RUO

For Professional Use Only

AmpliSens® *EBOV* Zaire 1-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® *EBOV* Zaire 1-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of the *EBOV* Zaire 1 RNA in the biological material (whole blood, saliva, urine, viscera biopsy material) using real-time hybridization-fluorescence detection of amplified products.

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

2. PRINCIPLE OF PCR DETECTION

EBOV Zaire 1 detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific *EBOV* Zaire 1 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.

EBOV Zaire 1 detection by the polymerase chain reaction (PCR) includes three stages: the phenolic RNA extraction from the test samples, combined stage of the RNA reverse transcription and the given microorganism cDNA fragment amplification and real-time hybridization-fluorescence detection.

AmpliSens® *EBOV* Zaire 1-FRT PCR kit is a qualitative test that contains the Internal Control (Internal Control STI-87-rec (IC)). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

AmpliSens® *EBOV* Zaire 1-FRT PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. In variant FRT-100 F, “hot-start” is guaranteed by the separation of nucleotides and Taq-polymerase by using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT

AmpliSens® *EBOV* Zaire 1-FRT PCR kit is produced in 2 forms:

AmpliSens® *EBOV* Zaire 1-FRT PCR kit variant FRT-100 F, **REF** R-V69-F-CE.

AmpliSens® *EBOV* Zaire 1-FRT PCR kit variant FRT-100 F includes:

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-FL *EBOV* Zaire 1	colorless clear liquid	1.2	1 tube
PCR-buffer-C	colorless clear liquid	0.6	1 tube
Polymerase (TaqF)	colorless clear liquid	0.06	1 tube
TM-Revertase (MMlv)	colorless clear liquid	0.03	1 tube
RT-G-mix-2	colorless clear liquid	0.015	2 tubes
Positive Control *EBOV* Zaire 1 (C+$_{EBOV\ Zaire\ 1}$)	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 STI-87-rec (IC)**	colorless clear liquid	0,5	2 tubes

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

** add 10 µl of Internal Control during the RNA extraction procedure directly to the sample/lysis mixture (see RIBO-zol-B, **REF** K2-3-50-CE protocol).

AmpliSens® *EBOV* Zaire 1-FRT PCR kit is intended for 110 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- RNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips with aerosol filters (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Real-time instruments (for example, Rotor-Gene Q (QIAGEN, Germany)).
- Disposable polypropylene PCR tubes (0.1- or 0.2-ml) when working with PCR kit variant FRT-100 F:

a) 0.2-ml PCR tubes with optical transparent domed or flat caps if a plate-type instrument is used;
b) 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.

- 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 barriers 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 samples and reagents contact with the skin, eyes, and mucous membranes. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- 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.

6. SAMPLING AND HANDLING

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® *EBOV* Zaire 1-FRT PCR kit is intended for analysis of the RNA extracted with RNA extraction kits from the biological material (whole blood, saliva, urine, viscera biopsy material).

6.1. *Whole blood*

Blood samples are taken after overnight fasting in tubes with 6 % EDTA solution (1:20). Closed tubes with blood are to be rotated gently several times (for mixing with the anticoagulant). Transfer 1.5 ml of whole blood with EDTA in the new tubes and centrifuge the tubes at 800 g (380 g if the rotor diameter is 50 mm) for 10 min. Then transfer the top layer (500-600 µl) of plasma with leucocytes to the next tube and centrifuge the tubes at 8000 g at 5 min. Transfer the supernatant (except for 200 µl of liquid over the cells sediment) into the container with disinfection solution. **Use the cells sediment and 100 µl of supernatant for RNA extraction.**

The whole blood samples can be stored at 20-25 °C for 2 hours, at 2- 8 °C for 12 hours.

6.2. *Saliva*

Saliva samples are taken (after 3 mouthwashes with boiled water) in sterile tubes (1.5 ml) in an amount of 0.2-1.0 ml. Saliva samples can be stored at 2- 8 °C for 1 day or at the temperature from minus 24 to minus 16 °C for 1 month or at not more than minus 68 °C for a long time.

The pretreatment of saliva samples is not required. **For extraction use 100 µl of obtained sample.**

6.3. *Urine*

The urine samples are taken in an amount of 15-25 ml into the dry sterile container (50-60 ml) or dry clean vessels. In case of it is no opportunity to examine the material, transfer the urine sample into the centrifuge tube of 20-ml or Eppendorf tube. Add glycerin (10% of sample volume), mix up and freeze for storage (at the temperature minus 20 °C – for 1 week, at the temperature minus 70 °C – for a long time).

Pretreatment

- Centrifuge the sample at 8000-9000 g for 10 min. Transfer the supernatant (except for 1 µl of liquid over the cells sediment) into the container with disinfection solution, the cells sediment and 1 ml of supernatant over it – into the tube. Then centrifuge the sample at 8000 g for 10 min. Transfer 900 µl of the supernatant into the container with disinfection solution. **Use the cells precipitate and 100 µl of the supernatant for RNA extraction.** In case of large amounts of saline for RNA extraction transfer 100 µl of supernatant into the separate tube.
- In case of absence of centrifuge for 20-ml tubes and with 8000-g acceleration, concentrate the pathogen only from 1 ml of urine sample (as described above). **Use the cells precipitate and 100 µl of the supernatant for RNA extraction.**

6.4. *Viscera biopsy material*

The viscera biopsy material is put using sterile tools into sterile disposable containers with tight-fitting lids or into the tubes (2 ml).

Store the samples at the temperature from minus 24 to minus 16 °C for 1 week, after that – at not more than minus 68 °C. The material may be frozen and thawed only once.

For RNA extraction take 30-50 µl of the material and homogenize it by trituration using precooled sterile porcelain mortar and pounder or using tissue grinder. Prepare suspension using grinded tissue and precooled sterile physiological solution or phosphate buffer (1:5). **Use 100 µl of suspension for RNA extraction.**

7. WORKING CONDITIONS

AmpliSens® *EBOV* Zaire 1-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. RNA extraction

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

– RIBO-zol-B, **REF** K2-3-50-CE - 2 items;

Extract the RNA according to the manufacturer's protocol.

The RNA extraction for each sample is carried out in the presence of **Internal Control STI-87-rec (IC).**
Add **10 µl** of **Internal Control STI-87-rec (IC)** to each tube with samples.
Add **100 µl** of **Negative Control (C–)** and **10 µl** of **Internal Control STI-87-rec (IC)** to the tube labeled C– (Negative Control of Extraction).

8.2. Preparing reverse transcription and PCR

8.2.1 Preparing tubes for RT-PCR

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

1. Calculate the required quantity of each reagent for one reaction:
   - **10 µl** of **PCR-mix-FL *EBOV* Zaire 1,**
   - **5 µl** of **PCR-bufer-C,**
   - **0.5 µl** of **Polymerase(TaqF),**
   - **0.25 µl** of **TM-Revertase (MMlv),**
   - **0.25 µl** of **RT-G-mix-2**. per one reaction.

Prepare the reaction mixture for required number of reactions (including clinical and control samples and one extra reaction).

Prepare the reaction mixture just before use

2. Thaw the required number of tubes with **PCR-mix-FL *EBOV* Zaire 1** and **RT-G-mix-2.** Thoroughly vortex all the reagents of the PCR-kit and sediment the drops from the caps of the tubes.
3. In a new sterile tube prepare the reaction mixture in accordance to the calculations.
4. Take the required number of the tubes taking into account the number of test samples and control samples.
5. Transfer **15 µl** of the prepared mixture to each tube. Discard the unused reaction mixture.
6. Add **10 µl** of **RNA samples** extracted from test or control samples of RNA extraction stage using tips with filter.

Avoid transferring of sorbent together with the RNA samples extracted by RIBO-sorb kit.

7. Carry out the control reactions:

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

C+$_{EBOV\ Zaire\ 1}$ - Add **10 µl** of **Positive Control *EBOV* Zaire 1 (C+$_{EBOV\ Zaire\ 1}$)** to the tube labeled (**C+$_{EBOV\ Zaire\ 1}$**) (Positive 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).

8.2.2. Reverse transcription and amplification

Make sure that the amplification run starts just after the addition of RNA to the reaction mixture

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

Table 1

AmpliSens unified amplification and detection program for rotor-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescent signal detection*	*Cycles*
1	50	30 min	–	1
2	95	15 min	–	1
3	95	10 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	10 s	–	40
	60	20 s	**FAM, JOE**	
	72	15 s	–	

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

2. Adjust the fluorescence channel sensitivity according to *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

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

– The signal of the IC cDNA amplification product is detected in the channel for the FAM fluorophore.

– The signal of the *EBOV* Zaire 1 cDNA amplification product is detected in the channel for the JOE fluorophore.

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at a specific level that corresponds to the presence (or absence) of a *Ct* value of a cDNA sample in the corresponding column of the result grid.

The threshold line is set in the middle of fluorescence growth area of Positive Control of Amplification (C+) in the logarithmic scale.

The results are interpreted in accordance with the Table 2.

Table 2

Results interpretation

***Ct* value in the channel for the fluorophore**		**Result**
FAM	**JOE**	
< boundary value	absent	*EBOV* Zaire 1 RNA **is not detected**
< boundary value	< boundary value	*EBOV* Zaire 1 RNA **is detected**
absent or > boundary value	absent or > boundary value	**Invalid** result Repeat the extraction and amplification

Principle of interpretation is the following:

– *EBOV* Zaire 1 RNA is **detected** if the *Ct* value determined in the results grid in the channel for the JOE fluorophore is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.

– *EBOV* Zaire 1 RNA is **not detected** in a sample if the *Ct* value is not determined (absent) in the channels for JOE fluorophores, whereas the *Ct* value determined in the channel for the FAM fluorophore is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.

– The result is **invalid** if the *Ct* value is not determined (absent) in the channel for JOE fluorophores, whereas the *Ct* value in the channel for the FAM fluorophore is not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the RNA extraction stage.

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

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (Table 3).

Table 3

Results for controls

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

10. TROUBLESHOOTING

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

1. If the *Ct* value determined for the Positive Control of Amplification (C+) in the channel for **JOE** fluorophore is greater than the boundary *Ct* value or absent, the amplification and detection (beginning with the RNA extraction stage) should be repeated for all samples in which the *EBOV* Zaire 1 RNA was not detected.
2. If the *Ct* value determined for the Negative Control of Extraction (C–) in the channel for **JOE** fluorophore is less than the boundary *Ct* value, PCR analysis (beginning with RNA extraction stage) should be repeated for all samples in which *EBOV* Zaire 1 RNA was detected.
3. If the *Ct* value determined for the Negative Control of Amplification (NCA) in the channel for **FAM** and/or **JOE** fluorophores, 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. If the *Ct* value 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 that threshold line or parameters of threshold line measurement are correct. If the result has been obtained with the correct threshold line level, the amplification and detection should be repeated for this sample.

11. TRANSPORTATION

AmpliSens® *EBOV* Zaire 1-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® *EBOV* Zaire 1-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-FL *EBOV* Zaire 1, PCR-buffer-C, RT-G-mix-2, polymerase (TaqF), TM-Revertase (MMlv)). All components of the **AmpliSens® *EBOV* Zaire 1-FRT** PCR kit are stable until the expiry date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

PCR-mix-FL *EBOV* Zaire 1, PCR-buffer-C, RT-G-mix-2, polymerase (TaqF) and TM-Revertase (MMlv) are to be stored at the temperature from minus 24 to minus 16 °C

PCR-mix-FL *EBOV* Zaire 1 is to be kept away from light

13. SPECIFICATIONS

13.1. Analytical specificity

The analytical specificity of **AmpliSens® *EBOV* Zaire 1-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.

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, Moscow, 2010.

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® *EBOV* Zaire 1-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code		Sufficient for
RUO	Research use only		Expiration Date
VER	Version		Consult instructions for use
	Temperature limitation		Keep away from sunlight
	Manufacturer		Date of manufacture
IC	Internal control	**C–**	Negative control of extraction
NCA	Negative control of amplification	**C+** ***EBOV*** **Zaire 1**	Positive control of amplification
EC REP	Authorised representative in the European Community		