AmpliSens® *Zika virus*-FRT PCR kit

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
IVD	*In vitro* diagnostic medical device		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limit	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**C+**	Positive control of amplification
EC REP	Authorized representative in the European Community	**IC**	Internal control
	Caution	**PCE**	Positive control of extraction

1. INTENDED USE

AmpliSens® *Zika virus*-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of RNA of *Zika virus* in the biological material (blood plasma, saliva, urine, spermatic fluid, histological material (autopsy/biopsy material, placenta), amniotic fluid, mosquitos, oropharyngeal swabs), taken from the persons suspected of Zika fever without distinction of form and presence of manifestation, using real-time hybridization-fluorescence detection of amplified products.

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

2. PRINCIPLE OF PCR DETECTION

Zika virus detection by the polymerase chain reaction (PCR) is based on the RNA extraction from the test samples and simultaneous carrying out the RNA reverse transcription and cDNA fragments amplification with hybridization-fluorescence detection.

The RNA reverse transcription using TM-revertase and the amplification of the pathogen genome specific region using specific *Zika virus* primers and Taq-polymerase are carried out with 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.

AmpliSens® *Zika virus*-FRT PCR kit is a qualitative test that contains the Internal Control (Internal Control ICZ-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® *Zika 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 amplification stage 2 reactions are carried out in one tube simultaneously: amplification of cDNA fragments of *Zika virus* as well as amplification of Internal Control ICZ-rec (IC) cDNA. The results of amplification of *Zika virus* cDNA and Internal Control ICZ-rec (IC) cDNA are registered in 2 different fluorescence channels.

Table 1

Channel for fluorophore	FAM	JOE
cDNA-target	IC cDNA	*Zika virus* cDNA
Target gene	Artificially synthesized sequence	NS3

3. CONTENT

AmpliSens® *Zika virus*-FRT PCR kit is produced in 1 form:
variant FRT-50 F REF H-2411-1-CE.

Variant FRT-50 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *ZIKV*	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+ *ZIKV* / *ICZ*	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Positive Control *ZIKV*	colorless clear liquid	0.1	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	7 tubes
Internal Control *ICZ*-rec (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 *ICZ*-rec (IC)** during the RNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep** protocol or **MAGNO-sorb** protocol).

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

4. ADDITIONAL REQUIREMENTS

- Transport medium.
- Reagent for pretreatment of viscous fluids (saliva, spermatic fluid, amniotic fluid).
- 0.9 % of sodium chloride (sterile saline solution) or PBS buffer solution (137 mM sodium chloride; 2,7 mM potassium chloride; 10 mM sodium monophosphate; 2 mM potassium diphosphate; pH=7,5±0,2).
- Glycerin for urine pretreatment.
- Flocked swab for collection, transportation and storage of biological samples.
- Vacuum blood collection system.
- Sterile plastic container (50-60 ml) for sampling, storage and transportation of biological samples.
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and mallet) or homogenizer for pretreatment of histological material and mosquitos.
- RNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips with filters (up to 100 µl, 200 µl, 1,000 µl, 5,000 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), CFX 96 (Bio-Rad, USA)).
- Disposable polypropylene tubes:
  a) tightly closed 2.0-ml tubes for sampling.
  b) screwed or tightly closed 1.5 tubes for pretreatment
  c) screwed or tightly closed 1.5-ml tubes for reaction mixture preparation.
  d) thin-walled 0.2-ml PCR tubes with optical transparent domed or flat caps or strips of eight 0.2-ml tubes with optical transparent caps if a plate-type instrument is used;
  e) thin-walled 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used.
- 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.
- 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® *Zika virus*-FRT PCR kit is intended for analysis of the RNA extracted with RNA extraction kits from the biological material (blood plasma, saliva, urine, spermatic fluid, histological material (autopsy/biopsy material, placenta), amniotic fluid, mosquitos, oropharyngeal swabs).

The test material is collected by clinical staff.

Accounting, storage, transferring and transportation of the biological material suspected of *Zika virus* should be carried out in accordance with local regulations.

Sampling

6.1. Blood plasma. Blood samples are taken after overnight fasting into a tube (special vacuum blood collection system) with EDTA as anticoagulant. The closed tube with blood is rotated several times. The tubes with the whole blood are to be centrifuged no later than 6 hour from the blood collection at 800-1,600 g (for example, 3,500-5,000 rpm for the Eppendorf microcentrifuge) for 20 min at room temperature. No less than 1 ml of the obtained plasma is transferred into the sterile 2.0-ml tubes using a new one filter tip for each sample.

The blood plasma samples can be stored before the PCR analysis:

– at the temperature from 2 to 8 °C – for 1 day,
– at the temperature from minus 24 to minus 16 °C – for 1 week,
– at the temperature not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

*6.2. Histological (*autopsy/biopsy material, placenta*) material.* The material is taken by 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 material samples can be stored:

– at room temperature – for 6 hour,
– 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 not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

6.3. *Urine.* The first morning specimen is collected in an amount of 15-25 ml into the dry sterile container (50-60 ml) after cleansing the urethral area. If there is no opportunity to analyze the material within 1 day after collection, it is necessary to carry out the pretreatment of the material.

The material samples can be stored:

– at the temperature from 2 to 8 °C – for 24 hour,

Freezing of the material is not allowed!

6.4. *Spermatic fluid.* The material is taken into the dry sterile container (50-60 ml).

The material samples can be stored:

– at room temperature – for 6 hour,
– 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 not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

6.5. *Amniotic fluid.* The material is taken in an amount of no less than 1.0 ml into the dry sterile disposable 2.0-ml tube. The tube should be closed tightly.

The material samples can be stored:

– at room temperature – for 6 hour,
– 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 not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

6.6. *Saliva.* The mouth should be rinsed three times prior to saliva collection. The material is taken in an amount of no less than 1.0 ml into the dry sterile disposable 2.0-ml tube. The tube should be closed tightly.

The material samples can be stored:

– at room temperature – for 6 hour,
– 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 not more than minus °C – for a long time.

Only one freeze-thawing cycle is required.

6.7. *Mosquitos.* The collected material is sorted into species, sex, places and dates of collection and placed into the dry sterile disposable 2.0-ml tube. Number of mosquitos in pool for analysis should not exceed 10.

The material samples can be stored after sorting and samples formation:

– at the temperature from minus 24 to minus 16 °C – for 1 month,
– at the temperature not more than minus 70 °C or in the Dewar flask with liquid nitrogen – for a long time.

Only one freeze-thawing cycle is required.

6.8. *Oropharyngeal swabs.* The material is taken with a sterile dry probe with a viscose tip. Rotate the probe over the tonsillar area, palatine arches, and posterior area of the oropharynx. Place the lower part of the probe (with the viscose tip) into the sterile disposable tube containing a special transport medium. Break off the probe, tightly close the cap.

The material samples can be stored:

– at room temperature – for 6 hour,
– at the temperature from 2 to 8 °C – for 3 days,
– at the temperature from minus 24 to minus 16 °C – for 1 month,
– at the temperature not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

Pretreatment

6.9. *The blood plasma samples, oropharyngeal swabs and clear urine samples.* The pretreatment is not required.

6.10. *Urine samples* are to be pretreated in case of cloudy urine. Transfer 1,200 µl of urine in 1.5 ml tubes. Centrifuge at 10,000 g (for example, 12,000 rpm for the Eppendorf microcentrifuge) for 1 min. 100 µl of obtained clarified suspension is used for RNA extraction by RIBO-prep nucleic acid extraction kit or 1,000 µl of obtained clarified suspension is used for RNA extraction by MAGNO-sorb nucleic acid extraction kit. If the material will be analyzed later than 1 day after sampling, it is necessary to transfer 1,100 µl of urine into several 1.5-ml tubes, and then add glycerin in amount of 10 % of sample volume (120 µl). Vortex the tubes for homogeneous mixing of glycerin.

The material samples with glycerin can be stored:

– at the temperature from minus 24 to minus 16 °C – for 1 week,
– at the temperature not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

6.11. *Histological (*autopsy/biopsy material, placenta*) material* is to be pretreated. For RNA extraction take 30-50 mg (µl) of the material and homogenize it by trituration using precooled sterile porcelain mortar and mallet or homogenizer. Prepare suspension using grinded tissue and precooled sterile physiological solution or phosphate buffer. For this, add 9 volumes of physiological solution to 1 volume of grinded tissure. Centrifuge at 10,000 g (for example, 12,000 rpm for the Eppendorf microcentrifuge) for 1 min. Use 50 µl of obtained clarified suspension for RNA extraction.

The pretreated histological (autopsy/biopsy material, placenta) material samples can be stored:

– at the temperature from minus 24 to minus 16 °C – for 1 week,
– at the temperature not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

6.12. *Spermatic fluid samples* are to be pretreated. Transfer 100 µl of spermatic fluid in 1.5 ml tubes. Add 900 µl of **Mucolysin**, into the tubes with spermatic fluid. Vortex the tubes thoroughly and incubate at room temperature (18-25 °C) within 10 min, thoroughly vortexing each 2-3 min. Use 50 µl of pretreated spermatic fluid for RNA extraction.

The pretreated spermatic fluid samples can be stored:

– at the temperature from minus 24 to minus 16 °C – for 1 week,
– at the temperature not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

6.13. *Mosquitoes samples* are to be pretreated. At first pools of mosquitoes should be formed (not more than 50 mosquitoes). Mosquitoes are homogenized in the saline solution in proportion 1 mosquito – 30 µl of solution. Centrifuge at 10,000 g (for example, 12,000 rpm for the Eppendorf microcentrifuge) for 1 min. Remove 100 µl of supernatant for RNA extraction.

For making mosquito suspension sterile porcelain cap and sterile pestle are used. If there is an automatic homogenizer TissueLyser LT (QIAGEN, Germany) the following homogenization parameters for mosquitoes should be used: balls' diameter – 5 mm, frequency – 50 Hz/s, time of homogenization – 5 min, buffer volume – 700 µl (pool of 25 mosquitoes), buffer volume – 1,500 µl (pool of 50 mosquitoes).

The pretreated mosquitoes samples can be stored:

– at the temperature from minus 24 to minus 16 °C – for 1 week,
– at the temperature not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

6.13. *Saliva/amniotic fluid samples* are to be pretreated. Prior to nucleic acids extraction reduce the viscosity of the material using **Mucolysin** reagent. Add **Mucolysin** into the tube with material in proportion 1:3 (1 part of the material and 3 parts of the **Mucolysin** reagent), guided by the graduations on the tube. In the case of very stiffness saliva use the proportion 1:5. Vortex the tube at times during the dissolution process. Use 100 µl dissolved sputum and 50 µl of dissolved amniotic fluid for the RNA extraction.

The pretreated saliva/amniotic fluid samples can be stored:

– at the temperature from minus 24 to minus 16 °C – for 1 week,
– at the temperature not more than minus 70 °C – for a long time.

Only one freeze-thawing cycle is required.

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 *ICZ*-rec (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 whole blood samples, collected in the tubes with heparin as anticoagulant are inapplicable for analysis.

7. WORKING CONDITIONS

AmpliSens® *Zika 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 extraction from blood plasma, saliva, urine, spermatic fluid, histological material (autopsy/biopsy material, placenta), amniotic fluid, mosquitos, oropharyngeal swabs;
- **MAGNO-sorb** for extraction from blood plasma and urine.

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

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 *ICZ*-rec (IC)** and positive control of extraction **Positive Control *ZIKV*.**
Add **10 µl** of **Internal Control *ICZ*-rec (IC)** to each tube with samples.
The volume of the test sample is **100 µl, (autopsy/biopsy material, placenta, spermatic fluid, amniotic fluid – 50 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 *ZIKV*** to the tube labeled PCE (Positive Control of Extraction).
The volume of elution is **50 µl.**

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

The RNA extraction for each sample is carried out in the presence of **Internal Control *ICZ*-rec (IC)** and positive control of extraction **Positive Control *ZIKV*.**
Add **10 µl** of **Internal Control *ICZ*-rec (IC)** to each tube with samples.
The volume of the test sample is **200 µl.**
Add **190 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 *ZIKV*** to the tube labeled PCE (Positive Control of Extraction).
The volume of elution is **50 µl,** when using RNA extraction automatic station – **100 µl.**

The volumes of reagents and samples when the RNA is extracted by the MAGNO-sorb reagent kit for 1,000 µl of sample:

The RNA extraction for each sample is carried out in the presence of **Internal Control *ICZ*-rec (IC)** and positive control of extraction **Positive Control *ZIKV*.**
Add **10 µl** of **Internal Control *ICZ*-rec (IC)** to each tube with samples.
The volume of the test sample is **1,000 µl.**
Add **1,000 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 *ZIKV*** to the tube labeled PCE (Positive Control of Extraction).
The volume of elution is **50 µl,** when using RNA extraction automatic station – **100 µl.**

8.2. Preparing reverse transcription and PCR

8.2.1 Preparing tubes for PCR

The total reaction volume is **25 µl**, the volume of the **RNA** sample is **10 µl**.
The type of tubes depends on the RT-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 one reaction:
   - **10 µl** of **PCR-mix-FL *ZIKV*,**
   - **5 µl** of **PCR-buffer-C,**
   - **0.5 µl** of **Polymerase (TaqF),**
   - **0.25 µl** of **TM-Revertase (MMlv),**
   - **0.25 µl** of **RT-G-mix-2**.

   Prepare the reaction mixture for the total number of test and control samples plus one extra reaction.

NOTE: Prepare the reaction mixture just before use.

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

7. Carry out the control reactions:

NCA	– Add **10 µl** of **TE-buffer** to the tube labeled **NCA** (Negative Control of Amplification).
C+$_{ZIKV\ /\ ICZ}$	– Add **10 µl** of **C+ *ZIKV* / *ICZ*** to the tube labeled **C+**$_{ZIKV\ /\ ICZ}$ (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).
PCE	– Add **10 µl** of **the sample extracted from the Positive Control *ZIKV* 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.

8.2.2. Reverse transcription and amplification

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

Table 2

Amplification and detection 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
	55	20 s	**FAM, JOE**	

Fluorescent signal is detected in the channels for the FAM, JOE and ROX fluorophores.
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 (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 RT-PCR instrument used by measuring fluorescence signal accumulation in two channels:

Table 3

Results interpretation

Channel for the fluorophore	FAM	JOE
Signal registration, indicating the amplification product accumulation	Internal Control *ICZ*-rec (IC) cDNA	*Zika virus* 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 RNA 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	*Zika virus* RNA **is not detected**
determined or absent	< boundary value	*Zika virus* RNA **is detected**
absent or > boundary value	absent or > boundary value	**Invalid** result*
< boundary value	> boundary value	**Equivocal** result**

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

** In case of equivocal result, the PCR analysis should be repeated for the corresponding test sample starting from the RNA extraction stage. If the same result is obtained, the sample is considered positive. If the negative result is obtained in the second run, 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 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**
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

10. TROUBLESHOOTING

The 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 channels 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 *Zika virus* 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 *Zika virus* 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 *Zika virus* 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® *Zika virus*-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.

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

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Zika virus*-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-FL *ZIKV*, PCR-buffer-C, RT-G-mix-2, polymerase (TaqF), TM-Revertase (MMlv)). All components of the **AmpliSens® *Zika virus*-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-FL *ZIKV*, PCR-buffer-C, RT-G-mix-2, polymerase (TaqF), TM-Revertase (MMlv) are to be stored at the temperature from minus 24 to minus 16 °C

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

13. SPECIFICATIONS

13.1. Analytical sensitivity

Table 6

Biological material	The volume of sample for extraction, µl	Nucleic acid extraction kit	PCR kit	Analytical sensitivity, copies/ml
Blood plasma	100	RIBO-prep	variant FRT-50 F	2,000
Mosquitos (homogenate)	100		variant FRT-50 F	2,000
Urine	100		variant FRT-50 F	2,000
Saliva	100		variant FRT-50 F	2,000
Oropharyngeal swabs	100		variant FRT-50 F	2,000
Histological material (autopsy/biopsy material, placenta)	50		variant FRT-50 F	10,000
Spermatic fluid	50		variant FRT-50 F	10,000
Amniotic fluid	50		variant FRT-50 F	10,000
Blood plasma	200	MAGNO-sorb	variant FRT-50 F	1,000
	1,000		variant FRT-50 F	100
Urine	1,000		variant FRT-50 F	500

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

Table 7

The results of AmpliSens® *Zika virus*-FRT PCR kit analytical sensitivity validation with using the material from healthy people and patients with another causation of disease and samples, contaminated by inactivated strain of Zika virus (MRS-Opy Martinique-PaRi-2015), or QCS (Quality control sample) no. 198 Positive Control *ZIKV*-rec

Type of sample	RNA concentration (copies per ml of the sample)	Number of replicates	Number of positives	Hit Rate, %
Blood plasma (RNA extraction from 100 µl)	$2x10^3$	18	18	100
Blood plasma (RNA extraction from 200 µl)	$2x10^3$	18	18	100
Blood plasma (RNA extraction from 1,000 µl)	$1x10^2$	18	18	100
Urine (RNA extraction from 100 µl)	$2X10^3$	18	18	100
Urine (RNA extraction from 1,000 µl)	$5x10^2$	18	18	100
Saliva	$2x10^3$	18	18	100
Mosquitos	$2x10^3$	18	18	100
Oropharyngeal swabs	$2x10^3$	18	18	100
Spermatic fluid	$1x10^4$	18	18	100
Histological material (autopsy/biopsy material)	$1x10^4$	18	18	100
Amniotic fluid	$1x10^4$	18	18	100

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *Zika virus*-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 specificity was proved on the follows strains of microorganisms and biological material obtained from healthy people or patients with another causation of disease:

Table 8

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

Organisms	The channel for the FAM fluorophore (Internal control)	The channel for the JOE fluorophore (*Zika virus*)
Dengue virus serotype 1	Valid	Negative
Dengue virus serotype 2	Valid	Negative
Dengue virus serotype 3	Valid	Negative
Dengue virus serotype 4	Valid	Negative
West Nile virus	Valid	Negative
Japanese encephalitis virus	Valid	Negative
Tick-borne encephalitis virus, Siberian genotype	Valid	Negative
Tick-borne encephalitis virus, Far-Eastern genotype	Valid	Negative
Tick-borne encephalitis virus, European genotype	Valid	Negative
Yellow fever virus	Valid	Negative
Omsk hemorrhagic fever virus	Valid	Negative
Langat virus	Valid	Negative
Powassan virus	Valid	Negative
Spondweni virus	Valid	Negative
Crimean-Congo hemorrhagic fever virus	Valid	Negative
Chikungunya virus	Valid	Negative
Rickettsia conorii	Valid	Negative
R.sibirica	Valid	Negative
R.raoultii	Valid	Negative
R.heilonjiangensis	Valid	Negative
R.slovaca	Valid	Negative
25 blood plasma samples from healthy people	Valid	Negative
25 urine samples from healthy people	Valid	Negative
25 saliva samples from healthy people	Valid	Negative
25 spermatic fluid samples from healthy people	Valid	Negative
25 amniotic fluid samples from the patients with another causation of disease	Valid	Negative
25 biopsy material samples from the patients with another causation of disease	Valid	Negative
25 oropharyngeal swabs samples from the patients with another causation of disease	Valid	Negative
25 samples of suspension of mosquitos (*Aedes albopictus*) from laboratory culture	Valid	Negative

13.3. Diagnostic characteristics

Table 9

Results of AmpliSens® *Zika virus*-FRT PCR kit testing in comparison with the reference assay

Samples type	Results of using AmpliSens® *Zika virus*-FRT PCR kit		Results of using the reference essay[3]	
			positive	negative
Blood plasma	128 samples was analyzed	**positive**	11	0
		negative	0	117
Urine	81 samples was analyzed	**positive**	30	1
		negative	1	49
Saliva	73 samples was analyzed	**positive**	13	0
		negative	1	59
Spermatic fluid	4 samples was analyzed	**positive**	2	0
		negative	0	2

286 clinical samples, obtained from the patients with symptoms not excluding *Zika* fever, were used, among them 27 blood plasma samples, 36 urine samples, 31 saliva samples and 3 spermatic fluid samples, obtained from 5 persons affected by Zika fever at different stage of the disease.

The **diagnostic sensitivity** of the **AmpliSens® *Zika virus*-FRT** PCR kit relatively to used reference essay is 96 % for the samples of blood plasma, urine, saliva and spermatic fluid.

The **diagnostic specificity** of the **AmpliSens® *Zika virus*-FRT** PCR kit relatively to used reference essay is 99 % for the samples of blood plasma, urine, saliva and spermatic fluid.

14. REFERENCES

1. Daniel Alzate, Esteban Marín, Jahir Orozco, Carlos Muskus, Differential detection of zika virus based on PCR, J Virol Methods. 2022 Mar;301:114459.

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

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.03.19 EM	3. Content	The colour of the reagents was specified
22.05.20 VA	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
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.
19.01.22 MM	Through the text	The reference numbers of nucleic acid extraction kits and transport mediums were deleted
29.08.22 KK	14. References	The section was updated

AmpliSens®

EC REP Ecoli Dx, s.r.o., Purkyňova 74/2
110 00 Praha 1, Czech Republic
Tel.: +420 325 209 912
Cell: +420 739 802 523

Federal Budget Institute of Science "Central Research Institute for Epidemiology"
3A Novogireevskaya Street
Moscow 111123 Russia

[3] RealStar® *Zika Virus* RT-PCR Kit 1.0 (Altona Diagnostics GmbH) was used as a reference essay.