



For *in Vitro* Diagnostic Use

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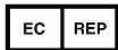
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AmpliSens[®] WNV-FRT

PCR kit

Instruction Manual

AmpliSens[®]



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1. INTENDED USE.

AmpliSens® WNV-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *West Nile virus* RNA in the clinical material (blood plasma, serum; white blood cells; cerebrospinal fluid), autopsy material of human and animals (brain tissue), and biological material (mosquitoes and ticks) by means of real-time RT-PCR with hybridization-fluorescence detection.

2. PRINCIPLE OF PCR DETECTION.

West Nile virus detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using special primers. In real-time PCR the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. The real-time PCR monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run. **AmpliSens® WNV-FRT PCR kit** is a qualitative test and contains the Internal Control (IC) which must be used in the isolation procedure in order to control the isolation process of each individual sample and to identify possible reaction inhibition. **AmpliSens® WNV-FRT PCR kit** uses “hot-start”, which greatly reduces frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) activates by heating at 95°C for 15 min.

3. CONTENT.

AmpliSens® WNV-FRT PCR kit is produced in 1 form:

AmpliSens® WNV-FRT PCR kit variant FRT (for use with RG, iQ, Mx)

REF R-V53(RG,iQ,Mx)-CE

AmpliSens® WNV-FRT PCR kit includes:

Reagent	Description	Volume (ml)	Amount
RT-PCR-mix-1-FRT WNV	colorless, clear liquid	0.6	1 tube
RT-PCR-mix-2-FEP/FRT	colorless, clear liquid	0.3	1 tube
RT-G-mix-2	colorless, clear liquid	0.015	1 tube
Polymerase (TaqF)	colorless, clear liquid	0.03	1 tube
TM-Revertase (MMIv)	colorless, clear liquid	0.015	1 tube
Positive Control cDNA WNV (C+)	colorless, clear liquid	0.1	1 tube
RNA-buffer	colorless, clear liquid	0.6	2 tubes

Negative Control (C-)*	clear liquid of stramineous color	1.6	1 tube
Positive Control WNV-rec	colorless, clear liquid	0.03	5 tubes
Internal Control STI-87-rec (IC)**	colorless, clear liquid	0.12	5 tubes

* must be used in the isolation procedure as Negative Control of Extraction.

** add 10 µl of Internal Control STI-87-rec during the RNA isolation procedure directly to the sample/lysis mixture (see RIBO-sorb **REF** K2-2-Et-50-CE, RIBO-zol-C **REF** K2-13-50-CE, or RIBO-prep **REF** K2-9-Et-50-CE protocols).

AmpliSens® WNV-FRT PCR kit is intended for 60 reactions, including controls.

4. ADDITIONAL REQUIREMENTS.

- RNA isolation kit.
- Disposable powder-free gloves.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers up to 200 µl.
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2 ml reaction tubes.
- PCR box.
- Rotor-Gene™ 3000 or Rotor-Gene™ 6000 (Corbett Research, Australia); iQ5 (BioRad, USA); or Mx3000 (Stratagene, USA) Instrument.
- Disposable polypropylene microtubes for PCR with 0.5 (0.2) ml capacity (for example, “Axygen”, USA).
- Refrigerator for 2–8 °C.
- Deep-freezer with temperature below minus 16 °C.
- Waste bin for used tips.

5. GENERAL PRECAUTIONS.

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. 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.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes and mucose membranes. If any of 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 the techniques of DNA amplification.
- Workflow in the laboratory must proceed in a one directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents to the area in which 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 of biological material samples for PCR-analysis, transportation and storage are described in manufacturer's handbook [1]. It is recommended that this handbook is read before starting of the work.

AmpliSens® WNF-FRT PCR kit is intended for the analysis of RNA extracted with RNA isolation kits from:

— blood plasma, blood serum; leukocytic fraction of blood; cerebrospinal fluid (CSF)

Take whole blood specimen in the morning after overnight fasting in a tube with 6% EDTA solution in the ratio 1:20. Invert closed tube several times. To collect plasma centrifuge the tube at 800-1600 g (3,000 rpm) for 20 minutes. Take 100 µl of plasma for the test.

To obtain leukocytic fraction of blood (diagnostic sensitivity of the test is increased if this clinical material is used) transfer 1.5 ml of the blood with EDTA in a tube (like Eppendorf tube) and centrifuge at 800 rpm for 10 minutes. Then collect 500-600 µl of plasma and centrifuge at 11,000 rpm for 10 minutes. After that remain cell pellet and 100 µl of supernatant for RNA extraction in the tube.

Centrifuge blood serum and CSF at 11,000 rpm for 10 minutes and use the cell pellet and 100 µl of supernatant above the sediment for the test.

— internal organs of animals and autopsy material

Homogenize internal organs of animals and autopsy material by means of a porcelain mortar and pestle and prepare 10% suspension with sterile saline solution or phosphate buffer. Obtain 30 µl of the suspension for RNA extraction.

— mosquitoes suspension

Prepare mosquitoes pools (up to 50 mosquitoes). Homogenize gnats in sterile saline solution or phosphate buffer calculating 30 µl of the solution per 1 mosquito. Centrifuge the samples at 13,000 rpm for 1 minute. Collect 100 µl or the supernatant for RNA extraction.

— ticks suspension

Form ticks pools: hungry ticks pool consists of 5-7 specimens; half-full ticks pool consists of 2-3 specimens; congested ticks pool consists of 1 tick. Place ticks into the tubes (like Eppendorf), add 1 ml

of 96% ethanol and shake on vortex. Centrifuge the tubes with ticks at 5,000 r/min for 3-5 sec then remove fluid by vacuum aspirator. To the tubes with ticks add 1 ml of 0.15 M sodium chloride solution, vortex, and spin at 5,000 rpm for 5 sec. Remove fluid by vacuum aspirator. To make ticks suspension use sterile porcelain mortar and a pestle. Prior to homogenization, congested ticks should be pierced to let blood out. Homogenize ticks in 500 µl (if the sample consists of 1 tick) or 1 ml (if the pool consists of several ticks) of 0.15 M sodium chloride solution. Add the solution by small portion while homogenizing. Centrifuge prepared suspension at 13,000 rpm for 1 min and use 100 µl of supernatant for RNA extraction.

7. PROTOCOL.

7.1. RNA Isolation

It's recommended that the following nucleic acid extraction kits are used:

Material	Extraction kit	REF
<ul style="list-style-type: none"> • blood plasma • blood serum • CSF 	RIBO-sorb	K2-1-Et-50-CE
<ul style="list-style-type: none"> • leukocytic fraction of blood • CSF • suspension of internal organs • suspension of mosquitoes • suspension of ticks • urine sediment (including salts) 	RIBO-zol-C (only for the stage I of RNA extraction)	K2-13-50-CE
	RIBO-sorb (only for the stage II of RNA extraction)	K2-1-Et-50-CE
<ul style="list-style-type: none"> • blood plasma • blood serum • leukocytic fraction of blood • CSF • brain tissue homogenates • mosquitoes homogenates • urine sediment (without salts) 	RIBO-prep	K2-9-Et-50-CE

7.1.1 RIBO-sorb

RNA is extracted from blood plasma, blood serum, and CSF omitting cell concentration stage (if only 200-300 µl of clinical material is available).



Carry out the RNA isolation according to the manufacturer's instructions



Into the tube of Positive Control of extraction add:

90 µl of Negative Control (C-)

10 µl of Positive Control RNA WNV-rec



The volume of the CSF sample should be 250 µl (200 µl of CSF +50 µl of Negative Control (C-))



Centrifuge the tubes at following time and speed:
 after sorbent adding – 30 sec, 9,000 rpm
 after washing with Washing Solution 1 – 30 sec, 9,000 rpm
 after washing with Washing Solution 3 – 45 sec, 9,000 rpm
 after washing with Washing Solution 4 – 1 min, 10,000 rpm

7.1.2 RIBO-zol-C and RIBO-sorb

RNA is extracted from leucocytic fraction of blood and CSF, suspensions of internal organs tissues, mosquitoes and ticks.

I stage. RIBO-zol-C



Carry out the RNA isolation according to the manufacturer's instructions



Volume of the brain tissue suspension sample should be 30 µl.
 Volume of the tick or mosquito suspension sample should be 100 µl

If extracting from leucocytic fraction of blood or CSF:
 add 300 µl of Solution D and 10 µl of IC STI-87-rec directly to the tubes with cell pellet.



Into the tube of Negative Control of extraction add 100 µl of Negative Control (C-) and 10 µl of IC STI-87-rec;

Into the tube of Positive Control of extraction add 10 µl of Positive Control WNV-rec, 10 µl IC STI-87-rec, 90 µl of Negative Control (C-)



Volume of the top phase collected at the final step should be:
 400 µl if extracting from CSF or urine sediments, homogenates of internal organs, mosquitoes, or ticks

300 µl if extracting from blood sediments

450 µl if extracting from Negative or Positive Controls

II stage. RIBO-sorb



Carry out the RNA isolation according to the manufacturer's instructions



Use 400 µl of Lysis Solution instead of 450 µl

Centrifuge the tubes at following time and speed:
 after sorbent adding – 30 sec, 5,000 rpm
 after washing with Washing Solution 1 – 30 sec, 5,000 rpm
 after washing with Washing Solution 3 – 45 sec, 9,000 rpm
 after washing with Washing Solution 4 – 1 min, 10,000 rpm



Store unsealed tube with RNA-buffer at temperature not more than minus 16°C



After adding RNA-buffer, incubate the samples at 56 °C for 5 minutes and spin the tubes in vortex every other minute.

7.1.3 RIBO-prep

RNA is extracted from blood plasma, blood serum, leucocytic fraction of blood, CSF, urine (without salt sediments), brain tissue homogenates, mosquitoes homogenates.



Carry out the RNA isolation according to the manufacturer's instructions



Volume of the tissue homogenates should be 30 µl



If extracting from cells pellets: add 300 µl of Solution for Lysis and 10 µl of IC STI-87-rec directly to the tubes with the samples



Into the tube of Negative Control of extraction add 300 µl of Solution for Lysis and 10 µl of IC STI-87-rec;

Into the tube of Positive Control of extraction add 10 µl of Positive Control WNV-rec, 10 µl IC STI-87-rec, 90 µl of Negative Control (C-)



Volume of RNA-buffer is 40 µl

7.2. Preparing the PCR.

Total reaction volume is **25 µl**, the volume of DNA sample is **10 µl**.

7.2.1 Preparing tubes for PCR.

Variant FRT

1. Prepare the required number (N) of the 0.2 ml tubes. Include negative and positive controls: N+2.
2. Prepare reaction mix for required number of reactions. To do this, mix in a clean tube RT-PCR-mix-1-FRT WNV, RT-PCR-mix-2 FEP/FRT, polymerase (TaqF), RT-G-mix-2, TM-Revertase (MMIv) calculating per each reaction:
 - **10 µl RT-PCR-mix-1-FRT WNV;**
 - **5 µl RT-PCR-mix-2-FEP/FRT;**
 - **0.5 µl polymerase (TaqF);**
 - **0.25 µl TM-Revertase (MMIv);**
 - **0.25 µl RT-G-mix-2;**

Take into account that each run includes at least for control points: Positive and Negative Controls of extraction (PCE, C-) as well as Positive and Negative Controls of amplification (C+, NCA).
3. Transfer **15 µl** of prepared reaction mix per each tube.



Do not store prepared mix.

- Using tips with aerosol barrier add **10 µl** of **RNA samples** obtained from clinical or control samples at the stage of RNA extraction into prepared tubes. Carefully mix by pipette.
- Carry out control amplification reactions:

NCA -Add **10 µl** of **RNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

C+ -Add **10 µl** of **Positive Control cDNA WNV** to the tube labeled C+ (Positive Control of Amplification).

Amplification should immediately follow after compounding of the reaction mix with RNA-samples and controls.

7.2.2. Amplification

7.2.2.1. RG

- Program the Rotor-Gene™ according to manufacturer's manual and Appendix 1.
- Create a temperature profile on your Rotor-Gene™ instrument as follows:

Step	Temperature	Time	Fluorescence detection	Repeats
1	50 °C	30 min	-	1
2	95 °C	15 min	-	1
3	95°C	5 sec	-	5
	56°C	25 sec	-	
	72°C	15 sec	-	
4	95°C	5 sec	-	40
	56 °C	25 sec	FAM, JOE	
	72 °C	15 sec	-	

- Make the adjustment of the fluorescence channel sensitivity according to Appendix 1.

7.2.2.2. iQ

- Program the iQ5 according to manufacturer's manual and Appendix 2.
- Create a temperature profile on your iQ5 instrument as follows:

Step	Temperature	Time	Fluorescence detection	Repeats
1	50 °C	30 min	-	1
2	95 °C	15 min	-	1
3	95°C	5 sec	-	5
	56°C	30 sec	-	
	72°C	15 sec	-	
4	95°C	5 sec	-	40
	56 °C	30 sec	FAM, JOE	
	72 °C	15 sec	-	

- Make the adjustment of the fluorescence channel sensitivity according to Appendix 2.

7.2.2.3. Mx

- Program the Mx3000 according to manufacturer's manual and Appendix 3.
- Create a temperature profile on your Mx3000 instrument as follows:

Step	Temperature	Time	Fluorescence detection	Repeats
1	50 °C	30 min	-	1
2	95 °C	15 min	-	1
3	95°C	5 sec	-	5
	56°C	30 sec	-	
	72°C	15 sec	-	
4	95°C	5 sec	-	40
	56 °C	30 sec	FAM, JOE	
	72 °C	15 sec	-	

- Make the adjustment of the fluorescence channel sensitivity according to Appendix 3.

8. DATA ANALYSIS.

Internal Control is detected in the FAM fluorescence channel, WNV RNA is detected in the JOE fluorescence channel.

See **Appendices 1, 2, 3** for data analysis settings for Rotor-Gene™ 3000/6000, iQ5 or Mx3000 instruments, respectively.

Results interpretation

The results are interpreted by the software of used device by the crossing (or not) of fluorescence curve with the threshold line.

Results for controls

Control	Stage for control	Ct in channel		Interpretation
		FAM	JOE	
C-	RNA isolation	Pos ($\leq Y^*$)	Neg	OK
PCE	RNA isolation	Pos ($\leq F^*$)	Pos ($\leq J^*$)	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	Neg	Pos ($\leq X^*$)	OK

*For F, J, X, Y, values see Appendices 1, 2, 3.

Results for clinical samples

- WNV RNA amplification (JOE channel)**
 - If Ct value of a sample does not exceed A** in JOE channel, the sample is considered positive for *West Nile virus* RNA.
 - If Ct value of a sample exceeds A in JOE channel, the result is considered equivocal. This RNA-sample should be re-examined in two runs.
- IC amplification (FAM channel)**
 - Ct value of a sample should not exceed B** in FAM channel.

- If Ct value of a sample exceeds B in FAM channel while Ct value in JOE channel is negative, the result is considered invalid. The test should be repeated for this sample starting from the extraction stage.

**For A, B, values see Appendices 1, 2, 3.

Results are accepted as relevant if both positive and negative controls of amplification along with negative and positive controls of extraction are passed.

9. TROUBLESHOOTING.

Results of analysis are not being registered in the following cases:

1. Presence of any Ct value either for negative control of extraction (C-) in JOE channel or for negative control of amplification (NCA) in FAM and JOE channels indicates the contamination of reagents or samples. In this case results of the analysis for all samples are considered invalid. It is necessary to repeat the analysis, and also to take measures to detect and eliminate the source of contamination.
2. If Ct value for negative control of extraction (C-) in FAM channel and/or for positive control of extraction (PCE) in FAM and JOE channels are absent, results of the analysis for all samples are considered invalid. It is necessary to repeat the analysis of all samples starting from the extraction stage.
3. If Ct value for positive control of amplification (C+) in JOE channel is absent, results of the analysis for all samples are considered invalid. It is necessary to repeat the RT-PCR for all samples.

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

10. STABILITY AND STORAGE.

All components of the **AmpliSens® WNV-FRT** PCR kit (except for Positive Control cDNA WNV, RNA-buffer, Negative Control, Positive Control WNV-rec, Internal Control STI-87-rec) are to be stored at not more than minus 16 °C, when not in use. They also must be stable until the expiry date stated on the label.



Positive Control cDNA WNV, RNA-buffer, Negative Control, Positive Control WNV-rec, Internal Control STI-87-rec should be stored between 2 and 8 °C

11. SPECIFICATIONS.

11.1. Sensitivity.

Analytical Sensitivity of **AmpliSens® WNV-FRT** PCR kit is no less than 1×10^3 copies per 1 ml of sample (copies/ml).



The claimed analytical features of **AmpliSens® WNV-FRT** PCR kit are guaranteed only when additional reagents kits, “RIBO-sorb”, “RIBO-zol-C”, or “RIBO-prep”, (manufactured by Federal State Institution of Science Central Research Institute of Epidemiology) are used.

11.2. Specificity.

Specificity of **AmpliSens® WNV-FRT** PCR kit is assured by selection of specific primers and probes, as well as the selection of strict reaction conditions. The primers and probes were checked for possible homologies to all in gene banks published sequences by sequence comparison analysis. Specificity of **AmpliSens® WNV-FRT** PCR kit was confirmed in laboratory clinical trials.

















12. REFERENCES.

1. Handbook “Sampling, transportation, storage of clinical material for PCR diagnostics”, developed by Federal State Institution of Science “Central Research Institute of Epidemiology” of Federal Service for Surveillance on Consumers’ Rights Protection and Human Well-Being, Moscow, 2008.

13. QUALITY CONTROL.

In compliance with Federal State Institution of Science “Central Research Institute of Epidemiology” ISO 13485 – certified Quality Management System, each lot of **AmpliSens® WNV-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

14. EXPLANATION OF SYMBOLS.

	Manufacturer		Temperature limitation
	Use by		Batch code
	For <i>in Vitro</i> Diagnostic Use		Version
	Catalogue number		Internal Control complex
	Contains sufficient for <n> tests		Authorized representative in the European Community.
	Consult instructions for use		Caution, consult accompanying documents
	For working with Rotor-Gene™ 3000/6000		For working with iQ5, iQ iCycler
	Positive control		Negative control