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eSens

CE

IVD

For Professional Use Only

eSens WNV QL PCR kit

REF ES3602B

Instructions for Use

1 INTENDED USE

eSens WNV QL PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *West Nile virus (WNV)* RNA in the clinical material (blood plasma, serum, leukocytic fraction of blood, cerebrospinal fluid, and urine) and human autopsy material (brain, liver, spleen, and lymph node tissue), animal material (brain tissue), mosquitoes and ticks 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

West Nile virus detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *WNV* primers. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

eSens WNV QL PCR kit is a qualitative test that contains the Internal Control (Internal Control STI-87-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.

eSens WNV QL PCR kit uses “hot-start,” which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase using a chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	**FAM**	**JOE**
cDNA-target	Internal Control (IC) STI-87-rec cDNA	*WNV* cDNA
Target gene	Artificially synthesized sequence	5' non-coding target and part of the genome encoding nucleoprotein (5'-UTR – C protein)

3 CONTENT

eSens WNV PCR kit (ES3602B) includes:

Reagent	**Description**	**Volume, ml**	**Quantity**
RT-G-mix-2	colorless clear liquid	0.015	1 tube
RT-PCR-mix-1-FRT *WNV*	clear liquid from colorless to light lilac colour	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 (MMlv)	colorless clear liquid	0.015	1 tube
Positive Control cDNA *WNV* / STI (C+$_{WNV/STI}$)	colorless clear liquid	0.1	1 tube
RNA-buffer	colorless clear liquid	0.6	2 tubes
Negative Control (C–)*	colorless clear liquid	1.6	8 tubes
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 extraction procedure as Negative Control of Extraction.

****** add **10 µl** of **Internal Control STI-87-rec (IC)** during the RNA extraction procedure directly to the sample/lysis mixture.

eSens WNV PCR kit is intended for 60 reactions (including controls).

4 ADDITIONAL REQUIREMENTS

- 0.15 M NaCl 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).
- Homogenizer for pretreatment of autopsy material, mosquitos and ticks.
- Stainless steel beads (5 and 7 mm diameter).
- RNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.

- Pipettes (adjustable).
- Sterile pipette tips with aerosol filters (up to 200 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 Touch, CFX 96 Opus (Bio-Rad, USA), QuantStudio 5 (Thermo Fisher Scientific), or equivalent).
- Disposable polypropylene tubes:
- screwed or tightly closed 1.5-ml tubes
- thin-walled 0.2-ml PCR tubes with domed caps if a plate-type instrument is used;
- thin-walled 0.2-ml PCR tubes with flat caps 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 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 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 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.
- 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

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

eSens WNV QL PCR kit is intended for analysis of the RNA extracted with RNA extraction kits from the clinical material:

- *blood plasma, blood serum, and CSF*

Take a whole blood specimen in the morning after overnight fasting in a tube with 6 % EDTA solution in the ratio 1:20. Invert the closed tube several times. To collect plasma, centrifuge the tube at 1600 g for 20 min. Obtain blood serum by standard methods. Take 200 µl of clinical material for the test.

- *leukocytic fraction of blood*

To obtain leukocytic fraction of blood (the clinical material is recommended to be used on the second week of the disease), transfer 1.5 ml of the blood with EDTA to an Eppendorf tube and centrifuge at 400 g for 10 min. Then collect 500–600 µl of plasma and centrifuge at 7,000 g for 10 min. Use the cell pellet and 200 µl of the supernatant for subsequent RNA extraction.

- *internal organs of animals and autopsy material*

Homogenize internal organs of animals and autopsy material in a porcelain mortar with a pestle and prepare 10 % suspension with sterile saline solution or phosphate buffer.

If a TissueLyser LT automated homogenizer is used, set the following parameters:

- the volume of PBS buffer or 0.15 M NaCl solution for homogenizing is defined by the volume of homogenized tissue. The tissue–buffer ratio is 1 : 9; therefore, a 10 % suspension is prepared;
- the total sample volume should not exceed 1 ml for 1.5-ml tubes;
- homogenization parameters

1. for brain tissues: bead size, 5 mm; frequency, 50 Hz/s; homogenization time, 2-3 min;
2. for liver, spleen, and lymph node tissues: bead size, 7 mm, frequency, 50 Hz/s; homogenization time, 10 min;
3. for lymph nodes: bead size, 5 mm; frequency, 50 Hz/s; homogenization time, 5 min.

Take 30 µl of the suspension for RNA extraction.

- *urine*

Collect a urine sample to a clean vessel. If the sample will not be analyzed within 1 day after taking, transfer the urine to a 30-ml centrifuge tube or an Eppendorf tube, add glycerol (10 % of the sample volume), stir, and freeze at the temperature minus 20 °C for storage up to 1 week or at the temperature minus 70 °C for storage for a long time.

If a cooling centrifuge (temperature, 4 °C; tube volume, 30 ml; acceleration, 8,000 g) is used, follow the pretreatment algorithm described below:

Centrifuge the sample at 8,000–9,000 g for 10 min and discard the supernatant. Transfer the pellet and 1 ml of the supernatant to an Eppendorf tube. Centrifuge the sample at 8,000 g for 10 min. Discard 900 µl of the supernatant. Use the pellet with the remaining 100 µl of the supernatant for RNA extraction. In the case of a high content of salts in the urine sample, transfer 100 µl of the supernatant to an Eppendorf tube and use it for RNA extraction.

If a cooling centrifuge for 30-ml tubes and 8,000 g is absent, centrifuge 1-ml sample as described below. Extract RNA from the pellet with 100 µl of the supernatant.

- *mosquito suspension*

To prepare mosquitoes suspension, use a porcelain mortar and pestle. If a TissueLyser LT automated homogenizer is used, set the following homogenizing parameters: bead size, 5 mm; frequency, 50 Hz/s; homogenization time, 5 min; buffer volume, 700 µl (a pooled sample of 25 mosquitoes) or 1,000–1500 µl (a pooled sample of 50 mosquitoes).

Prepare mosquito 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 10,000 g for 1 minute. Collect 100 µl or the supernatant for RNA extraction.

- *tick suspension*

Form tick pools: pool of hungry ticks consists of 5-7 specimens; pool of half-full ticks consists of 2-3 specimens; pool of blood-filled ticks consists of 1 tick.

If a TissueLyser LT automated homogenizer is used, set the following homogenizing parameters for the *Hyalomma* genus ticks: bead size, 7 mm; frequency, 50 Hz/s; homogenization time, 10–12 min; buffer volume, 700 µl (hungry tick) or 1,000-1500 µl (blood-filled tick and tick pools).

To prepare tick suspension a sterile porcelain mortar and pestle can be used as well. Prior to homogenization, blood-filled ticks should be pierced to let blood out. Homogenize ticks in 700 µl (if the sample consists of 1 hungry tick) or 1-1.5 ml (if a blood-filled tick or tick pools are homogenized) of 0.15 M sodium chloride solution. Add the solution in small portions while homogenizing. Centrifuge the prepared suspension at 10,000 rpm for 1 min and use 100 µl of the supernatant for RNA extraction.

The samples are to be stored at 2–8 °C for 1 day or at the temperature not more than minus 16 °C for 1 week.

Internal organ tissues and mosquitoes are to be stored at the temperature not more than minus 16 °C for 1 week or at the temperature minus 70 °C for a long time.

Ticks are to be stored alive at most 1 month or at the temperature not more than minus 16 °C for 1 week, or at the temperature minus 70 °C for a long time.

7 WORKING CONDITIONS

eSens WNV QL PCR kit should be used at 18–25 °C.

8 PROTOCOL

8.1 RNA extraction

Any commercial nucleic acid extraction kit, if IVD-CE validated for the indicated specimen types, could be used.

Ecoli Dx, s.r.o. recommends:

- For the manual extraction

- **RIBO-prep** (K2-9-Et-100-CE)

- For the automatic extraction

- **ePure Viral Nucleic acid Extraction Kit** (E2003)

8.1.1 RIBO-prep

RNA is extracted from blood plasma, blood serum, leukocytic fraction of blood, CSF, urine (without salt sediments), homogenates of internal organ tissues, and mosquito homogenates.

NOTE:

- Extract RNA according to the manufacturer's protocol taking into account next additions and improvements:
- The volume of the tissue homogenate sample is 30 µl. The volume of the suspension of mosquitoes is 100 µl. The volume of the blood plasma, blood serum, CSF is 100 µl.
- If extracting from leukocytic fraction of blood and urine sediment: add 300 µl of Solution for Lysis and 10 µl of Internal Control STI-87-rec (IC) directly to the tubes with the samples.
- Add 300 µl of Solution for Lysis and 10 µl of Internal Control STI-87-rec (IC) to the tube labeled C– (Negative Control of Extraction);
- Add 10 µl of Positive Control *WNV*-rec, 10 µl of Internal Control STI-87-rec (IC), and 300 µl of Solution for Lysis to the tube labeled PCE (Positive Control of Extraction).

Centrifuge the tubes under the following conditions:

- after Solution for Precipitation addition, at 10,000 g for 5 min,
- after washing with Washing Solution 3, at 10,000 g for 2 min,
- after washing with Washing Solution 4, at 10,000 g for 2 min,
- after RNA-buffer addition, at 10,000 g for 1 min.

8.2 Preparing reverse transcription and PCR

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

8.2.1 Preparing tubes for PCR

The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, RNA and control samples into tubes.

1. Prepare the required number of tubes for PCR.
2. Prepare the reaction mixture for the required number of reactions. To do this, mix in a new tube RT-PCR-mix-1-FRT *WNV*, RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), RT-G-mix-2, and TM-Revertase (MMlv) calculating per <u>each</u> reaction:

- **10 µl of RT-PCR-mix-1-FRT *WNV*;**
- **5 µl of RT-PCR-mix-2-FEP/FRT;**
- **0.5 µl of polymerase (TaqF);**
- **0.25 µl of TM-Revertase (MMlv);**
- **0.25 µl of RT-G-mix-2;**

Take into account that each run includes at least four control points: Positive and Negative Controls of extraction (PCE, C–) as well as Positive and Negative Controls of RT-PCR ($C+_{WNV\,/\,STI}$, NCA).

3. Transfer **15 µl** of the prepared reaction mixture to each tube.

NOTE: Do not store the prepared mixture.

4. Using filter tips add **10 µl** of **RNA samples** obtained at the RNA extraction stage into prepared tubes. Carefully mix by pipette.
5. Carry out the control amplification reactions:

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

C+*WNV / STI* Add **10 µl of Positive Control cDNA *WNV* / STI** to the tube labeled **C+*WNV / STI*** (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 *WNV*-rec reagent** to the tube labeled **PCE** (Positive control of Extraction)

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

8.2.2 Reverse transcription and amplification

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

Table 2

	Rotor-type instruments (e.g Rotor-Gene Q or equivalent)			**Plate-type instruments (e.g CFX 96 Touch, CFX 96 Opus, QuantStudio 5 or equivalent)**		
Step	**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**	**5 s**	**5**	**95**	**5 s**	**5**
	56	**25 s**		**56**	**30 s**	
	72	**15 s**		**72**	**15 s**	
4	**95**	**5 s**	**40**	**95**	**5 s**	**40**
	56	**25 s** ***fluorescent signal detection***		**56**	**30 s** ***fluorescent signal detection***	
	72	**15 s**		**72**	**15 s**	

Fluorescent signal is detected in the channels for the FAM and JOE fluorophores.

2. Adjust the fluorescence channel sensitivity.
3. Insert tubes into the reaction module of the device.
4. Run the amplification program with fluorescence detection.
5. Analyze results after the amplification program is completed.

8.3 Instrument Settings

Test settings for rotor-type instruments

Channel	Calibrate/Gain Optimisation	Threshold	Dynamic tube	Slope Correct	More Settings/ Outlier Removal	Eliminate cycles before
FAM/Green	from 5 FI to 10 FI	0.03	On	On	5%	5
JOE/Yellow	from 5 FI to 10 FI	0.03	On	On	5%	5

Test settings for plate-type instruments

Set the heating/cooling ***Ramp Rate* 2,5 °C/s**.

Channel	Threshold
FAM JOE/HEX	For each channel in ***Log Scale*** set the threshold line at the level of 10% (FAM) and 5% (HEX) of maximum fluorescence obtained for the Positive Control of Amplification (C+) in the last amplification cycle

9 DATA ANALYSIS

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

- The signal of the IC cDNA amplification product is detected in the channel for the FAM fluorophore.
- The signal of the *WNV* cDNA amplification product is detected in the channel for the JOE fluorophore.

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

The results are interpreted according to the Table 3.

Table 3

Correspondence of targets and channels

PCR-mix-1	Detection in the channel for the fluorophore	
	FAM	**JOE**
RT-PCR-mix-1-FRT *WNV*	IC	*WNV*

Principle of interpretation is the following:

- *WNV* cDNA is **detected** in a sample if the *Ct* value determined in the results grid in the channel for the JOE fluorophore is less than the boundary *Ct* value. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
- *WNV* cDNA is **not detected** in a sample if the *Ct* value determined in the result grid in the channel for the FAM fluorophore is less than the specified boundary value, whereas the *Ct* value in the channel for the JOE fluorophore is not determined or greater than the specified boundary value.
- The result is **invalid** if the *Ct* value is not determined (absent) in the channel for JOE fluorophore, whereas the *Ct* value in the channel for the FAM fluorophore is not determined (absent) or

greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the RNA extraction stage.

NOTE: Boundary *Ct* values are specified in the table below.

The result of the analysis is considered reliable only if the results obtained for the Positive and Negative Controls of amplification as well as for the Positive and Negative Control of extraction are correct (see Table 4 and 5).

Table 4

Results for controls

Control	**Stage for control**	***Ct* value in the channel for the fluorophore**	
		JOE	**FAM**
C–	RNA extraction	Absent	<boundary value
PCE	RNA extraction	<boundary value	<boundary value
NCA	PCR	Absent	Absent
C+*WNV / STI*	PCR	<boundary value	<boundary value

Table 5

Boundary *Ct* values

	Rotor-type instrument		**Plate-type instrument**	
Sample	**Channel for fluorophore**			
	FAM	**JOE**	**FAM**	**JOE**
C-	28	Ct is absent	31	Ct is absent
PCE	28	30	31	33
C+	28	28	31	31
Test samples	28 (for blood serum, CSF) 31 (for homogenized mosquitos, ticks, internal organs, urine)	38	31 (for blood serum, CSF) 33 (for homogenized mosquitos, ticks, internal organs, urine)	39
NCA	Ct is absent		Ct is absent	

10 TROUBLESHOOTING

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

1. If the *Ct* value determined for the Positive Control of Amplification (C+) in the channel for the JOE fluorophore is greater than the boundary Ct value or absent, the amplification should be repeated for all samples in which the specific cDNA 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 extraction should be repeated for all samples in which the specific cDNA was not detected.

3. If the *Ct* value is determined for the Negative Control of Extraction (C–) in the channel for the JOE fluorophore, the PCR-analysis should be repeated for all samples in which cDNA was detected in the channel for the JOE fluorophore.
4. If the *Ct* value is determined for the Negative Control of Amplification (NCA) in the channels for the FAM and JOE fluorophores, the amplification should be repeated for all samples in which cDNA was detected in the channel for the JOE fluorophore, with simultaneously running NCA in triplicate.

11 TRANSPORTATION

eSens WNV QL PCR kit should be transported at 2–8 °C for no longer than 5 days.

12 STABILITY AND STORAGE

All components of the **eSens WNV QL PCR kit** (except for RT-G-mix-2, RT-PCR-mix-1-FRT *WNV*, RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), and TM-Revertase (MMlv)) are to be stored at 2–8 °C when not in use. All components of the **eSens WNV QL PCR kit** are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: RT-G-mix-2, RT-PCR-mix-1-FRT *WNV*, RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), and TM-Revertase (MMlv) are to be stored at temperature from minus 24 to minus 16 °C when not in use.

NOTE: RT-PCR-mix-1-FRT *WNV* is to be kept away from light.

13 SPECIFICATIONS

13.1 Analytical sensitivity

The analytical sensitivity of **eSens WNV QL PCR kit** is specified in the table below.

Type of biological material (test sample volume)	**RNA extraction kit**	**PCR kit**	**Analytical sensitivity, copies/ml**	**Pretreatment of biological material**
blood serum (200 µl), CSF (200 µl), leukocytic fraction of blood (200 µl), 10 % brain tissue homogenate (30 µl), mosquitoes (100 µl) leukocytic fraction of blood (200 µl), 10 % brain tissue homogenate (30 µl), mosquitoes (100 µl) blood plasma, blood serum, CSF (1 ml for all)	**RIBO-prep** **ePure Viral Nucleic acid Extraction Kit** (E2003)	eSens WNV QL PCR kit	5×10^3	Indicated sensitivity can be reached only if the specified pretreatment instructions are followed and the specified specimen volume is used

13.2 Analytical specificity

The analytical specificity of **eSens WNV QL 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 deposited in gene banks by sequence comparison analysis. The analytical specificity was assayed using the following microorganisms:

- *flaviviruses* (*tick-borne encephalitis virus, Langat, Powassan, Japanese encephalitis, and Omsk hemorrhagic fever viruses*);
- *herpes viruses* (I and II types, *CMV, EBV, VZV*, IV type), *enteroviruses* (*ECHO, Coxsackie*);
- *rickettsiae* of spotted fever group (*Rickettsia conorii spp. caspia and R.heilongiangensis; Coxiella burnetii*; and *Bartonella henselae* and *B.quintana*).
- spirochaetes (*Borrelia miyamotoi; Treponema pallidum; Leptospira interrogans, L.kirshneri*; and *L.borgpetersenii*);

No false-positive results were observed during examination of RNA/DNA of the above-mentioned organisms, human DNA, DNA of birds, DNA of ticks and mosquitoes, DNA of rodents.

The clinical specificity of **eSens WNV QL PCR kit** was confirmed in laboratory clinical trials.

14 QUALITY CONTROL

The production process, including batch release, is carried out in accordance with an established quality management system certified according to ISO 13485.

15 KEY TO SYMBOLS USED

REF	Catalogue number	⚠	Caution
LOT	Batch code	Σ	Contains sufficient for <n> tests
IVD	In vitro diagnostic medical device	⌛	Use-by Date
VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer	NCA	Negative control of amplification
	Date of manufacture	C–	Negative control of extraction
PCE	Positive Control of Extraction	C+WNV / STI	Positive control of Amplification
		IC	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
01_04/2022		

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