AmpliSens® *Yellow fever virus*-FRT PCR kit

Instruction Manual

For Professional Use Only

KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code	Σ	Sufficient for
IVD	*In vitro* diagnostic medical device		Use-by Date
VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer		Keep dry
	Date of manufacture	C–	Negative control of extraction
EC REP	Authorized representative in the European Community	C+	Positive control of amplification
		PCE	Positive control of extraction
		IC	Internal control

1. INTENDED USE

AmpliSens® *Yellow fever virus*-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Yellow fever virus* (*YFV)* RNA in the biological material (blood plasma, saliva, urine, tissue (autopsy, biopsy), mosquitoes) using real-time hybridization-fluorescence detection of amplified products. The material for RT-PCR is RNA-samples extracted from test material.

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

2. PRINCIPLE OF PCR DETECTION

The principle of testing is based on the RNA extraction from test samples together with the exogenous internal control (Internal Control-FL (IC)) and simultaneous RNA reverse transcription and amplification of DNA fragments of the detected microorganism and DNA of the internal control 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.

Yellow fever virus detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region 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.

AmpliSens® *Yellow fever virus*-FRT PCR kit is a qualitative test that contains the Internal Control (Internal Control-FL (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® *Yellow fever virus*-FRT PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by the separation of nucleotides and Taq-polymerase using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

At the RT-PCR stage 2 reactions are carried out in one tube simultaneously: amplification of *Yellow fever virus* cDNA as well as amplification of Internal Control-FL (IC) cDNA. The results of amplification of *Yellow fever virus* cDNA and Internal Control-FL (IC) cDNA are registered in 2 different fluorescence channels.

Table 1

Channel	FAM	JOE
DNA-target	Internal Control-FL (IC) cDNA	*Yellow fever virus* cDNA
Target gene	Artificially synthesized sequence	5'-noncoding region and part of the genome encoding the nucleoprotein (5'-UTR-C-protein)

3. CONTENT

AmpliSens® *Yellow fever virus*-FRT PCR kit is produced in 2 forms:

variant FRT-50 F REF H-2461-1-CE.

variant FRT-L REF H-2462-1-4-CE.

Variant FRT-50 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *YFV*	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+ *YFV*	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Positive Control *YFV**	colorless clear liquid	0.1	1 tube
Internal Control-FL (IC)****	colorless clear liquid	0.5	1 tube
Negative Control (C–)*****	colorless clear liquid	1.2	7 tubes

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

** 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** or **MAGNO-sorb** protocols).

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

Variant FRT-L includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix *YFV*-Lyo	white powder	-	48 tubes of 0.2 ml
C+ *YFV*	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Positive Control *YFV**	colorless clear liquid	0.1	1 tube
Internal Control-FL (IC)****	colorless clear liquid	0.5	1 tube
Negative Control (C–)*****	colorless clear liquid	1.2	7 tubes

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

** 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** or **MAGNO-sorb** protocols).

Variant FRT-L is intended for 48 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- Vacuette® blood collection system.
- Plastic container (50-60 ml) for sampling, storage and transportation of biological samples.
- Medical centrifuge with accessories.
- Reagent for pretreatment of saliva.
- 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 the longterm storage of biological material (feces) at severely cold conditions.
- Microcentrifuge for Eppendorf tubes (RCF max. 12,000 x g).
- Vortex mixer.
- Vacuum aspirator with flask for removing supernatant.
- RNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free and pipette tips with filters (up to 100 µl, 200 µl, 1000 µl and 5000 µl).
- Tube racks.
- PCR box.
- Real-time instruments with 2 (or more) independent detection channels (for example, Rotor-Gene Q (QIAGEN, Germany), CFX96 (Bio-Rad, USA)).
- Disposable polypropylene PCR tubes:
  a) tightly closed 2.0-ml tubes for sampling;
  b) screwed or tightly closed 1.5-ml tubes for pretreatment and reaction mixture preparation;
  c) 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;
  d) 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.
- Refrigerator with the range from 2 to 8 °C.
- Deep-freezer with the range 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 (samples, 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 afterward.
- 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 samples and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance 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 the 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 to 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® *Yellow fever virus*-FRT PCR kit is intended for the analysis of RNA extracted with RNA extraction kits from the biological material (blood plasma, saliva, urine, tissue (autopsy, biopsy), mosquitoes).

Sampling

6.1*Blood plasma.*

To obtain blood plasma one should be taken blood after overnight fasting into a disposable tube with EDTA (special vacuum system). After blood sampling the tube should be gently inverted several times for the thoroughly mixing with the anticoagulant. Within 6 hours after blood collection, plasma should be taken and transferred to a new tube. To do this, centrifuge tubes with whole blood at 800-1600 g for 20 minutes at room temperature. Transfer the obtained plasma (at least 1 ml) using individual tips with aerosol barrier into sterile dry 2.0-ml tubes.

The pretreated samples of blood plasma can be stored before PCR analysis:

- at the temperature from 2 to 8 °C - for 1 day;
- at the temperature from minus 24 to minus 16 °C – for 2 days;
- at the temperature no more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

6.2*Tissue (autopsy, biopsy) material.*

The material should be taken using sterile tool (for example, a pair of tweezers) into the disposable 50-ml containers with wide mouth or 2.0-ml tubes. Tightly close the tube.

The material samples can be stored:

- at the temperature from 18 to 25 °C - for 6 hours;
- at the temperature from 2 to 8 °C - for 3 days;
- at the temperature from minus 24 to minus 16 °C – for 1 week;
- at the temperature no more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

6.3*Urine.*

The first portion of first void urine is taken for analysis in an amount 15-25 ml into the dry sterile container (50-60 ml). Collect urine after thorough toilet of external genitals. If it is impossible to examine the material within 1 day after the sampling, then it is necessary to pretreat the material.

The material samples can be stored:

- at the temperature from 2 to 8 °C – for 24 hours.

NOTE: Freezing of urine samples is unacceptable!

6.4*Saliva.*

Saliva samples are taken (after 3 mouthwashes with physiological solution) in sterile 2.0-ml tubes in an amount of not less than 1.0 ml. Tightly close the tube.

The material samples can be stored:

- at the temperature from 18 to 25 °C - for 6 hours;
- at the temperature from 2 to 8 °C - for 1 day;
- at the temperature from minus 24 to minus 16 °C – for 1 week;
- at the temperature no more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

6.5*Mosquitoes.*

The collected material should be sorted in laboratory according to species, gender, places and dates of collection and placed into the dry sterile 2.0-ml tube. Number of mosquitoes in pool for analysis should not exceed 50.

The material samples can be stored after sorting and sample forming:

- at the temperature from minus 24 to minus 16 °C – for 1 month;
- at the temperature no more than minus 68 °C or in a Dewar flask with liquid nitrogen – for a long time.

Only one freeze-thawing cycle is acceptable.

Pretreatment

6.6Pretreatment of *blood plasma samples* is not required.

6.7*The urine samples* are to be pretreated if the samples of muddy urine were given for analysis. In such case transfer 1200 µl of urine into the 1.5-ml tube. Centrifuge at 10,000 g (for example, 12,000 for the MiniSpin Eppendorf microcentrifuge) for 1 min. Use 100 µl of obtained clarified urine for RNA extraction using **RIBO-prep** or 1000 µl of clarified urine for RNA extraction using **MAGNO-sorb**. If the material will be tested later than 1 day after collection, it is necessary to transfer 1100 µl of urine into few 1.5-ml tubes. If it is expected the RNA extraction using **RIBO-prep** add glycerin (10% of sample volume) into the tubes with urine, vortex for uniformly mixing of glycerin. If it is expected the RNA extraction using **MAGNO-sorb** do not add glycerin, the urine samples must be freezed without glycerin.

The urine samples with/without glycerin can be stored:

- at the temperature from minus 24 to minus 16 °C – for 1 week;
- at the temperature no more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

6.8*Tissue (autopsy, biopsy) material.*

Take 30-50 mg (µl) of material for RNA extraction and homogenize it using precooled sterile porcelain mortar and mallet or homogenizer. Prepare 10% solution using ground tissue and cooled 0.9 % sodium chloride solution (sterile saline solution) or phosphate buffer (PBS) (1:9 ratio).

Centrifuge the samples at 10,000 g (for example, 12,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 1 min. Use 100 µl of the obtained clarified suspension RNA extraction.

The pretreated tissue samples can be stored before PCR analysis:

- at the temperature from minus 24 to minus 16 °C – for 1 week;
- at the temperature no more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

6.9*The mosquitoes* are to be pretreated

Preliminary form mosquitoes pools (no more than 50). Homogenize mosquitoes in 0.9 % sodium chloride solution (sterile saline solution) or phosphate buffer (PBS) at the rate of 1 mosquito – 30 µ of solution. Centrifuge the samples at 10,000 g (for example, 12,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 1 min. Take 100 µ of supernatant for RNA extraction.

Use sterile porcelain mortar and mallet for preparing mosquito suspension. In case of using an automatic homogenizer TissueLyser LT (QIAGEN, Germany) the following homogenization parameters for mosquitoes should be applied: balls’ diameter – 5 mm, frequency – 50 Hz/s, time of homogenization – 5 min, buffer volume – 700 µl (pool of 25 mosquitoes), 1500 µl (pool of 50 mosquitoes).

The urine samples with/without glycerin can be stored:

- at the temperature from minus 24 to minus 16 °C – for 1 week;
- at the temperature no more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

6.10 *The saliva samples* are to be pretreated

It is necessary to deliquate salive using **Mucolysin** reagent before RNA extraction. Add **Mucolysin** to the container with saliva samples (1 part of saliva to 3 parts of Mucolysin). Stir occasionally the container for 10 min. Use 100 µl of deliquated salive for RNA extraction.

The samples of deliquated salive can be stored before PCR analysis:

- at the temperature from minus 24 to minus 16 °C – for 1 week;
- at the temperature no more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

Interfering substances and limitations of using test material samples

The whole blood samples collected in the tubes with heparin as anticoagulant are not applicable for analysis as the heparin is PCR inhibitor. Information about other interfering substances are absent providing that the rules for sampling and handling of the test material specified in the instruction manual are observed.

To reduce the risk of obtaining a false negative result due to the presence of interfering substances in the sample 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.

7. WORKING CONDITIONS

AmpliSens® *Yellow fever virus*-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-prep** for RNA extraction from blood plasma, saliva, urine, tissue (autopsy, biopsy) material, mosquitoes,
- **MAGNO-sorb** for RNA extraction from blood plasma and urine.

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

The volumes of reagents and samples when the RNA is extracted by **RIBO-prep** nucleic acid extraction 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 with samples.

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

NOTE: 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 *YFV*** to the tube labeled PCE (Positive Control of Extraction).

The volume of elution is **50 µl.**

In case of using PCR kit variant FRT-L for the amplification carry out the RNA elution in **100 µl** of **Buffer for elution.**

The volumes of reagents and samples when the RNA is extracted by **MAGNO-sorb** nucleic acid extraction kit for 200 µl of the sample:

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 with samples.

The volume of the test sample is **200 µl.**

NOTE: Add **200 µl** of **Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).

Add **190 µl** of **Negative Control (C–)** and **10 µl** of **Positive Control *YFV*** to the tube labeled PCE (Positive Control of Extraction).

The volume of elution is **50 µl,** in case of using automatic station for RNA extraction **– 100 µl.**

In case of using PCR kit variant FRT-L for the amplification carry out the RNA elution in **100 µl** of **Buffer for elution.**

The volumes of reagents and samples when the RNA is extracted by **MAGNO-sorb** nucleic acid extraction kit for 1000 µl of the sample:

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 with samples.

The volume of the test sample is **1000 µl.**

NOTE: Add **1000 µl** of **Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).

Add **990 µl** of **Negative Control (C–)** and **10 µl** of **Positive Control *YFV*** to the tube labeled PCE (Positive Control of Extraction).

The volume of elution is **50 µl,** in case of using automatic station for RNA extraction **– 100 µl.**

In case of using PCR kit variant FRT-L for the amplification carry out the RNA elution in **100 µl** of **Buffer for elution.**

It is recommended to perform RT-PCR reaction immediately after RNA samples obtaining.

NOTE: It is allowed to store RNA samples at the temperature from 2 to 8 °C for no more than 30 minutes, at the temperature from minus 24 to minus 16 °C for not more than one week and at the temperature no more than minus 68 °C for a year. Only one freeze-thawing cycle for RNA samples is allowed.

8.2. Preparing reverse transcription and PCR

8.2.1. Preparing tubes for PCR

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 *YFV*,**
   - **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**.
2. Prepare the reaction mixture for the total number of test and control samples plus several extra reactions. See numbers of control samples in item 4.

NOTE: Prepare the reaction mixture just before use.

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

NOTE: Avoid transferring of sorbent together with the RNA samples extracted by reagent kit with magnetic separation method.

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

8. **Carry** out the control amplification reactions:

C+ – Add **10 µl** of **C+ *YFV*** 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).

PCE – Add **10 µl** of **the sample extracted from the Positive Control *YFV* 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.

Variant FRT-L

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 *YFV*-Lyo** for RT-PCR of RNA from test and control samples (see numbers of control samples in point 3).
2. Add **25 µl** of **RNA samples** extracted from test samples into the prepared tubes.

NOTE: Avoid transferring of sorbent together with the RNA samples extracted by reagent kit with magnetic separation method.

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

3. Carry out the control reactions:

C+ – Add **25 µl** of **C+ *YFV*** 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 *YFV* 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-[1] and plate-type[2] instruments

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

NOTE: Any combination of the tests including test 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.

2. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin* and Guidelines [2].
3. Insert tubes into the reaction module of the device.

NOTE: It is recommended to sediment drops from walls of tubes by short centrifugation 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 fluorescence signal accumulation in two channels:

Table 3

Channel for the fluorophore	FAM	JOE
Signal registration, indicating the amplification product accumulation	Internal Control-FL (IC) cDNA	*Yellow fever virus* cDNA

Results are interpreted by the crossing (or not-crossing) the 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 4

Results interpretation

***Ct* value in the channel for the fluorophore**		**Result**
FAM	**JOE**	
< boundary value	absent	*Yellow fever virus* RNA **is not detected**
determined or absent *	< boundary value	*Yellow fever virus* RNA **is detected**
absent or > boundary value	absent or > boundary value	**Invalid result****
< boundary value	> boundary value	**Equivocal*****

* The signal absence in the channel for FAM fluorophore is irrelevant if *Yellow fever virus* RNA is detected.

** In case of **invalid result**, it is necessary to repeat PCR-study of the corresponding test sample, starting from the RNA extraction stage.

*** In case of **equivocal result** it is necessary to repeat PCR-study of the corresponding test sample, starting from the RNA extraction stage. If the same result was obtained once again, the sample is considered positive. If the negative result was obtained, the sample is considered equivocal and re-sampling of the material for analysis is recommended.

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

The results of the analysis is considered reliable only if the results obtained for controls of RT-PCR and extraction stages are correct (according to Table 5 and the *Important Product Information Bulletin* enclosed to the PCR kit).

Table 5

Results for controls

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

[1] For example, Rotor-Gene Q (QIAGEN, Germany).
[2] For example, CFX96 (Bio-Rad, USA).

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 the FAM and/or JOE fluorophore is greater than the boundary *Ct* value or absent. The amplification and detection should be repeated for all samples in which the specific RNA was not detected.
2. If the *Ct* value determined for the Positive Control of Extraction (PCE) in the channel for the JOE fluorophore 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. If the *Ct* value is determined for the Negative Control of Extraction (C–) in the channel for the JOE fluorophore. 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 the specific RNA was detected.
4. If the *Ct* value is determined for the Negative Control of Amplification (NCA) in the channels for the 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 the specific RNA was detected.
5. If 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 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.

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

11. TRANSPORTATION

AmpliSens® *Yellow fever virus*-FRT PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Yellow fever virus*-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-FL *YFV*, PCR-buffer-C, Polymerase (TaqF), TM-Revertase (MMlv), RT-G-mix-2). All components of the **AmpliSens® *Yellow fever virus*-FRT** PCR kit are stable until labeled expiration date. The shelf life of opened reagents is the same as that of unopened reagents, unless otherwise stated.

NOTE: PCR-mix-FL *YFV*, 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-FL *YFV* is to be kept away from light

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

13. SPECIFICATIONS

13.1. Analytical sensitivity (limit of detection)

Test material	Sample volume for extraction, µl	Nucleic acid extraction kit	PCR kit	Analytical sensitivity (limit of detection), copies/ml
Blood plasma	100	RIBO-prep	variant FRT-50 F, FRT-L	1000
Mosquitoes (homogenate)	100		variant FRT-50 F, FRT-L	1000
Urine	100		variant FRT-50 F, FRT-L	1000
Saliva	100		variant FRT-50 F, FRT-L	1000
Tissue (autopsy, biopsy) material	100		variant FRT-50 F, FRT-L	5000
Blood plasma	200	MAGNO-sorb	variant FRT-50 F, FRT-L	1000
	1000		variant FRT-50 F, FRT-L	100
Urine	1000		variant FRT-50 F, FRT-L	500

Table 6

The results of AmpliSens® *Yellow fever virus*-FRT PCR kit analytical sensitivity validation with using the material from healthy people and patients with another causation of disease and mosquitoes, contaminated by strains 17-D, Kintampo, Bwamba 1441 Nyamande 207

Type of sample	Sample volume, µl	RNA concentration (copies per ml of the sample)	Strain/ Number of replicates	Number of samples	Number of positives	Hit Rate, %
Blood plasma	100	$1x10^3$	17-D	20	20	100
			Kintampo	20	20	100
			Bwamba 1441 Nyamande 207	20	20	100
	200	$1x10^3$	Bwamba 1441 Nyamande 207	20	20	100
	1000	$1x10^2$	Bwamba 1441 Nyamande 207	20	20	100
Urine	100	$1x10^3$	17-D	20	20	100
			Kintampo	20	20	100
			Bwamba 1441 Nyamande 207	20	20	100
	1000	$5x10^2$	Bwamba 1441 Nyamande 207	20	20	100
Saliva	100	$1x10^3$	17-D	20	20	100
			Kintampo	20	20	100
			Bwamba 1441 Nyamande 207	20	20	100
Mosquitoes	100	$1x10^3$	17-D	10	10	100
			Kintampo	10	10	100
			Bwamba 1441 Nyamande 207	10	10	100
Tissue (autopsy, biopsy) material	100	$1x10^3$	17-D	10	10	100
			Kintampo	10	10	100
			Bwamba	10	10	100

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® *Yellow fever virus*-FRT** PCR kit is ensured by 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 specificity was proved on the follows strains of microorganisms and biological material obtained from healthy people or patients with another causation of disease:

Table 7

The results of AmpliSens® *Yellow fever virus*-FRT PCR kit analytical specificity validation

Organisms	The channel for the FAM fluorophore (Internal control)	The channel for the JOE fluorophore (*Yellow fever virus*)
Dengue virus	Valid	Negative
Langat virus	Valid	Negative
Powassan virus	Valid	Negative
West Nile virus	Valid	Negative
Japanese encephalitis virus	Valid	Negative
Omsk hemorrhagic fever virus	Valid	Negative
Tick-borne encephalitis virus	Valid	Negative
Chikungunya virus	Valid	Negative
50 urine samples from the patients with another causation of disease	Valid	Negative
50 blood saliva from the patients with another causation of disease	Valid	Negative
50 biopsy material samples from the patients with another causation of disease	Valid	Negative
50 cerebral fluid samples from the patients with another causation of disease	Valid	Negative
50 samples of *Aedes albopictus* pools	Valid	Negative

14. REFERENCES

1. Guidelines Gisela Freitas Trindade, Sheila Maria Barbosa de Lima, Constança Britto, Alice Gomes Fernandes-Monteiro, Detection of Yellow Fever Virus by Quantitative Real-Time PCR (qPCR) . 2020;2065:65-77.

15. QUALITY CONTROL

In accordance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Total Quality Management System, each lot of **AmpliSens® *Yellow fever virus*-FRT** PCR kit is tested against predetermined specifications to ensure consistent product quality.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
05.04.18 PM	3. Content	The colour of the reagent was specified
12.05.20 MA	Through the text	The text formatting was changed
	Footer	The phrase "Not for use in the Russian Federation" was added
12.03.21 MM	—	The name, address and contact information for Authorized representative in the European Community was changed
29.08.22 KK	Through the text	The reference numbers of nucleic acid extraction kits and transport mediums were deleted
	14. References	The section was updated

AmpliSens®

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