.

Only one freeze-thawing cycle is acceptable.

The material can be transported at 2–8 °C for 3 days.

Tissue (biopsic, autopsy) material is taken with a sterile tool (for example, tweezers) into a sterile plastic 50-ml container with tightly closed cap or 2 ml tube. The tube is to be closed tightly.

The tissue (biopsic, autopsy) material samples can be stored:
- at the temperature from 2 to 8 °C – no more than 1 day,
- at the temperature from minus 24 to minus 16 °C – no more than 1 week,
- at the temperature not more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

The material can be transported at 2–8 °C for 1 day.

Pretreatment

Pretreatment of *water 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.

Sputum or *tracheal aspirate* is to be pretreated.

Use reagent **Mucolysin** manufactured by CRIE for sputum and aspirate pretreatment. See the Instruction manual to **Mucolysin** for a proper use. The pretreated sputum (100 µl) is used for RNA extraction.

The pretreated samples can be stored before the PCR analysis:
- at the temperature from 2 to 8 °C – no more than 1 day,
- at the temperature not more than minus 68 °C – for a long time.

Swabs from respiratory tract are to be pretreated.

Vortex the tube, then centrifuge it at 5,000 rpm for 5 s to sediment drops from the interior wall of the tube lid. 100 µl of sample is taken for RNA extraction.

The pretreated samples can be stored before the PCR analysis:
- at the temperature from 2 to 8 °C – for 1 day,
- at the temperature not more than minus 68 °C – for a long time.

Tissue (biopsic, autopsy) material is to be pretreated.

Tissue (biopsic, autopsy) material from human is homogenized using sterile porcelain mortars and pestles. Then, prepare a 10 % suspension in 0.9 % sodium chloride solution (sterile saline solution) or phosphate buffer (PBS). Transfer the suspension to a 1.5-ml tube and centrifuge at 10,000 rpm for 5 min. The supernatant (100 µl) is used for RNA extraction.

The pretreated samples of tissue material can be stored before the PCR analysis:
- at the room temperature – no more than 6 hours;
- at the temperature from minus 24 to minus 16 °C – no more than 1 week,
- at the temperature not more than minus 68 °C – for a long time.

Interfering substances and limitations of using test material samples

In order to control the efficiency of RNA, RT and 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.

Potential interfering substances

Endogenous and exogenous substances that may be present in the biological material (cerebrospinal fluid, feces, swabs from respiratory tract, sputum or tracheal aspirate, tissue (biopsic, autopsy) material) used for the study were selected to assess potential interference.

Model samples of cerebrospinal fluid, feces, swabs from respiratory tract, sputum or tracheal aspirate, tissue (biopsic, autopsy) material were tested without adding and with the addition of potentially interfering substances. Concentration of each potentially interfering substance is represented in table 2. Model samples with quality control sample (QCS) containing *Enterovirus*-rec RNA and *Parechovirus*-rec RNA in concentration $5x10^3$ GE/ml for cerebrospinal fluid, swabs from respiratory tract, $1x10^4$ GE/ml for feces, autopsy material and $3x10^4$ GE/ml for sputum or tracheal aspirate.

Table 2

Type of test material	Type of potential interferent	Potential interferent	Tested concentration in sample	Interference
Feces	Endogenous substances	Whole blood	Up to 40%	Not observed
		Fecal fat	Up to 40%	Not observed
		Mucin (mucus)	Up to 3%	Not observed
	Exogenous substances	Enterofuryl, suspension for ingestion	Up to 4.25 mg/ml	Not observed
		Enterosgel, oral paste	Up to 174.75 mg/ml	Not observed
		Dextrin (Russia)	Up to 68.6 mg/ml	Not observed
Cerebrospinal fluid	Endogenous substances	Whole blood	Up to 4 %	Not observed
	Exogenous substances	Ceftriaxone, Powder for solution for injection/infusion	Up to 8 mg/ml	Not observed
		Iodium, solution for external use alcohol 5%	Up to 0.03%	Not observed
Swabs from respiratory tract	Endogenous substances	Whole blood	Up to 4%	Not observed
		Mucin (mucus)	Up to 5%	Not observed
	Exogenous substances	Miramistin	Up to 5%	Not observed
		Lugol solution with glycerin	Up to 1%	Not observed
Sputum	Endogenous substances	Whole blood	Up to 4%	Not observed
		Mucin (mucus)	Up to 5%	Not observed
	Exogenous substances	Fluditec, syrup	Up to 10 mg/ml	Not observed
Tissue material	Endogenous substances	Whole blood	Up to 40%	Not observed
	Exogenous substances	Formalin solution, neutral buffered, 10%	Up to 10%	Not observed
		Dexamethasone, solution for injection	Up to 0.32 mg/ml	Not observed

7. WORKING CONDITIONS

AmpliSens® *Enterovirus / Parechovirus*-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 kits:
– **RIBO-prep**.

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

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–)** into the tube labeled C– (Negative Control of Extraction).
The volume of elution is **50 µl**.

NOTE: It is recommended to carry out the reverse transcription reaction just after the obtaining the RNA samples. It is allowed to store the 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 is acceptable.

8.2. Reverse transcription

It is recommended to use the following kit for the complementary DNA (cDNA) synthesis from the RNA:
– **REVERTA-L**.

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

8.3. Preparing PCR

8.3.1 Preparing tubes for PCR

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 / Parechovirus*** 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 / Parechovirus.***
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 / Parechovirus*** 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 sample** obtained by extraction and reverse transcription of the **Negative Control (C–) reagent** to the tube labeled C– (Negative Control of Extraction).

8.3.2. Amplification

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

Table 3

AmpliSens unified amplification program for rotor-type[1] and plate-type[2] 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, ROX	

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. 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. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin*.
3. 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.

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

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring and indicating the amplification product accumulation in 3 channels:

Table 4

Channel for the fluorophore	FAM	JOE	ROX
Amplification product	Internal Control-FL (IC) cDNA	*Enterovirus* cDNA	*Parechovirus* 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 cDNA sample in the corresponding column of the results grid.
Principle of interpretation is the following:

Table 5

Results interpretation

***Ct* value in the channel for the fluorophore**			**Result**
FAM	**JOE**	**ROX**	
< boundary value	absent or > boundary value	absent or > boundary value	*Enterovirus / Parechovirus* RNA **is NOT detected**
> boundary value or < boundary value	< boundary value	> boundary value or < boundary value	*Enterovirus* RNA **is detected**
> boundary value or < boundary value	> boundary value or < boundary value	< boundary value	*Parechovirus* RNA **is detected**
absent or > boundary value	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 PCR analysis is considered reliable only if the results obtained for the controls of amplification and extraction are correct (see Table 5).

Table 6

Results for controls

Control	Stage for control	***Ct* value in the channel for fluorophore**		
		FAM	**JOE**	**ROX**
C–	RNA extraction	< boundary value	absent or > boundary value	absent or > boundary value
NCA	PCR	absent or > boundary value	absent or > boundary value	absent or > boundary value
C+	PCR	< boundary value	< 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 any of the channels for the FAM, JOE, ROX fluorophores (see Table 6) is greater than the boundary value or absent. The amplification and detection should be repeated for all samples in which the specific RNA was not detected.
2. The *Ct* value determined for the Negative Control of Extraction (C–) in the channels for the JOE and/or ROX fluorophores 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 any of the channels for the FAM, JOE, ROX fluorophores (see Table 6) 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.

If you have any further questions or if you encounter problems, please contact our Authorized representative in the European Community.

11. TRANSPORTATION

AmpliSens® *Enterovirus / Parechovirus*-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® *Enterovirus / Parechovirus*-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-buffer-K).
All components of the **AmpliSens® *Enterovirus / Parechovirus*-FRT** PCR kit are stable until the expiry date stated on the label. PCR kit **variant FRT-50-0,2** can be stored without unpacking at 2 to 8 °C for 3 months from the date of manufacture before opening. Once opened, PCR kit **variant FRT-50-0,2** should be unpacked in accordance with the storage temperatures for each component. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-buffer-K is to be stored at the temperature from minus 24 to minus 16 °C

NOTE: PCR-mix-FL *Enterovirus / Parechovirus* is to be kept away from light

13. SPECIFICATIONS

13.1. Analytical sensitivity (limit of detection)

Table 7

Biological material	Nucleic acid extraction kit	Reverse transcription kit	PCR kit	Analytical sensitivity (limit of detection)[3], GE/ml
Sputum	RIBO-prep	REVERTA-L	variant FRT-50-0,2	$3x10^4$
Feces				$1x10^4$
Tissue material				$1x10^4$
Water sample concentrates				$5x10^3$
Cerebrospinal fluid				$5x10^3$
Swabs from respiratory tract				$5x10^3$

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

[1] For example, Rotor-Gene Q (QIAGEN, Germany).
[2] For example, CFX 96 (Bio-Rad, USA).
[3] Number of genome equivalents (GE) of the microorganism per 1 ml of the test material sample.

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *Enterovirus* / *Parechovirus*-FRT** PCR kit is ensured by the selection of specific primers and probes as well as stringent reaction conditions. The primers and probes have been checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The reagent kit detects cDNA-fragments of the established microorganisms. Analytical specificity of the reagent kit is proved by the examination of following nucleic acid sample panels:

1. *Human enteroviruses* RNA (the representatives of different genetic clusters: cluster A – *Human coxsackievirus* A4, A5, A6, A9, A16 ; cluster B – *Human echovirus* 2, 6, 9, 11, 14, 16, 17, 18, 30 and *Human coxsackievirus* B4, B5 (the clinical samples; the species affiliation was proved by the direct sequencing of the nucleotide sequences); cluster C – *Human poliovirus* 1, 2, 3 (the vaccinal strains); cluster D – *Enterovirus* D68 (ATCC® VR-1825PQ™)); *Parechovirus* A and B (the clinical samples; the species affiliation was proved by the direct sequencing method of the nucleotide sequences) in concentration not less than $1x10^4$ GE/ml. Positive results were obtained while testing RNA samples of the strains / clinical samples listed above.
2. *Influenza viruses* A RNA (H3N2 (NCBI:txid2029290), H1N1 (NCBI:txid1898984),) and B, *Rhinoviruses*, *RS viruses*, *Human adenoviruses* DNA – 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 concentration not less than $1x10^4$ GE/ml (clinical specimens, species affiliation was proved by the direct sequencing of the nucleotide sequences).
3. Nucleic acid samples extracted from strains from ATCC collection in concentration not less than $1x10^6$ microbial cells/ml (American Type Culture Collection, USA): of *Acinetobacter baumannii* 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 minutissimum* ATCC® 23348™ CL1904 3, *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™, *Gardnerella vaginalis* ATCC® 14018™, *Haemophilus influenzae* ATCC® 33930™, *Haemophilus influenzae* ATCC® 9006™, *Haemophilus influenzae* ATCC® 10211™, *Haemophilus parainfluenzae* ATCC® 7901™, *Klebsiella oxytoca* ATCC® 49131™, *Klebsiella pneumoniae* ATCC® 27736™, *Listeria grayi* (*murrayi*) ATCC® 25401™, *Listeria innocua* ATCC® 33090™, *Listeria monocytogenes* ATCC® 7644™, *Moraxella* (*Branhamella*) *catarrhalis* ATCC® 25238™, *Moraxella catarrhalis* ATCC® 25240™, *Moraxella* (*Branhamella*) *catarrhalis* ATCC® 8176™, *Neisseria gonorrhoeae* ATCC® 49926™, *Neisseria gonorrhoeae* ATCC® 19424™, *Neisseria lactamica* ATCC® 23970™, *Neisseria meningitidis* ATCC® 13102™ (Serogroup C), *Neisseria meningitidis* ATCC® 13090™ (Serogroup B), *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™, *Streptococcus pneumoniae* ATCC® 6303™, *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™, *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® 27336™, *Streptococcus pneumoniae* ATCC® 6305™, *Streptococcus pyogenes* ATCC® 19615™, *Streptococcus salivarius* ATCC® 13419™, *Streptococcus uberis* ATCC® 700407™, *Vibrio parahaemolyticus* ATCC® 17802™.

The nonspecific reactions were absent while testing 2[d] and 3[d] panels as well as human DNA samples (0.2 mg/ml) (Sigma-Aldrich, USA).

The clinical specificity of **AmpliSens® *Enterovirus* / *Parechovirus*-FRT** PCR kit was confirmed in laboratory clinical trials.

The information about interfering substances is specified in the *Interfering substances and limitations of using test material samples*.

13.3. Repeatability and reproducibility

Repeatability and reproducibility were determined by testing of positive and negative model samples. Positive samples was a quality control sample (QCS) containing *Enterovirus* RNA, *Parechovirus* RNA in concentration $5x10^3$ GE/ml, Negative Control (C–) was used as 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 in two independent laboratories, by different operators, on different days, using different equipment. The results are presented in Table 8.

Table 8

Samples type	Repeatability		Reproducibility	
	Number of samples	Agreement of results, %	Number of samples	Agreement of results, %
Positive	10	100	40	100
Negative	10	100	40	100

13.4. Diagnostic characteristics

Table 9

The results of testing AmpliSens® *Enterovirus* / *Parechovirus*-FRT PCR kit in comparison with the reference assay

Detectable pathogen	Type of test material	The results of application of AmpliSens® *Enterovirus* / *Parechovirus*-FRT PCR kit		Results of using the reference assay [4]	
				Positive	Negative
Enterovirus	Feces	281 samples were tested	**Positive**	103	5
			Negative	0	173
	Water sample concentrates	137 samples were tested	**Positive**	36	0
			Negative	0	101
	Cerebrospinal fluid	341 samples were tested	**Positive**	182	4
			Negative	0	155
	Swabs from respiratory tract	202 samples were tested	**Positive**	38	0
			Negative	1	163
	Sputum samples	147 samples were tested	**Positive**	65	0
			Negative	0	82
	Tissue material	136 samples were tested	**Positive**	39	0
			Negative	0	97
Parechovirus	Feces	281 samples were tested	**Positive**	59	0
			Negative	0	222
	Water sample concentrates	137 samples were tested	**Positive**	31	0
			Negative	0	106
	Cerebrospinal fluid	341 samples were tested	**Positive**	45	0
			Negative	0	296
	Swabs from respiratory tract	202 samples were tested	**Positive**	40	0
			Negative	0	162
	Sputum samples	147 samples were tested	**Positive**	62	0
			Negative	0	85
	Tissue material	136 samples were tested	**Positive**	39	1
			Negative	0	97

To assess the diagnostic characteristic were used:

- 281 samples of feces obtained from the patients with serous meningitis and clinically healthy individuals (ambulatory vaccinated children) in the period 2013-2021.
- 341 samples of cerebrospinal fluid from patients with serous and purulent meningitis in the period 2007-2011.
- 202 samples of swabs from respiratory tract obtained from the patients with serous meningitis and acute respiratory infections in the period 2014-2021.
- 85 samples of sputum obtained from the patients with serous meningitis and from the patients acute respiratory infections of various etiologies as well as 62 model samples contaminated with dilutions of QCS[5] no. 47 Positive Control *Enterovirus*-rec and QCS no. 243 Positive Control *Parechovirus*-rec in concentration of $3x10^4$ GE/ml.
- 113 samples of autopsy material obtained from the patients with a fatal outcome in the period 2014-2020 as well as 23 model samples contaminated with QCS no. 47 Positive Control *Enterovirus*-rec and QCS no. 243 Positive Control *Parechovirus*-rec in concentration of $1x10^4$ GE/ml.
- 137 samples of water concentrates (106 samples received from the subjects of the Russian Federation as part of the activities of the Russian Reference Center for monitoring pathogens of intestinal infections in 2015-2020 and 31 model samples contaminated with dilutions of QCS no. 47 Positive Control *Enterovirus*-rec and QCS no. 243 Positive Control *Parechovirus*-rec in concentration of $5x10^3$ GE/ml).

Table 10

Diagnostic characteristics of AmpliSens® *Enterovirus* / *Parechovirus*-FRT PCR kit

Detectable pathogen	Type of test material	Diagnostic sensitivity[6] (with a confidence level of 95 %)	Diagnostic specificity[7] (with a confidence level of 95 %)
Enterovirus	Feces	100 (97.1-100) %	97.2 (94.8-100) %
	Water sample concentrates	100 (92.02-100) %	100 (97.1-100) %
	Cerebrospinal fluid	100 (98.4-100) %	97.5 (95.0-100) %
	Swabs from respiratory tract	97.4 (92.3-100) %	100 (98.2-100) %
	Sputum	100 (95.5-100) %	100 (96.4-100) %
	Tissue material	100 (92.6-100) %	100 (97.0-100) %
Parechovirus	Feces	100 (95.0-100) %	100 (98.7-100) %
	Water sample concentrates	100 (90.8-100) %	100 (97.2-100) %
	Cerebrospinal fluid	100 (93.6-100) %	100 (99.0-100) %
	Swabs from respiratory tract	100 (92.8-100) %	100 (98.2-100) %
	Sputum	100 (95.3-100) %	100 (96.5-100) %
	Tissue material	100 (92.6-100) %	100 (97.0-100) %

14. REFERENCES

1. Enterovirus surveillance guidelines. Guidelines for enterovirus surveillance in support of the Polio Eradication Initiative//World Health Organization, 2015.
2. Human Parechoviruses. Aizawa Y, Saitoh A. Uirusu. 2015;65(1):17-26. doi: 10.2222/jsv.65.17. Review.

[4] **AmpliSens® *Human enterovirus*-FRT** PCR kit was used as a reference assay for *Enterovirus* detection. The *Parechovirus* detection was proved by the direct sequencing of amplification products.

[5] QCS - quality control sample.

[6] Relative sensitivity in comparison with applied reference assay.

[7] Relative specificity in comparison with applied reference assay.

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 the **AmpliSens® *Enterovirus* / *Parechovirus*-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
30.08.19 DV	Through the text	The text formatting was changed
	1. Intended use	The subsection Indications and contra-indications for use of the reagent kit was added
	6. Sampling and handling	The information about transportation of biological material was added
	13. Specifications	The section was rewritten
	14. References	The section was actualized
27.05.20 MA	Footer	The phrase "Not for use in the Russian Federation" was added
17.11.20 MA	Through the text	The clarification "biopsic, autopsy" was added for tissue material
	1. Intended use	The sections were actualized
	2. Principle of PCR detection	
	4. Additional requirements	Transport medium for storage and transportation of respiratory swabs was added
	6. Sampling and handling	The subsection "Interfering substances and limitations of using test material samples" was rewritten The subsection "Potential interfering substances" was added
	13.2. Analytical specificity	The list of strains was actualized
	13.3. Repeatability and reproducibility	The section was added
	13.4. Diagnostic characteristics	Quantity of tested samples and their diagnostic sensitivity and specificity parameters were changed
04.03.21 MM	1. Intended use	The examples of clusters A, B, C, D for Human enterovirus was removed. The cluster C for RNA of human parechovirus was deleted
	13.2. Analytical specificity	The clusters A, B, C, D for Human enterovirus was added
12.03.21 EM	—	The name, address and contact information for Authorized representative in the European Community was changed
04.10.21 EM	1. Intended use	The section was updated
	13.2. Analytical specificity	Microorganism strains and RNA/DNA samples were replaced with nucleic acid samples
	12. Stability and storage	The information about storage conditions for 3 months from the date of manufacture and subsequent unpacking was added
20.10.21 KK	3. Content 8. Protocol 8.1.RNA extraction	The RIBO-prep, REF K2-9-Et-50-CE was change to RIBO-prep, REF K2-9-Et-100-CE.
20.01.22 KK	Through the text	The reference numbers of nucleic acid extraction kits and transport mediums were deleted
27.04.22 KK	4. Additional requirements	The section was actualized
	13.4. Diagnostic characteristics	The results of application of AmpliSens® *Enterovirus* / *Parechovirus*-FRT PCR kit and results of using the reference assay were changed

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