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For Professional Use Only

AmpliSens[®] *Dengue virus type-FRT*

PCR kit

Instruction Manual

AmpliSens[®]



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1. INTENDED USE

AmpliSens[®] Dengue virus type-FRT PCR kit is an *in vitro* nucleic acid amplification test for detection and differentiation of RNA of *Dengue virus* type 1-4 in the clinical materials (blood plasma, blood serum) and in the human autopsy materials (brain, liver, spleen tissues), in animals materials (brain, spleen tissues), in mosquitoes by using real-time hybridization-fluorescence detection of amplified products.



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

2. PRINCIPLE OF PCR DETECTION

DV RNA detection by the polymerase chain reaction (PCR) contains two stages: RNA extraction from the biological material samples, reverse transcription of RNA and amplification of the part of DV cDNA with real-time hybridization-fluorescence detection which takes place during the PCR. RNA extraction from the biological material is carried out with the presence of the internal control sample (Internal Control STI-87-rec (IC)) which allows to control the research procedure for each sample. Afterwards the RNA reverse transcription is carried out with the obtained test samples with the help of TM-revertase enzyme and amplification of the parts of DV 1-4 types cDNA by using specific primers for the cDNA parts and Taq-polymerase enzyme. In the reaction mixture fluorescent marked oligonucleotide probes which hybridize with the complementary parts of the amplified cDNA targets. As a result the fluorescence intensity grows. This allows to register the accumulation of the specific amplification products by measuring of the fluorescence signal intensity. Fluorescent signal detection by using real-time monitoring FRT is carried out during the PCR with the help of thermocycler with real-time hybridization-fluorescence detection.

3. CONTENT

AmpliSens[®] Dengue virus type-FRT PCR kit is produced in 1 form:

AmpliSens[®] Dengue virus type-FRT PCR kit variant FRT-50 F, **REF** R-V63(RG,CFX)-CE;

AmpliSens[®] Dengue virus type-FRT PCR kit variant FRT-50 F includes:

Reagent	Description	Volume, ml	Quantity
RT-G-mix-2	colorless clear liquid	0,015	1 tube
RT-PCR-mix-1-FRT DV	colorless clear liquid	0,6	1 tube
RT-PCR-mix-2-FEP/FRT	colorless clear liquid	0,3	1 tube

Polymerase (TaqF)	colorless clear liquid	0,03	1 tube
TM-Revertase (MMIv)	colorless clear liquid	0,015	1 tube
Positive Control DV 1-4 types / STI (C+_{DV 1-4 types / STI})	colorless clear liquid	0,2	1 tube
TE-buffer	colorless clear liquid	0,2	1 tube
Negative Control (C-)	colorless clear liquid	1,2	8 tubes
Internal Control STI-87-rec (IC)	colorless clear liquid	0,12	5 tubes

AmpliSens[®] Dengue virus type-FRT PCR kit is intended for 60 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- 0,15 M NaCl or phosphate buffer solution (PBS) (sodium chloride, 137 mM; potassium chloride, 2,7 mM; sodium monophosphate, 10 mM; potassium diphosphate, 2 mM; pH=7,5±0,2).
- Homogenizer TissueLyser LT (QIAGEN, Germany). Is recommended to use for autopsy materials and mosquitoes homogenization.
- Stainless steel balls with 5 mm and 7 mm diameter.
- RNA/DNA extraction kits (depending on the type of the biomaterial).
- Additional requirements for RNA/DNA extraction.
- PCR box.
- Vortex mixer.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips with filter (up to 100 µl and to 200 µl).
- Tube racks.
- Disposable screwed up or tightly closed 1.5 ml polypropylene PCR tubes.
- 1.5 ml tubes and tips racks.
- Refrigerator for 2–8 °C.
- Deep-freezer at temperature from minus 24 to minus 16 °C.
- Disposable powder-free gloves and laboratory coat.
- Reservoir for used tips.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia); Rotor-Gene Q (QIAGEN, Germany), CFX 96 (Bio-Rad, USA)).

- Disposable polypropylene PCR tubes (0.1- or 0.2-ml):
 - a) 0.2-ml thin walled PCR tubes with optically transparent domed or flat cap if a plate-type instrument is used;
 - b) 0.2-ml thin walled PCR tubes with flat caps if a rotor-type instrument is used;

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use protective gloves and laboratory cloths, and protect eye while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet compliance with appropriate biosafety practices.
- Clean and disinfect all specimens or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid specimens and reagents contact with the skin, eyes, and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where the previous step was performed.



Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING



Obtaining samples of biological materials for PCR-analysis, transportation, and storage are described in the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens[®] Dengue virus type-FRT PCR kit is intended for analysis of RNA/DNA extracted with RNA/DNA extraction kits from the clinical/biological material blood plasma, blood serum, human's autopsy materials (brain, liver, spleen tissues), animal's materials (brain, spleen tissues) and mosquitoes.

Sampling

- 6.1 *Blood plasma, blood serum.* Fasting draw of the whole peripheral blood is carried out in the morning to the tube with 6 % EDTA solution in proportion 1:20. Closed tube with the whole peripheral blood should be overturned several times. For obtaining blood plasma the tube should be centrifuged for 20 min on 1600 g. For obtaining blood serum fasting draw of the whole peripheral blood is carried out in the morning to the dry tube. To form the clot blood should be defecated for 30 min at 37 °C, after that the tube should be centrifuged at 1600 g for 20 min. For the test 100 µl of the clinical materials must be taken during the RNA extraction using RIBO-prep or 1 µl of the clinical materials using MAGNO-sorb.
- 6.2 *Autopsy materials (brain, liver, spleen tissues).* This material is homogenized by using sterile porcelain mortars and pestles, after that 10 % suspension is made on the sterile saline solution or phosphate buffer. If there is an automatic homogenizer TissueLyser LT the following homogenization parameters for internal tissues should be used: PBS-buffer volume or 0,15 M of NaCl solution volume for homogenization depends on the volume of homogenizing tissue, the proportion tissue/buffer is 1:9, so 10 % suspension is made. The total sample volume for the 1.5 ml tubes mustn't exceed 1 ml. Homogenization conditions for the brain tissues: balls' diameter – 5 mm, frequency – 50 Hz/s, time of homogenization – 2-3 min, for liver and spleen tissues: balls' diameter – 7 mm, frequency – 50 Hz/s, time of homogenization – 10 min. For RNA extraction 30 µl of suspension should be taken.
- 6.3 *Mosquitoes.* For making mosquito suspension sterile porcelain cap and sterile pestle are used. If there is an automatic homogenizer TissueLyser LT the following homogenization parameters for mosquitoes should be used: balls' diameter – 5 mm, frequency – 50 Hz/s, time of homogenization – 5 min, buffer volume – 1000 µl (pool of 25 mosquitoes). At first pools of mosquitoes should be formed (not more than 25 species of mosquitoes of *Aedes* class). Mosquitoes are homogenized in the saline

solution or in the phosphate buffer in proportion 1 mosquito – 40 µl solution. The samples are centrifuged at 10 000 g for 1 min. After that 100 µl of supernatant is taken away for RNA extraction.

The above-mentioned biological material can be stored for 24 hour period before the test at 2-8 °C or for a week at temperature from minus 24 to minus 16 °C. For autopsy materials and mosquitoes the following storage conditions are provided: internal tissues and mosquitoes are stored for a week at temperature from minus 24 to minus 16 °C, for longtime storage period at temperature minus 68 °C.

7. WORKING CONDITIONS

AmpliSens® Dengue virus type-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. DNA/RNA extraction

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

- RIBO-prep, **REF** K2-9-Et-100-CE;
- MAGNO-sorb, **REF** K2-16-1000-CE;



Extract RNA according to the manufacturer's protocol.

- If extracting with RIBO-prep reagent kits, put **30 µl** of testing homogenized brain tissues or internal tissues suspensions or 100 (possibly 200 µl) of blood plasma (or blood serum) or 100 µl of mosquito suspension into the tubes with lysis solution and Internal Control STI-87-rec (IC).
- Put **10 µl** of **Internal Control STI-87-rec (IC)** and **300 µl** of **lysis solution** to the tube with NCA.
- The tube's content should be mixed properly on vortex mixer and heated for **5 min** at **65 °C** in thermostat.
- Centrifuge the tubes for **5 s** at **1500 g** to remove the drops from the internal part of the cap.
- Add to the tubes **400 µl** of **precipitation solution** and mix on the vortex mixer.
- Centrifuge the tubes for **5 min** at **10 000 g**.
- Carefully remove the supernatant without disturbing the pellet using a vacuum aspirator and a new tip for every sample.
- Add **500 µl** of **Washing Solution 3** to each tube, tightly close the tubes and turn them

- carefully upside down and back from 3 to 5 times to wash the pellet.
- Centrifuge all tubes at **10 000 g** for **2 min**.
 - Carefully remove the supernatant without disturbing the pellet using a vacuum aspirator and a new tip for every sample.
 - Add **200 µl** of **Washing Solution 4** to each tube, tightly close the tubes and turn them carefully upside down and back from 3 to 5 times to wash the pellet.
 - Centrifuge all tubes at **10 000 g** for **2 min**.
 - Carefully remove the supernatant without disturbing the pellet using a vacuum aspirator and a new tip for every sample.
 - Incubate all tubes with open caps at **65 °C for 5 min** (to dry the pellet).
 - Add **50 µl** of **RNA buffer** into each tube. Mix the tubes by vortex. Then incubate them at **65 °C** for 5 min occasionally stirring by vortex.
 - Centrifuge all tubes at **10 000 g** for **1 min**. The supernatant contains purified RNA and DNA. Samples are ready for reverse transcription reaction or PCR amplification.

8.2. Preparing PCR

8.2.1 Preparing tubes for PCR

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

1. Mix in the separate tube **RT-PCR-mix-1-FRT DV**, **RT-PCR-mix-2-FEP/FRT**, **polymerase (TaqF)**, **TM-Revertase (MMIv)** and **RT-G-mix-2** based on the volume of each reaction:
 - **10 µl** of **RT-PCR-mix-1-FRT DV**;
 - **5 µl** of **RT-PCR-mix-2-FEP/FRT**;
 - **0.5 µl** of **polymerase (TaqF)**;
 - **0.25 µl** of **TM-Revertase (MMIv)**;
 - **0.25 µl** of **RT-G-mix-2**.

While counting it is necessary to take into account the fact that there must be the amplification of three control samples: negative control of extraction (C⁻), positive and negative controls of RT-PCR (C⁺_{DV 1-4 types / STI} and NCA).

Put into each tube **15 µl** of the prepared mixture.



Do not store the prepared mixture.

2. Put **10 µl** of the extracted **RNA samples** into the prepared tubes. Mix carefully by

pipetting.

3. Carry out the control amplification reactions:

NCA - Add **10 µl** of **TE-buffer**

C+_{DV 1-4 types / STI} - Add **10 µl** of **Positive Control DV 1-4 types / STI (C+_{DV 1-4 types / STI})**

C- - Add **10 µl** of **the sample extracted from the Negative Control reagent** to the tube labeled C-



The samples should be amplified right after the mixing of the reaction mixture with RNA samples and controls.

8.2.2. Reverse transcription of RNA and amplification of cDNA with real-time hybridization-fluorescence detection

1. Program thermocycler for running the necessary program of reverse transcription and amplification using real-time hybridization-fluorescence detection (see Table 1).

Table 1

Amplification program

Step	Rotor-type instruments ¹			Plate-type instruments ²		
	Temperature, °C	Time	Cycles	Temperature, °C	Time	Cycles
1	50	30 min	1	50	30 min	1
2	95	15 min	1	95	15 min	1
3	95	10 s	5	95	10 s	5
	56	35 s		56	40 s	
	72	15 s		72	20 s	
4	95	10 s	40	95	10 s	40
	54	35 s Fluorescence acquiring		54	40 s Fluorescence acquiring	
	72	15 s		72	20 s	

Fluorescent signal is detected in the channels for the FAM, JOE, ROX, Cy5 and Cy5.5 fluorophores.

- Put the tubes into the cells of the reaction module. **Well 1** must be filled with the test tube.
- Run the amplification program.
- Analyze results after the amplification program is completed.

¹ For example, Rotor-Gene 6000 (Corbett Research, Australia), Rotor-Gene Q (QIAGEN, Germany).

² For example, iCycler iQ, iQ5 (Bio-Rad, USA).

9. DATA ANALYSIS

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

- The signal of the Dengue virus 1 part of cDNA amplification product is detected in the channel for the FAM fluorophore.
- The signal of the Dengue virus 2 part of cDNA amplification product is detected in the channel for the JOE fluorophore.
- The signal of the Dengue virus 3 part of cDNA amplification product is detected in the channel for the ROX fluorophore.
- The signal of the Dengue virus 4 part of cDNA amplification product is detected in the channel for the Cy5 fluorophore.
- The signal of the IC part of cDNA amplification product is detected in the channel for the Cy5.5 fluorophore.

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

Results are interpreted according to the table 2.

Table 2

Correspondence of the targets and detection channels

Detection through the channel				
FAM	JOE	ROX	Cy5	Cy5.5
<i>DV 1</i>	<i>DV 2</i>	<i>DV 3</i>	<i>DV 4</i>	BKO

Principle of interpretation is the following:

- cDNA *DV* is **detected** if the *Ct* value determined in the results grid in the channel for the FAM and/or JOE and/or ROX and/or Cy5 fluorophores is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
- cDNA *DV* is **not detected** in a sample if *Ct* value determined in the results grid in the channel Cy5.5 fluorophore is less than the boundary *Ct* value, and through the corresponded *DV* types channels FAM, JOE, ROX, Cy5 *Ct* value is not detected or greater than specified.
- The result is **invalid** if *Ct* value is not determined (absent) in the channel for FAM, JOE, ROX, Cy5 fluorophores, whereas the *Ct* value in the channel for the Cy5.5

fluorophore is not determined (absent) or greater than the specified boundary Ct value. In such cases, PCR analysis should be repeated starting from RNA extraction stage. If the same result is obtained in the second run, re-sampling of material is recommended.



Boundary Ct values are specified in the *Important Product Information Bulletin* enclosed in the PCR kit. See also Guidelines [2]

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

Table 3

Results for controls

Control	Stage for control	Ct value in the channel for fluorophore				
		FAM	JOE	ROX	Cy5	Cy5.5
NCA	RNA extraction	Absent	Absent	Absent	Absent	<boundary value
C-	PCR	Absent	Absent	Absent	Absent	Absent
C ^{+DV 1-4} types / STI	PCR	<boundary value	<boundary value	<boundary value	<boundary value	<boundary value

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^{+DV 1-4} types / STI) in the channels for **FAM, JOE, ROX, Cy5** fluorophores is greater than the boundary Ct value or absent, amplification and detection should be repeated for all samples in which the specific cDNA was not detected in the respective channel.
2. If a Ct value is determined for the Negative Control of extraction (NCA) in the channels for **FAM, JOE, ROX, Cy5** fluorophores, PCR analysis should be repeated for all samples in which cDNA was detected in the respective channel.
3. If a Ct value is determined for the Negative Control of Amplification (C-) in the channels for **FAM, JOE, ROX, Cy5, Cy5.5** fluorophores, PCR analysis should be

repeated for all samples in which cDNA was detected in the respective channel with C-not less than in three repeats.

11. TRANSPORTATION

AmpliSens® Dengue virus type-FRT PCR kit should be transported at 2–8 °C for no longer than 5 days. PCR kit variant 50 F must be de-kitted according to the temperatures of storage.

12. STABILITY AND STORAGE

All components of the **AmpliSens® Dengue virus type-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for RT-G-mix-2, RT-PCR-mix-1-FRT DV, RT-PCR-mix-2-FEP/FRT, Revertase (MMLv) and polymerase (TaqF)). All components of the **AmpliSens® Dengue virus type-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.



RT-PCR-mix-1-FRT DV, RT-PCR-mix-2-FEP/FRT, Revertase (MMLv), RT-G-mix-2 and polymerase (TaqF) are to be stored at the temperature from minus 24 to minus 16 °C



RT-PCR-mix-1-FRT DV is to be kept away from light

13. SPECIFICATIONS

13.1. Sensitivity

Biological material	Pathogen agent	Nucleic acid extraction kit	PCR kit	Sensitivity, copies/ml
Blood plasma/blood serum, mosquito suspension	<i>Dengue virus</i> type 1-4	RIBO-prep	PCR kit variant FRT-50 F	5 x 10 ³
Blood plasma, blood serum	<i>Dengue virus</i> type 1-4	MAGNO-sorb	PCR kit variant FRT-50 F	5 x 10 ²

13.2. Specificity

The analytical specificity of **AmpliSens® Dengue virus type-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 analytical specificity was tested on:

- *flavivirus* (TBEV, Japanese encephalitis B virus, Omsk hemorrhagic fever);

- rickettsiae of spotted fever group (*Rickettsia conorii* ssp. *caspia*, *R.hejlonjiangensis*);
- *Coxiella burnetii*;
- *Bartonell quintana*;
- *Hantaviruses: Puumala, Dobrava*;
- *Leptospira interrogans, L.kirshneri, L.borgpetersenii*.

During the work with the RNA of the above-mentioned microorganisms and with human DNA, mosquito DNA, rodent DNA false positive results were not detected.

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













14. REFERENCES

1. Handbook “Sampling, Transportation, and Storage of Clinical Material for PCR diagnostics”, developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology” of Federal Service for Surveillance on Consumers’ Rights Protection and Human Well-Being, Moscow, 2010.
2. Guidelines to the **AmpliSens® Dengue virus type-FRT** PCR kit for qualitative detection of RNA of *Dengue virus* type 1-4 in the clinical materials (blood plasma, blood serum) and in the human’s autopsy materials (brain, liver, spleen tissues), in animal’s materials (brain, spleen tissues) and in mosquitoes by real-time hybridization-fluorescence detection of amplified products developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology”.

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

16. KEY TO SYMBOLS USED

	Catalogue number		Caution
	Batch code		Sufficient for
	Research use only		Expiration Date
	Version		Consult instructions for use
	Temperature limitation	NCA	Negative control of amplification
	Manufacturer	C-	Negative control of extraction
	Date of manufacture	C+_{DV 1-4 types / STI}	Positive control of amplification
	Keep away from sunlight	IC	Internal control