AmpliSens® FiloA-screen-FRT PCR kit

RUO

For Professional Use Only

Instruction Manual

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
PCE	Positive control of extraction	IC	Internal control

1. INTENDED USE

AmpliSens® FiloA-screen-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of RNA of Ebola virus variant Zaire (*EBOV* Zaire), Ebola virus variant Sudane *(SUDV)* and Marburg virus *(MARV)* of *Filoviridae* family in the human biological material (whole blood or its components: leucocytes and plasma), taken from the persons suspected of filoviral haemorrhagic fevers without distinction of form and presence of manifestation, using real-time hybridization-fluorescence detection of amplified products.

NOTE: For research use only. Not for diagnostic procedures

2. PRINCIPLE OF PCR DETECTION

The method is based on the RNA extraction from the test material with the exogenous Internal Control (Internal Control-FL (IC)) and simultaneous RNA reverse transcription reaction and amplification of the cDNA fragments of the detected viruses and Internal Control-FL (IC) cDNA with hybridization-fluorescence detection. The Internal Control-FL (IC) allows to control all the stages of the PCR analysis of each sample and evaluate the effect of inhibitors on the results of the PCR analysis.

The detection by the polymerase chain reaction (PCR) is based on the RNA reverse transcription using TM-Revertase enzyme and amplification of the pathogens genome specific regions using specific 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.

At the amplification stage 4 reactions are carried out in one tube simultaneously: amplification of *EBOV* Zaire cDNA, *SUDV* cDNA, *MARV* cDNA as well as amplification of Internal Control-FL (IC) cDNA. The results of amplification of *EBOV* Zaire cDNA, *SUDV* cDNA, *MARV* cDNA and Internal Control-FL (IC) cDNA are registered in 4 different fluorescence channels.

Table 1

Channel for fluorophore	FAM	JOE	ROX	Cy5
cDNA-target	*EBOV* Zaire cDNA	*SUDV* cDNA	*MARV* cDNA	IC cDNA
Target gene	Polymerase	Transcription Factor	Nucleoprotein	Artificial nucleotide sequence

3. CONTENT

AmpliSens® FiloA-screen-FRT PCR kit is produced in 1 form:
variant FRT-L, REF H-2781-1-4-CE.
Variant FRT-L includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix FiloA-Lyo	white powder	–	96 tubes of 0.2 ml
C+ FiloA	colorless clear liquid	0.5	1 tube
TE-buffer	colorless clear liquid	0.5	1 tube
Positive Control FiloA	colorless clear liquid	0.1	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	8 tubes
Internal Control-FL (IC)**	colorless clear liquid	1.0	1 tube

* must be used in the extraction procedure as Negative Control of Extraction.
** add **10 µl** of **Internal Control-FL (IC)** during the RNA extraction procedure directly to the sample/lysis mixture (see **RIBO-zol-B**, **RIBO-prep** protocol).
Variant FRT-L is intended for 96 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- Vacuum blood collection system.
- Disposable polypropylene tightly closed 1.5 and 2-ml tubes for pretreatment.
- RNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips with aerosol filters (up to 100, 200, 1,000 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)).
- 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 positive material (specimens, controls) away from all other reagents and add it to the reaction mix in a distantly separated facility. Store and handle amplicons away from all other reagents.
- 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® FiloA-screen-FRT PCR kit is intended for analysis of the RNA extracted with RNA extraction kits from the biological material (whole blood or its components).

Sampling

Whole peripheral blood. Blood samples are taken in the morning after overnight fasting in a tube with 6 % EDTA in a ratio 1:20. After blood sampling the tube should be smoothly rotated several times for the thoroughly mixing with the anticoagulant. (Otherwise, blood will coagulate and DNA extraction will be impossible!). Place the tube in the rack after rotating. The RNA extraction is performed from 100 µl of whole blood.

Blood samples can be stored before the PCR analysis or pretreatment.

– at the temperature from 20 to 25 °C – for 2 hour,
– at the temperature from 2 to 8°C – for 12 hour,

Freezing of whole blood samples is unallowable.

If it is necessary to carry out the analysis of blood components (leucocytes and plasma), the additional pretreatment should be performed (see the section "Pretreatment")

Perform the pretreatment of the samples not later than the above mentioned time.

It is allowed to transport the material at the temperature from 2 to 8 ° C for 12 hours.

Pretreatment

Whole blood samples. The pretreatment is not required.

For obtaining the *leucocytes and plasma* the pretreatment is required for blood samples.

Using a filter tip transfer 1.5 ml of blood with EDTA into a sterile disposable 2.0-ml tube. Centrifuge at 40 g (for example, 800 rpm for the MiniSpin Eppendorf microcentrifuge) for 10 min. Using a new one filter tip transfer 500-600 µl of supernatant (plasma with leucocytes) into sterile disposable 1.5-ml tube (do not take the pellet with erythrocytes).

The supernatant contain plasma and leucocytes and can be used for the RNA extraction procedure form 100 µl.

For the separation of plasma and leucocytes centrifuge the tubes with supernatant at 7,000 g (for example, 10,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 5 min. The lower fraction contains blood leukocytes, the upper fraction contains blood plasma. The RNA extraction is performed from 100 µl of blood leucocytes suspension or 100 µl of blood plasma (in case of extraction from 200 µl increase volume to 200 µl by the Negative Control (C–) reagent).

Blood plasma and/or leucocytes (pellet) 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.

Interfering substances and limitations of using test material samples

In order to control the RNA extraction efficiency and possible reaction inhibition 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.

The next samples are inapplicable for analysis:

– the whole blood samples, collected in the tubes with heparin as anticoagulant,
– the whole blood samples, containing blood clot or which has been exposed to freezing.

7. WORKING CONDITIONS

AmpliSens® FiloA-screen-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-zol-B**;
- **RIBO-prep**.

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

The volumes of reagents and samples when the RNA is extracted by the RIBO-zol-B, RIBO-prep nucleic acid extraction kits:

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.

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

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

Add **90 µl** of **Negative Control (C–)** and **10 µl** of **Positive Control FiloA** to the tube labeled PCE (Positive Control of Extraction).

The volume of elution is **50 µl.**

NOTE: It is recommended to carry out the reverse transcription and amplification 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 ≤ – 68 °C for 1 year. Only one freeze-thaw cycle is allowed.

8.2. Preparing PCR/RT-RCR

8.2.1. Preparing tubes for RT-RCR

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

Use disposable filter tips for adding reagents, RNA and control samples into tubes.

1. Take the required number of the tubes with ready-to-use lyophilized reaction mixture **PCR-mix FiloA-Lyo** for RT-PCR of RNA from test and control samples (see the number of control samples in point 3).
2. Add **25 µl** of **RNA samples** extracted from test samples into the prepared tubes.

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

3. Carry out the control amplification reactions:

C+ – Add **25 µl** of **C+ FiloA** 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).

PCE – Add **25 µl** of **the sample extracted from the Positive Control FiloA reagent** to the tube labeled **PCE** (Positive 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.

8.2.2. Amplification

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

Table 2

AmpliSens unified amplification program for rotor-type[1] instruments

Step	Temperature, °C	Time	Fluorescent signal detection	Cycles
1	50	15 min	–	1
2	95	15 min	–	1
3	95	10 s	–	42
	60	20 s	**FAM, JOE, ROX, Cy5**	

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

9. DATA ANALYSIS

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

Table 3

Channel for the fluorophore	FAM	JOE	ROX	Cy5
Signal registration, indicating the amplification product accumulation	*EBOV* Zaire cDNA	*SUDV* cDNA	*MARV* cDNA	IC 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 4

Results interpretation

***Ct* value in the channel for the fluorophore**				**Result**
FAM	**JOE**	**ROX**	**Cy5**	
absent	absent	absent	< boundary value	*EBOV* Zaire, *SUDV* and *MARV* RNA **is not detected**
< boundary value	absent	absent	determined or absent	*EBOV* Zaire RNA **is detected**
absent	< boundary value	absent	determined or absent	*SUDV* RNA **is detected**
absent	absent	< boundary value	determined or absent	*MARV* RNA **is detected**

* In case of **invalid** result, the PCR analysis should be repeated for the corresponding test sample starting from the RNA extraction stage. It is recommended, if possible, to repeat sampling of the test material.

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 5).

Table 5

Results for controls

Control	Stage for control	***Ct* value in the channel for fluorophore**			
		FAM	**JOE**	**ROX**	**Cy5**
PCE	RNA extraction	< boundary value	< boundary value	< boundary value	< boundary value
C–	RNA extraction	Absent	Absent	Absent	< boundary value
NCA	RT-PCR	Absent	Absent	Absent	Absent
C+	RT-PCR	< boundary value	< 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 reverse transcription and amplification (C+) in any of the channels for fluorophores (see table 5) is greater than the boundary *Ct* value or absent. The amplification and detection should be repeated for all samples.
2. The *Ct* value determined for the Positive Control of Extraction (PCE) in the channels for the FAM and/or JOE and/or ROX fluorophores is greater than the boundary *Ct* value or absent. The PCR analysis (beginning with the RNA extraction stage) should be repeated for all samples.
3. For the Negative Control of Extraction (C–):
   a) The *Ct* value is determined in any of the channels for the FAM and/or JOE and/or ROX 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 PCR analysis (beginning with the RNA extraction stage) should be repeated for all samples in which specific RNA was detected.
   b) The *Ct* value determined in the channel for the Cy5 fluorophore is greater than the boundary *Ct* value or absent. It means that the C– sample did not perform the function of contamination control. The PCR analysis (beginning with the RNA extraction stage) should be repeated for all samples in which specific RNA was detected.
4. The *Ct* value is determined for the Negative Control of reverse transcription and amplification (NCA) in the channels for the FAM and/or JOE and/or ROX 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.
5. 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.

[1] For example, Rotor-Gene Q (QIAGEN, Germany).

11. TRANSPORTATION

AmpliSens® FiloA-screen-FRT PCR kit should be transported at 2–8 °C for no longer than 5 days. PCR kit can be transported at 2–25 °C for no longer than 3 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® FiloA-screen-FRT** PCR kit are to be stored at 2–8 °C when not in use. All components of the **AmpliSens® FiloA-screen-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.

NOTE: PCR-mix FiloA-Lyo is to be stored in a desiccated pack and is to be kept away from light

13. SPECIFICATIONS

13.1. Analytical sensitivity (limit of detection)

Table 6

Biological material	The volume of sample for extraction, µl	Nucleic acid extraction kit	PCR kit	Analytical sensitivity (limit of detection), copies/ml
Whole blood or its components	100	RIBO-zol-B, RIBO-prep	variant FRT-L	10^3

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® FiloA-screen-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 PCR kit detects the RNA fragments of the claimed viruses. In order to confirm the PCR kit analytical specificity, samples containing genomic DNA/RNA of viruses/microorganisms capable of causing diseases similar in clinical manifestations with filovirus fevers were examined:

- *Bunyaviruses* (*Bunyaviridae* family): *CCHFV*.
- *Flaviviruses* (*Flaviviridae* family): *Yellow fever virus, Dengue virus, WNV, TBE, JEV.*
- *Togaviruses* (*Togaviridae* family): *Chikungunya virus*.
- *Rhabdoviruses* (*Rhabdoviridae* family): *Rabies virus*.
- *Rickettsia* DNA *(R. prowazekii, R. canadensis, R. sibirica).*

Also samples containing DNA/RNA of viruses causing acute and chronic infections, that could be present in the test samples and produce false positive results on filoviruses, were examined:

- *Flaviviruses* (*Flaviviridae* family): *HCV.*
- *Retroviruses* (*Retrovoridae* family): *HIV*.
- *Hepadnaviruses* (*Hepadnaviridae* family): *HBV*.

Human genomic DNA was used to prove the absence of cross reactions with DNA-material of the patient.

The nonspecific responses were not observed while testing the DNA/RNA samples of the above mentioned organisms, as well as human DNA. The information about the known interfering substances is specified in the section “Sampling and handling”.

14. REFERENCES

1. VG Dedkov, Magassouba NF, Safonova MV, Deviatkin AA, Dolgova AS, Pyankov OV, Sergeev AA, Utkin DV, Odinokov GN, Safronov VA, Agafonov AP, Maleev VV, Shipulin GA. 2015. Development and evaluation of a real-time RT-PCR assay for the detection of Ebola virus (Zaire) during an Ebola outbreak in Guinea in 2014-2015. J Virol Methods. 2015 Nov 18;228:26-30. doi: 10.1016/j.jviromet.2015.11.007

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® FiloA-screen-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

AmpliSens®

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