ECOLI

eSens

CE

IVD

For Professional Use Only

eSens Dengue virus type QL PCR kit

REF ES3601B

Instructions for Use

1 INTENDED USE

eSens Dengue virus type QL PCR kit is an *in vitro* nucleic acid amplification test for detection and differentiation of RNA of *Dengue virus* type 1-4 in the human clinical (blood plasma, blood serum) and autopsy material (brain, liver, spleen tissues), in animal material (brain, spleen tissues), in mosquitoes 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

The method is based on the RNA extraction from the biological material samples, RNA reverse transcription and amplification of the part of *DV* cDNA with real-time hybridization-fluorescence detection.

Dengue virus (DV) RNA detection by the polymerase chain reaction (PCR) is based on the RNA reverse transcription using TM-revertase enzyme and amplification of the fragments of *DV* 1-4 types cDNA using specific primers for the cDNA parts and Taq-polymerase enzyme. 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 Dengue virus type 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 Dengue virus type QL PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by the separation of nucleotides and Taq-polymerase using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

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

Table 1

Channel for fluorophore	**FAM**	**JOE**	**ROX**	**Cy5**	**Cy5.5**
cDNA-target	*Dengue virus* 1 type cDNA	*Dengue virus* 2 type cDNA	*Dengue virus* 3 type cDNA	*Dengue virus* 4 type cDNA	Internal Control STI-87-rec (IC) cDNA
Target gene	5'-noncoding region and part of the genome encoding the nucleoprotein (5'-UTR-C-protein)	5'-noncoding region and part of the genome encoding the nucleoprotein (5'-UTR-C-protein)	5'-noncoding region and part of the genome encoding the nucleoprotein (5'-UTR-C-protein)	5'-noncoding region and part of the genome encoding the nucleoprotein (5'-UTR-C-protein)	Artificially synthesized sequence

3 CONTENT

eSens Dengue virus type QL PCR kit (ES3601B) includes:

Reagent	**Description**	**Volume, ml**	**Quantity**
RT-G-mix-2	colorless clear liquid	0.015	1 tube
RT-PCR-mix-1-FRT *DV*	clear liquid from colorless to blue grey 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 *DV* 1-4 types / STI (C+$_{DV\ 1\text{-}4\ types\ /\ STI}$)	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

***** 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 Dengue virus type QL 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 is recommended to use for autopsy materials and mosquitoes homogenization.
- Stainless steel balls with 5 mm and 7 mm diameter.
- RNA extraction kits.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips with aerosol filters (up to 200 µl).
- Disposable screwed up or tightly closed 1.5 ml polypropylene PCR tubes.
- 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 PCR tubes:
  a) 0.2-ml PCR tubes with optical transparent domed or flat caps if a plate-type instrument is used;
  b) 0.2-ml PCR tubes with flat caps if a rotor-type instrument is used.
- Refrigerator at the temperature range from 2 to 8 °C.
- Deep-freezer at the temperature range 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

eSens Dengue virus type QL PCR kit is intended for analysis of RNA extracted with RNA extraction kits from the human clinical (blood plasma, blood serum) and autopsy material (brain, liver, spleen tissues), animal material (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** (K2-9-Et-100-CE).

6.2 *Autopsy materials (brain, liver, spleen tissues).*

This material is homogenized using sterile porcelain mortars and pestles, after that 10% suspension is made on the sterile saline solution or phosphate buffer. In case of using an automatic homogenizer TissueLyser LT the following homogenization parameters for internal tissues should be applied: 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*.

Use sterile porcelain cap and sterile pestle for preparing mosquito suspension. In case of using an automatic homogenizer TissueLyser LT (QIAGEN, Germany) the following homogenization parameters for mosquitoes should be applied: 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 of 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 at 2-8 °C for 24 hours before the test or for a week at the temperature from minus 24 to minus 16 °C. For autopsy material and mosquitoes the following storage conditions are provided: internal tissues and mosquitoes are stored at temperature from minus 24 to minus 16 °C for a week, at temperature minus 68 °C - for a long time.

7 WORKING CONDITIONS

eSens Dengue virus type 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 RNA extraction from blood plasma and serum, internal and brain tissues homogenates, mosquitoes.

- For the automatic extraction

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

NOTE: The RNA extraction of each sample is carried out in the presence of **Internal Control STI-87-rec (IC).**
Extract the RNA according to the manufacturer's protocol.
Positive control of extraction (PCE) is not carried out in the analysis.

8.2 Preparing reverse transcription and PCR

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, DNA and control samples into tubes.

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

1. Prepare the reaction mixture for necessary number of reactions. Mix in the separate tube **RT-PCR-mix-1-FRT *DV*, RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), TM-Revertase (MMlv)** and **RT-G-mix-2.** For one reaction add:

   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 (MMlv);**
   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+ and NCA).

2. Add into each tube **15 µl** of the prepared mixture.

NOTE: Do not store the prepared mixture.

3. Add **10 µl** of the **RNA samples** extracted from the test and control samples into the prepared tubes. Mix carefully by pipetting.
4. Carry out the control amplification reactions:

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

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

8.2.2 Amplification

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

Table 2

Amplification program

	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**	**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, Cy** and **Cy5.5** fluorophores.

2. Insert tubes into the reaction module of the device. **Well 1** must be filled with the test tube.
3. Run the amplification program with fluorescence detection.
4. 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 5FI to 10FI	0.03	on	on	10 %	5
JOE/Yellow	from 5FI to 10FI	0.03	on	on	10 %	5
ROX/Orange	from 5FI to 1OFI	0.03	on	on	10 %	5
Cy5/Red	from 5FI to 10FI	0.03	on	on	15 %	5
Cy5.5/Crimson	from 5FI to 10FI	0.03	on	on	5 %	5

Note: If the fluorescence curves in the FAM/Green channel do not correspond to the exponential growth set the value of the threshold of negative samples (***NTC threshold***) equal to **20 %**.

If the fluorescence curves in the JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson channels do not correspond to the exponential growth set the value of the threshold of negative samples (***NTC threshold***) equal to **15-20 %**.

Test settings for plate-type instruments

Note: Set ***Ramp Rate* 2,5 °C/s** by clicking the ***Step Options*** button for each step of cycling.

Channel	Threshold
FAM, Cy5	Set the threshold line level at 20 % from the maximum fluorescence level of the Positive Control samples in the last amplification cycle.
HEX, ROX, Cy5.5	Set the threshold line level at 1O % from the maximum fluorescence level of the Positive Control samples in the last amplification cycle.

Note: The fluorescence curve of Positive Control samples is to cross the threshold line in the area of the fluorescence typical exponential rise which becomes a linear rise.

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 cDNA fragment amplification product **is detected** in the channel for the FAM fluorophore.
- The signal of the *Dengue virus* 2 cDNA fragment amplification product **is detected** in the channel for the JOE fluorophore.
- The signal of the *Dengue virus* 3 cDNA fragment amplification product **is detected** in the channel for the ROX fluorophore.
- The signal of the *Dengue virus* 4cDNA fragment amplification product **is detected** in the channel for the Cy5 fluorophore.
- The signal of the IC cDNA fragment 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 the cDNA sample in the corresponding column of the results grid.

Results are interpreted according to the Table 3.

Table 3

Correspondence of the targets and detection channels

Detection through the channel				
FAM	**JOE**	**ROX**	**Cy5**	**Cy5.5**
DV 1	*DV* 2	*DV* 3	*DV* 4	IC

Principle of interpretation is the following:

- *DV* cDNA **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. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
- *DV* cDNA 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 in the corresponded to *DV* types channels for the FAM, JOE, ROX, Cy5 fluorophores *Ct* value is not determined (absent) or greater than specified.
- The result **is invalid** if *Ct* value is not determined (absent) in the channel for the 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.

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 Negative Control of extraction are correct (see Table 4 and Table 5).

Table 4

Results for controls

Control	**Stage for control**	***Ct* value in the channel for fluorophore**				
		FAM	**JOE**	**ROX**	**Cy5**	**Cy5.5**
C–	RNA extraction	Absent	Absent	Absent	Absent	<boundary value
NCA	PCR	Absent	Absent	Absent	Absent	Absent
C+	PCR	<boundary value	<boundary value	<boundary value	<boundary value	<boundary value

Table 5

Boundary *Ct* values

Sample	Rotor-Gene Q					CFX96				
	The channel for fluorophore									
	FAM	**JOE**	**ROX**	**Cy5**	**Cy5.5**	**FAM**	**JOE**	**ROX**	**Cy5**	**Cy5.5**
	DV1	*DV2*	*DV3*	*DV4*	Detection of IC	*DV1*	*DV2*	*DV3*	*DV4*	Detection of IC
NCA	-	-	-	-	-	-	-	-	-	-
C-	-	-	-	-	<29	-	-	-	-	<31
C+	<25	<25	<25	<25	<25	<28	<28	<28	<28	<28
Test samples	<38	<38	<38	<38	<29 (for blood serum and blood plasma) <31 (for homogenates of brain tissues, internals, mosquitoes)	<38	<38	<38	<38	<31 (for blood serum and blood plasma) <31 (for homogenates of brain tissues, internals, mosquitoes)

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+) in the channels for the FAM, JOE, ROX, Cy5 fluorophores is greater than the boundary *Ct* value or absent, the amplification should be repeated for all the samples in which the specific cDNA was not detected in the respective channel.
2. If the *Ct* value is determined for the Negative Control of extraction (C-) in the channels for the FAM, JOE, ROX, Cy5 fluorophores, PCR analysis should be repeated for all the samples in which cDNA was detected in the respective channel.
3. If the *Ct* value is determined for the Negative Control of Amplification (NCA) in any of the channels for the FAM, JOE, ROX, Cy5, Cy5.5 fluorophores, the amplification should be repeated not less than in three repeats for all the samples in which cDNA was detected in the respective channel with NCA.

11 TRANSPORTATION

eSens Dengue virus type 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 Dengue virus type QL 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, TM-Revertase (MMlv) and polymerase (TaqF)). All components of the **eSens Dengue virus type QL 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: RT-PCR-mix-1-FRT *DV*, RT-PCR-mix-2-FEP/FRT, TM-Revertase (MMlv), RT-G-mix-2 and polymerase (TaqF) are to be stored at the temperature from minus 24 to minus 16 °C

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

13 SPECIFICATIONS

13.1 Analytical sensitivity

Biological material	Volume of test sample	Nucleic acid extraction kit	PCR kit	Analytical sensitivity, copies/ml	Material pretreatment
Blood plasma/blood serum, mosquito suspension	100 µl	RIBO-prep	ES3601B	5×10^3	The claimed sensitivity is achieved while respecting the rules and using the recommended volume of test sample
Blood plasma, blood serum	1 ml	ePure Viral Nucleic Acid Extraction Kit	ES3601B	5×10^2	

13.2 Analytical specificity

The analytical specificity of **eSens Dengue virus type 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 published in gene banks by sequence comparison analysis.

The analytical specificity was tested on:

- *Flaviviruses* (*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 **eSens Dengue virus type 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
LOT	Batch code
IVD	*In vitro* diagnostic medical device
VER	Version
	Temperature limit
	Manufacturer
	Date of manufacture
EC REP	Authorized representative in the European Community
	Caution
	Contains sufficient for <n> tests
	Use-by Date
	Consult instructions for use
	Keep away from sunlight
NCA	Negative control of amplification
C-	Negative control of extraction
C+	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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