# AmpliSens® *Dengue virus*-FRT PCR kit

IVD

For Professional Use Only

# Instruction Manual

## KEY TO SYMBOLS USED

| | | | |
|---|---|---|---|
| REF | Catalogue number | | Use-by Date |
| LOT | Batch code | | Consult instructions for use |
| IVD | *In vitro* diagnostic medical device | | Keep away from sunlight |
| VER | Version | | Keep dry |
| | 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 |
| | Contains sufficient for <n> tests | | |

## 1. INTENDED USE

**AmpliSens® *Dengue virus*-FRT** PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Dengue virus* (types 1-4) RNA in the biological material (blood plasma, histological (autopsy, biopsy) material (brain and visceral tissues), urine, saliva, mosquitoes (homogenate)) using real-time hybridization-fluorescence detection of amplified products.

The PCR kit is used for studying the biological material, taken from the persons suspected of dengue fever.

According to literature data, the diagnostic sensitivity of the PCR method used for detection of *Dengue virus* RNA has the highest rates in the first week of the disease and ranges from 87 to 63%[1]. Own data on the diagnostic sensitivity of the reagent kit at the first and second weeks of the disease are given in Table 9.

According to literature data[2], in the case of researching autopsy material from people who died from the acute form of hemorrhagic or shock dengue fever, the virus RNA is detected by PCR (in order of frequency decrease): in the liver, spleen, lymph nodes, kidneys, bone marrow, lungs, thymus and brain.

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

## 2. PRINCIPLE OF PCR DETECTION

*Dengue 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 *Dengue 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® *Dengue virus*-FRT** PCR kit is a qualitative test that contains an exogenous 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® *Dengue 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 by 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 simultaneously in one tube – amplification of the *Dengue virus* cDNA sequence and also amplification of the Internal Control *ICZ*-rec cDNA sequence. The amplification results of *Dengue virus* cDNA and Internal Control *ICZ*-rec cDNA are registered in 2 different fluorescence detection channels:

Table 1

| Channel for fluorophore | FAM | JOE |
|---|---|---|
| cDNA-target | Internal Control *ICZ*-rec cDNA | *Dengue virus* cDNA |
| Target gene | Artificially synthesized sequence | 3'-UTR |

[1] Wright W., Pritt B. Update: The diagnosis and management of dengue virus infection in North America//Diagn Microbiol Infect Dis. 2012. – Vol.73. – P.215-220.

[2] Martina BE, Koraka P, Osterhaus AD. Dengue virus pathogenesis: an integrated view. Clin. Microbiol. Rev. 22 (4): 564–81. doi:10.1128/CMR.00035-09.

## 3. CONTENT

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

**variant FRT-50 F**, REF H-2391-1-CE.

**variant FRT-L**, REF H-2392-1-4-CE.

**Variant FRT-50 F** includes:

| Reagent | Description | Volume, ml | Quantity |
|---|---|---|---|
| **PCR-mix-FL *DV*** | colorless clear liquid | 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 |
| **Positive Control *DV* / *ICZ* (C+$_{DV\,/\,ICZ}$)** | colorless clear liquid | 0.2 | 1 tube |
| **TE-buffer** | colorless clear liquid | 0.2 | 1 tube |
| **Positive Control *DV**** | colorless clear liquid | 0.1 | 1 tube |
| **Internal Control *ICZ*-rec (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.

** add **10 µl** of **Internal Control *ICZ*-rec (IC)** during the RNA extraction procedure directly to the sample/lysis mixture.

*** must be used in the extraction procedure as Negative Control of Extraction (see **RIBO-prep** or **MAGNO-sorb** protocol).

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

**Variant FRT-L** includes:

| Reagent | Description | Volume, ml | Quantity |
|---|---|---|---|
| **PCR-mix *DV*-Lyo** | white powder | - | 48 tubes of 0.2 ml |
| **Positive Control *DV* / *ICZ* (C+$_{DV\,/\,ICZ}$)** | colorless clear liquid | 0.2 | 1 tube |
| **TE-buffer** | colorless clear liquid | 0.2 | 1 tube |
| **Positive Control *DV**** | colorless clear liquid | 0,1 | 1 tube |
| **Internal Control *ICZ*-rec (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.

** add **10 µl** of **Internal Control *ICZ*-rec (IC)** during the RNA extraction procedure directly to the sample/lysis mixture.

*** must be used in the extraction procedure as Negative Control of Extraction (see **RIBO-prep** or **MAGNO-sorb** protocol).

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

## 4. ADDITIONAL REQUIREMENTS

- Sterile bilateral needles for vacuum tubes intended for venous blood collection for in vitro study.
- Vacuette® blood collection system.
- Sterile plastic container (50-60 ml) for sampling, storage and transportation of biological samples.
- Reagent for pretreatment of viscous fluids (saliva).
- 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 the storage of pretreated urine.
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and mallet) or homogenizer for pretreatment of tissue material.
- RNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips (up to 200 µl, 1,000 µl) and pipette tips with filters (up to 100 µl, 200 µl, 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), CFX 96 (Bio-Rad, USA)).
- Disposable polypropylene 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 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 samples 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® *Dengue virus*-FRT** PCR kit is intended for analysis of the RNA extracted with RNA extraction kits from the biological material (blood plasma, histological (autopsy, biopsy) material (brain and visceral tissues), saliva, urine, mosquitoes).
The test material is collected by clinical staff.
Accounting, storage, transferring and transportation of the biological material suspected of *Dengue 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 for thoroughly mixing with the anticoagulant. It can be stored at 2–8 °C. 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 68 °C – for a long time.
Only one freeze-thawing cycle is required.

6.2. *Histological (autopsy, biopsy) material* (brain and visceral tissues). 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 histological (autopsy, biopsy) material (brain and visceral tissues) samples can be stored:
– at the temperature from 2 to 8 °C – for 24 hours,
– 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.
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 hours,
Freezing of the material is not allowed!
6.4. *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 the temperature 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 68 °C - for a long time.
Only one freeze-thawing cycle is required.
6.5. *Mosquitoes.* 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 50.
The material can be stored after sorting and samples forming:
– at the temperature from minus 24 to minus 16 ° C - for 1 month;
– at the temperature not more than minus 68 °C or in Dewar flask with liquid nitrogen - for a long time.
Only one freeze-thawing cycle is required.

***Pretreatment***

6.6. *The blood plasma* and *clear urine samples*. The pretreatment is not required.
6.7. *Urine sample* are to be pretreated in case of muddy 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 urine is used for RNA extraction by RIBO-prep nucleic acid extraction kit or 1,000 µl of obtained clarified urine 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. If RIBO-prep nucleic acid extraction kit is meant to be used for the RNA extraction, add glycerin into the tubes with 1,100 µl of urine in amount of 10 % of sample volume (120 µl), vortex the tubes for homogeneous mixing of glycerin. If MAGNO-sorb nucleic acid extraction kit is meant to be used for the RNA extraction, glycerin must not be added to urine, the urine is frozen without glycerin.
The sample material can be stored with glycerin and without glycerin:
– at the temperature from minus 24 to minus 16 °C - for 1 week;
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thawing cycle is required.

*6.8. Histological (autopsy*, *biopsy) material (brain and visceral tissues)* is to be pretreated. For RNA extraction 30-50 µl of the material is taken and homogenized by trituration using precooled sterile porcelain mortar and mallet or homogenizer. The suspension is prepared using grinded tissue and precooled sterile saline solution or phosphate buffer. For this, 9 volumes of saline solution are to be added to 1 volume of grinded tissue. Use 100 µl of suspension for RNA extraction.
The pretreated biopsy material samples 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.
*6.9*. *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 Tissue Lyser 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 before the PCR analysis:
– at the temperature from minus 24 to minus 16 °C – for 1 week,
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thawing cycle is required.
*6.10. Saliva 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 of dissolved saliva for the RNA extraction.
The pretreated saliva samples can be stored before the PCR analysis:
– at the temperature from minus 24 to minus 16 °C – for 1 week,
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thawing cycle is required.

***Interfering substances and limitations of using test material samples***

The blood plasma samples collected into the tubes with heparin as anticoagulant are inapplicable for the analysis. Heparin is an inhibitor of PCR.
Data about other interfering substances are absent while respecting the rules for sampling and pretreatment of the test material specified in the instruction manual.
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.

## 7. WORKING CONDITIONS

**AmpliSens® *Dengue 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, histological (autopsy, biopsy) material (brain and visceral tissues), urine, saliva, mosquitos;
– **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 the **RIBO-prep** reagent kit :
The RNA extraction for each sample is carried out in the presence of **Internal Control *ICZ*-rec (IC).**
Add **10 µl** of **Internal Control *ICZ*-rec (IC)** to each tube with samples.
**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 *DV*** 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 from 200 µl of the sample:
The RNA extraction for each sample is carried out in the presence of **Internal Control *ICZ*-rec (IC).**
Add **10 µl** of **Internal Control *ICZ*-rec (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 *DV*** to the tube labeled PCE (Positive control of extraction).
The volume of elution is **50 µl.**
The volume of elution is **100 µl** (if automatic stations for RNA extraction are used).

The volumes of reagents and samples when the RNA is extracted by the **MAGNO-sorb** reagent kit from 1,000 µl of the sample:
The RNA extraction for each sample is carried out in the presence of **Internal Control *ICZ*-rec (IC).**
Add **10 µl** of **Internal Control *ICZ*-rec (IC)** to each tube with samples.
The volume of the test sample is **1,000 µl.**
**NOTE:** 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 *DV*** to the tube labeled PCE (Positive control of extraction).
The volume of elution is **50 µl.**
The volume of elution is **100 µl** (if automatic stations for RNA extraction are used).

**NOTE:** It is recommended to carry out the reverse transcription reaction just after the obtaining the RNA samples. It is allowed to store the RNA samples at the temperature from 2 to 8 °C for 30 min, at the temperature from minus 24 to minus 16 °C for 1 week and at the temperature not more than minus 68 °C for 1 year. Only one freeze-thawing cycle is required.

## 8.2. Preparing reverse transcription and PCR

### 8.2.1 Preparing tubes for RT-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 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 *DV*,**
   **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. See the number of control samples in item 7.

**NOTE:** Prepare the reaction mixture just before use.

2. Thaw the tube with **PCR-mix-FL *DV*.** 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 *DV*, 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 for RT-PCR of RNA of test 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 at the RNA extraction stage using tips with filter.

**NOTE:** Avoid transferring the 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:
   **C+** – Add **10 µl** of **Positive Control *DV* / *ICZ* (C+$_{DV\,/\,ICZ}$)** 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 *DV* 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.

**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 *DV*-Lyo** for RT-PCR of RNA from test and control samples (the number of control samples see 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 **Positive Control *DV* / *ICZ* (C+$_{DV\,/\,ICZ}$)** 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 *DV* 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. Reverse transcription and amplification

1. Create a temperature profile on your instrument as follows (tables 1, 2)[3]:

Table 1

**AmpliSens unified amplification program for rotor-type[4] and plate type[5] instruments**

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

**NOTE:** Any combination of the tests 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.

Table 2

**Amplification and detection program for rotor-type[4] and plate type[5] instruments**

| Step | Temperature, °C | Time | Fluorescent signal detection | Cycles |
|---|---|---|---|---|
| 1 | 50 | 15 min | – | 1 |
| 2 | 95 | 15 min | – | 1 |
| 3 | 95 | 10 s | – | 45 |
| | 60 | 30 s | FAM, JOE | |

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.

[3] Use the amplification program specified in the table 2, if there is no need to use the unified amplification program.
[4] For example, Rotor-Gene Q (QIAGEN, Germany).
[5] For example, CFX 96 (Bio-Rad, USA).

## 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 2 channels:

Table 3

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

* The signal absence in the channel for FAM fluorophore is irrelevant if *Dengue virus* 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.

*** 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 as 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 in 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

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

1. The *Ct* value determined for the Positive Control of Amplification (C+) in 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 *Dengue virus* RNA was not detected.
2. 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. 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 *Dengue virus* RNA was detected.
4. 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 *Dengue virus* 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 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® *Dengue 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.

## 12. STABILITY AND STORAGE

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

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

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

## 13. SPECIFICATIONS

### 13.1. Analytical sensitivity (limit of detection)

Table 6

| 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 | 1,000 |
| Mosquitoes (homogenate) | 100 | | variant FRT-50 F, FRT-L | 1,000 |
| Urine | 100 | | variant FRT-50 F, FRT-L | 1,000 |
| Saliva | 100 | | variant FRT-50 F, FRT-L | 1,000 |
| Histological (autopsy, biopsy) material | 100 | | variant FRT-50 F, FRT-L | 5,000 |
| Blood plasma | 200 | MAGNO-sorb | variant FRT-50 F, FRT-L | 1,000 |
| | 1000 | | variant FRT-50 F, FRT-L | 100 |
| Urine | 1000 | | variant FRT-50 F, FRT-L | 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® *Dengue 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 PCR kit detects the RNA fragments of claimed viruses: dengue virus type 1 (strain DENV-1/8/Thailand/01/2013), dengue virus type 2 (strain DENV-2/131/Philippines/12/2013), dengue virus type 3 (strain DENV-3/25/Thailand/01/2013), dengue virus type 4 (strain DENV-4/122/Vietnam/11/2013).

The analytical specificity was proved by testing the samples containing genomic cDNA of heterologous strains of flaviviruses, which can be present in test samples and give false-positive results on flaviviruses: *WNV* (*West Nile virus*; strains Eg-101 and LEIV-Vlg99-27889-human), *JEV* (*Japanese encephalitis virus*, strain Nakayama), *TBEV* (*Tick-borne encephalitis virus*, strains of Ain, Obor-4, Absettarov), *LGT* (*Langat virus*, strain TP-21), *POWV* (*Powassan virus*, Bayer strain), *USUV* (*Usutu virus*), *LIV* (*Louping ill virus*, Adra-7 strain), *OHFV* (*Omsk hemorrhagic fever virus*, strains Goloshubin, Krutinka-73/640, Veselovka-752).

Also, the samples containing cDNA/DNA of the microorganisms that produce similar clinical symptoms and occur in the areas of dengue virus circulation, were investigated: *CCHFV* (*Crimean-Congo hemorrhagic fever virus*, strain IbAr10200), *CHIKV* (*Chikungunya virus*, strain *Ross late*), *Leptospira spp* (subtypes group *grippotyphosa* - strain MV5; subtypes group *bataviae* - strain HS26, subtypes group *sejroe* – strain 3705), rickettsia of the tick-borne spotted fever group (*Rickettsia sibirica* (strain Netsvetaev), *R. conorii* (strain M-1), *R. raoultii* (Elanda 23/95, Kyzyl-Orda 7/14), *R. heilongjiangensis* (strain Primorye 25/81), *R. slovaca* (strain Karpunino 19/69), *R. canadensis* (strain 2678), *A.phagocytophillum* (sample from *I.ricinus* tick), *Babesia microti* (DNA isolate from rodents), *Bartonella henselae* (DNA isolate from *I.persulcatus* tick) , *Yersinia pestis* (vaccine strain EV RIEH).

The human genomic DNA extracted from placenta, blood plasma samples, saliva and urine, and DNA samples of *Aedes albopictus* mosquitoes were used to prove the absence of cross reactions with the patient's DNA material and mosquitoes.

The positive result was obtained while testing RNA samples strains of dengue virus types 1-4 in all cases.

Nonspecific responses were absent in tests of cDNA/DNA samples of above mentioned organisms, human DNA and mosquitoes.

The clinical specificity of **AmpliSens® *Dengue virus*-FRT** PCR kit was confirmed in laboratory clinical trials.

### 13.3. Diagnostic characteristics

Table 7

**The results of testing AmpliSens® *Dengue virus*-FRT PCR kit in comparison with the reference assay and PCR kit**

| Samples type | The results of studying the AmpliSens® *Dengue virus*-FRT PCR kit | | Results of comparison with the reference assay[6] | | Results of comparison with the PCR kit[7] | |
|---|---|---|---|---|---|---|
| | | | Positive | Negative | Positive | Negative |
| Blood plasma | 179 samples were tested | **Positive** | 124 | 0 | 122 | 2 |
| | | **Negative** | 5 | 50 | 1 | 54 |
| Urine | 110 samples were tested | **Positive** | 48 | 0 | 45 | 3 |
| | | **Negative** | 12 | 50 | 0 | 62 |
| Saliva | 90 samples were tested | **Positive** | 33 | 0 | 32 | 1 |
| | | **Negative** | 7 | 50 | 0 | 57 |
| Mosquitoes (homogenate) | 50 samples were tested | **Positive** | — | — | 25 | 0 |
| | | **Negative** | — | — | 0 | 25 |
| Tissue (autopsy, biopsy) material, namely, brain and visceral tissues | 50 samples were tested | **Positive** | — | — | 25 | 0 |
| | | **Negative** | — | — | 0 | 25 |

The following samples were used:

- 179 blood plasma samples obtained from the patients with the fevers of different etiology. For 129 samples, the diagnosis of "dengue fever" was confirmed by the presence of seroconversion of specific antibodies to the dengue virus using the reagents kit for in vitro diagnostics of viral infections by indirect immunofluorescence method (in the following variants: Mosaic Dengue virus types 1-4 IIFT (IgG), Mosaic Dengue virus types 1-4 IIFT (IgM) (EUROIMMUN AG, Germany).
- 110 urine samples from patients with the fevers of different etiology. For 60 samples diagnosed the diagnosis of "dengue fever" was confirmed as described above, and 50 urine samples from patients with diagnoses excluding dengue fever.
- 90 saliva samples from patients with the fevers of different etiology. For 40 samples, the diagnosis of "dengue fever" was confirmed as described above, and 50 saliva samples from patients with diagnoses excluding dengue fever.

The OM-Screen-Dengue/JL-RV reagent kit (ZAO Sintol, Russia) for detection and identification of *Dengue* and *Yellow fever* viruses RNA by real-time polymerase chain reaction was used as the kit for comparison for the same test samples.

Table 8

**Diagnostic characteristics of AmpliSens® *Dengue virus*-FRT PCR kit**

| Samples type | In comparison with PCR kit[8] | | In comparison with reference assay[9] | |
|---|---|---|---|---|
| | Diagnostic sensitivity, % | Diagnostic specificity, % | Diagnostic sensitivity, % | Diagnostic specificity, % |
| Blood plasma | 99 | 96 | 96 | 100 |
| Urine | 100 | 95 | 80 | 100 |
| Saliva | 100 | 98 | 83 | 100 |
| Tissue (autopsy, biopsy) material, namely, brain and visceral tissues | 100 | 100 | — | — |
| Mosquitoes (homogenate) | 100 | 100 | — | — |

Table 9

**Diagnostic sensitivity of AmpliSens® *Dengue virus*-FRT PCR kit while studying the biological material, depending on sampling time since disease onset**

| Samples type | In the first 7 days of the disease | | | From the 8th to the 15th day of the disease | | |
|---|---|---|---|---|---|---|
| | Test samples | Positive | % | Test samples | Positive | % |
| Blood plasma | 103 | 102 | 99 | 26 | 22 | 84 |
| Urine | 38 | 33 | 87 | 22 | 15 | 68 |
| Saliva | 25 | 22 | 88 | 15 | 11 | 73 |

## 14. REFERENCES


1. Myung-Jin Mun, Joon-Yong Bae, Jin Hyuck Kim, Soo Bok Kim, Ilseob Lee, Jin Il Kim, Mee Sook Park, Man-Seong Park, Yong Suk Nam Mol Cell Probes, One-step multiplex real-time RT-PCR for detection and typing of dengue virus. 2019 Feb;43:86-91.


## 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® *Dengue 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 |
|---|---|---|
| 20.11.15 PM | 8.1. RNA extraction | The volume of elution was specified |
| 25.04.17 DV | Throughout the text | The new biological material (urine, saliva, biopsy material and homogenates of mosquitoes) was added |
| | Additional requirements | Reagent for pretreatment of viscous fluids, Glycerine was added |
| | Sampling and handling | The material urine, saliva, mosquitoes was added |
| | Analytical sensitivity | The analytical characteristics of urine, saliva, biopsy material, mosquitoes was added |
| | Reproducibility and repeatability | Subsection was deleted |
| | Diagnostic characteristics | Data about diagnostic sensitivity for urine and saliva samples was added. Data about diagnostic sensitivity for histological material was deleted |
| 14.03.18 DV | Sampling and handling | Adjustments were made in the text (urine samples) |
| | Diagnostic characteristics | Changes in Table 7, 8 and 9 were added |
| | Throughout the text | Variant FRT-L was added |
| | Footer | The catalog number of REF R-V68-F-CE was changed to REF H-2391-1-CE. The catalog number REF H-2392-1-4-CE was added |
| 16.04.20 MM | Through the text | The text formatting was changed |
| | Footer | The phrase "Not for use in the Russian Federation" was added |
| 09.03.21 EM | — | The name, address and contact information for Authorized representative in the European Community was changed |
| 20.10.21 KK | 3. Content<br>8. Protocol<br>8.1.RNA extraction | The RIBO-prep, REF K2-9-Et-50-CE was change to RIBO-prep, REF K2-9-Et-100-CE. |
| 20.01.22 KK | Through the text | The reference numbers of nucleic acid extraction kits were deleted |
| 29.08.22 KK | 14. References | The section was updated |


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[6] The reagent kit for in vitro diagnostics of viral infections by indirect immunofluorescence method in the following variants: Mosaic Dengue virus types 1-4 IIFT (IgG), Mosaic Dengue virus types 1-4 IIFT (IgM) (EUROIMMUN AG, Germany) was used as the reference assay.

[7] The OM-Screen-Dengue/JL-RV reagent kit (ZAO Sintol, Russia) for detection and identification of *Dengue* and *Yellow fever* viruses RNA by real-time polymerase chain reaction was used as the kit for comparison.

[8] The OM-Screen-Dengue/JL-RV reagent kit (ZAO Sintol, Russia) for detection and identification of *Dengue* and *Yellow fever* viruses RNA by real-time polymerase chain reaction.

[9] The reagents kit for in vitro diagnostics of viral infections by indirect immunofluorescence method (in the following variants: Mosaic Dengue virus types 1-4 IIFT (IgG), Mosaic Dengue virus types 1-4 IIFT (IgM) (EUROIMMUN AG, Germany).