



For Professional Use Only

eSens Plasmodium sp./falciparum/vivax QL PCR kit

REF ES3640B

Instructions for Use

1 INTENDED USE

eSens Plasmodium sp./falciparum/vivax QL PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of DNA of all malaria plasmodium species (*Plasmodium* spp.) and differentiation of DNA of malignant tertian (*P.falciparum*) and tertian (*P.vivax*) malaria pathogens in the biological material (whole blood, mosquitoes) using real-time hybridization-fluorescence detection of amplified products. The material for PCR is DNA samples extracted from test material.

Indications and contra-indications for use of the reagent kit

The reagent kit is used in clinical laboratory diagnostics for the analysis of biological material, taken from persons with suspected diseases caused by malaria plasmodium species without distinction of form and presence of disease manifestation.

There are no contra-indications with the exception of cases when the material cannot be taken for medical reasons.

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

2 PRINCIPLE OF PCR DETECTION

Principle of testing is based on the DNA extraction from the samples of test material with the exogenous internal control sample (Internal Control-FL (IC)), and simultaneous amplification of DNA fragments of the detected microorganisms (*Plasmodium* spp., *P.falciparum*, *P.vivax*) and DNA of the internal control with hybridization-fluorescence detection. Exogenous internal control (Internal Control-FL (IC)) allows to control all PCR-analysis stages of each individual sample and to identify possible reaction inhibition.

Amplification of DNA fragments with the use of specific primers and Taq-polymerase enzyme are performed with the DNA 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.

eSens Plasmodium sp./falciparum/vivax QL PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

eSens Plasmodium sp./falciparum/vivax QL PCR kit contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and deoxyuridine triphosphate (dUTP).

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

Table 1

Channel for fluorophore	FAM	JOE	ROX	Cy5
DNA-target	<i>Plasmodium</i> spp. DNA	<i>P. falciparum</i> DNA	<i>P. vivax</i> DNA	IC DNA
Target gene	Non-transcribed area between cox3 and cox1	Cox1	Cox1	Artificially synthesized sequence

3 CONTENT

eSens Plasmodium sp./falciparum/vivax QL PCR kit (ES3640B) includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL <i>Plasmodium</i> spp.	clear liquid from colorless to light lilac colour	0.6	1 tube
PCR-buffer-H	colorless clear liquid	0.3	1 tube
C+ <i>Plasmodium</i> spp.	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Negative Control (C-)*	colorless clear liquid	1.2	1 tube
Internal Control-FL (IC)**	colorless clear liquid	0.5	1 tube

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

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

eSens Plasmodium sp./falciparum/vivax QL PCR kit is intended for 55 reactions (including controls).

4 ADDITIONAL REQUIREMENTS

For sampling and pretreatment

- 0.9 % of sodium chloride (sterile saline solution) or phosphate buffered saline (PBS) (137 mM sodium chloride; 2,7 mM potassium chloride; 10 mM sodium monophosphate; 2 mM potassium diphosphate; pH=7,5±0,2).
- Vacuum tubes for sampling, storage and transportation of blood samples.
- Disposable tightly closed polypropylene 1.5 and 2.0-ml tubes for sampling and pretreatment.
- Sterile pipette tips with aerosol filters (up to 100, 200, 1,000 µl).
- Tube racks.
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and pestle) or homogenizer for pretreatment of tissue material and mosquitoes.
- Desktop centrifuge up to 12,000 g (suitable for Eppendorf tubes).
- Vortex mixer.
- Vacuum aspirator with flask for removing supernatant.
- Pipettes (adjustable).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir to throw off and inactivate the material.
- Disposable powder-free gloves and a laboratory coat.

For DNA extraction and amplification


- DNA extraction kit or the automated station for DNA extraction.
- Sterile pipette tips with aerosol filters (up to 100, 200 µl).
- Tube racks.
- Vortex mixer.
- 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) screwed or tightly closed 1.5-ml tubes for reaction mixture preparation.
 - b) 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;
 - c) 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.
- Pipettes (adjustable).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.
- Disposable powder-free gloves and a laboratory coat.

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 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 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.
- While observing the conditions of transportation, operation and storage, there are no risks of explosion and ignition.
- 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

eSens Plasmodium sp./falciparum/vivax QL PCR kit is intended for analysis of the DNA extracted with DNA extraction kits from the biological material (whole blood, mosquitoes).

Sampling

6.1 Whole blood.

Whole blood should be taken after overnight fasting or in 3 hours after eating by a disposable 0.8-1.1 mm diameter needle into the tube (special vacuum system) with EDTA or sodium citrate as anticoagulant. After blood sampling the tube should be smoothly rotated several times for the thoroughly mixing with the anticoagulant. (Otherwise, blood will coagulate and DNA extraction will be impossible!). Place the tube in the rack after rotating.

Whole blood samples can be stored before pretreatment:

- at the temperature from 20 to 25 °C – for 2 hours;
- at the temperature from 2 to 8 °C – for 12 hours;
- at the temperature not more than minus 16 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

6.2 Mosquitoes.

The collected material should be sorted in laboratory according to species, gender, places and dates of collection and placed into the dry sterile 2.0-ml tubes. Number of mosquitoes in pool for analysis should not exceed 50.

The material samples can be stored after sorting and sample forming:

- at the temperature from minus 24 to minus 16 °C – for 1 month;
- at the temperature no more than minus 68 °C or in a Dewar flask with liquid nitrogen – for a long time.

Only one freeze-thawing cycle is acceptable.

Pretreatment

6.3 Whole blood.

Pretreatment of whole blood is not required.

6.4 Mosquitoes.

Mosquitoes are to be pretreated.

To prepare the suspension, form mosquito pools of no more than 50 specimens into a sterile porcelain mortar, add 0.9 % sodium chloride solution (sterile saline solution) or phosphate buffer solution (PBS) at the rate of 1 mosquito – 30 µl of solution and homogenize the sample with a sterile pestle. The following parameters should be applied for mosquito homogenization when an automatic homogenizer is used: beads' diameter – 5 mm, frequency – 50 Hz/s, homogenization time – 5 min, buffer volume – 700 µl (pool of 25 mosquitoes), 1500 µl (pool of 50 mosquitoes). Transfer the sample with a filter tip to a 1.5 ml microcentrifuge tube and centrifuge for 1 min at 10,000 g (for example, 12,000 rpm for the MiniSpin Eppendorf microcentrifuge). Take 100 µl of supernatant for DNA extraction.

The pretreated mosquitoes can be stored before the PCR analysis:

- at the temperature from minus 24 to minus 16 °C – for 1 week;
- at the temperature no more than minus 68 °C or in a Dewar flask with liquid nitrogen, preliminary formed into pools of 50 specimens – for a long time.

Only one freeze-thawing cycle is acceptable.

Interfering substances and limitations of using test material samples

The whole blood samples, collected in the tubes with heparin as anticoagulant are inapplicable for analysis.

In order to control the DNA extraction efficiency and possible reaction inhibition the Internal Control (Internal Control-FL (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.

Potential interfering substances

Endogenous and exogenous substances that may be present in the biological material (whole blood) used for the study were selected to assess potential interference.

Samples of whole blood without adding and with the addition of endogenous and exogenous potential interfering substances were tested. Concentration of each potential interfering substance is specified in the Table 2.

Model samples of whole blood contained *Plasmodium* spp., *P.falciparum*, *P.vivax* DNA at concentrations of 1×10^4 copies/ml.

Table 2

Type of potential interferent	Potential interferent	Tested concentration in a sample	Interference presence
Endogenous substances	Hemoglobin	250 g/l (upper limit of normal – 170 g/l)	Not detected
	Total bilirubin	210 µmol/l (upper limit of normal – 21 µmol/l)	Not detected
	Total cholesterol	78 mmol/l (upper limit of normal – 7.8 mmol/l)	Not detected
	Triglycerids	37.0 mmol/l (upper limit of normal – 3.7 mmol/l)	Not detected
Exogenous substances	Lithium heparin	from 12 to 30 IU/ml	<u>Detected</u>
	Potassium EDTA	2.0 µg/ml	Not detected

7 WORKING CONDITIONS

eSens Plasmodium sp./falciparum/vivax QL PCR kit should be used at the temperature from 20 to 28 °C and relative humidity from 15 to 75 %.

8 PROTOCOL

8.1 DNA 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 DNA extraction from whole blood and mosquitoes

- For the automatic extraction

- **ePure Blood DNA Extraction Kit** (E2001)

NOTE: Extract the DNA according to the manufacturer's protocol.

The DNA extraction for each sample is carried out in the presence of **Internal Control-FL (IC)**.

The purified DNA can be stored at 2–8 °C for 1 week, at the temperature from minus 24 to minus 16 °C for 6 months, and at the temperature not more than minus 68 °C for 1 year. To do this, transfer the supernatant into a sterile tube, without disturbing the magnetized silica.

8.2 Preparing 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 the **DNA** sample is **10 µl**.

1. Calculate the required quantity of each reagent for reaction mixture preparation. For one reaction:

10 µl of PCR-mix-FL *Plasmodium* spp.,
5 µl of PCR-buffer-H.

Prepare the reaction mixture for the total number of test and control samples plus some extra reaction. See numbers of control samples in item 7.

NOTE: Prepare the reaction mixture just before use.

2. Thaw the tubes with **PCR-mix-FL *Plasmodium* spp.** and **PCR-buffer-H**. 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 *Plasmodium* spp.** and **PCR-buffer-H**. Sediment the drops by vortex.
4. Take the required number of the tubes or strips for PCR of DNA 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 **DNA samples** obtained by extraction of the test samples.
7. Carry out the control amplification reactions:

NCA	–	Add 10 µl of TE-buffer to the tube labeled NCA (Negative Control of Amplification)
C+	–	Add 10 µl of C+ <i>Plasmodium</i> spp. to the tube labeled C+ (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).

NOTE: Carry out the PCR just after the mix of reaction mixture and DNA-samples and controls.

8.2.2 Amplification

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

Table 3

eSens unified amplification program

Step	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)	
	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, ROX, Cy5	

NOTE: Any combination of the tests (including tests with reverse transcription and amplification) 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. If only the tests for DNA detection are performed in one instrument then the first step of reverse transcription (50 °C – 15 min) can be omitted for time saving.

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

2. Adjust the fluorescence channel sensitivity.
3. Insert tubes into the reaction module of the device.

NOTE: It is recommended to sediment drops from walls of tubes by short centrifugation 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.

9 DATA ANALYSIS

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

Table 4

Channel for the fluorophore	FAM	JOE	ROX	Cy5
Amplification product	<i>Plasmodium</i> spp. DNA	<i>P. falciparum</i> DNA	<i>P. vivax</i> DNA	Internal Control-FL (IC) DNA

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 DNA sample in the corresponding column of the results grid.

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 channels for the FAM and/or JOE, and/or ROX, and/or Cy5 fluorophores is absent. The amplification and detection should be repeated for all samples in which the specific DNA was not detected.
2. The *Ct* value is determined for the Negative Control of Extraction (C-) in the channels for the FAM and/or JOE, and/or ROX 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 PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected.
3. The *Ct* value is determined for the Negative Control of Amplification (NCA) in the channels for the FAM and/or JOE, and/or ROX, and/or Cy5 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 specific DNA was detected.

4. 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.

11 TRANSPORTATION

eSens Plasmodium sp./falciparum/vivax QL 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 **eSens Plasmodium sp./falciparum/vivax QL PCR kit** are to be stored at 2–8 °C when not in use (except for PCR-mix-FL *Plasmodium* spp. and PCR-buffer-H).

All components of the **eSens Plasmodium sp./falciparum/vivax 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: PCR-mix-FL *Plasmodium* spp. and PCR-buffer-H are to be stored at the temperature from minus 24 to minus 16 °C

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

13 SPECIFICATIONS

13.1 Analytical sensitivity (limit of detection)

Table 5

Test material	The volume of sample for extraction, µl	Nucleic acid extraction kit	PCR kit	Analytical sensitivity (limit of detection), copies/ml
Whole blood	50	RIBO-prep	ES3640B	2x10 ³
Mosquitoes	100	RIBO-prep	ES3640B	10 ³

13.2 Analytical Specificity

The analytical specificity of **eSens Plasmodium sp./falciparum/vivax QL 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 DNA fragments of claimed microorganisms. The analytical specificity was proved on the following microorganisms/strains:

Table 6

Pathogen	Strain	Concentration
<i>Japanese encephalitis virus (JEV)</i>	Pekin-1	Titre 10 ⁶ TCID ₅₀ /ml
<i>Rickettsia heilongjiangensis</i>	Primorye-25/81	No less than 10 ⁵ copies/ml
<i>Dengue virus (DENV)</i>	DENV-1/8/Taiand/01/2013	No less than 10 ⁵ GE/ml
<i>Leptospira</i> spp. (<i>Icterohaemorrhagiae</i> serogroup)	M20 (Copenhageni)	No less than 10 ⁷ microorganisms/ml
<i>Leptospira</i> spp. (<i>Javanica</i> serogroup)	Poi	
<i>Rickettsia prowazekii</i>	Madrid-E	
<i>Rickettsia raoultii</i>	DnS-14-Shaiman	
<i>Chikungunya virus (CHIKV)</i>	Ross late	
<i>Tick-borne encephalitis virus (TBEV)</i>	Komarovo	
<i>Zika virus (ZIKV)</i>	MRS-Opy Martinique-PaPi 2015	
<i>Rickettsia sibirica</i> subsp. <i>sibirica</i>	Baevo-105-87	No less than 10 ⁸ copies/ml
<i>Rickettsia conorii</i> subsp. <i>conorii</i>	M1	
<i>Borrelia miyamotoi</i>	Izh-4	
<i>Yellow fever virus (YFV)</i>	I7D	
<i>West Nile virus (WNV)</i>	Leiv-VLG99-27889 human	

The nonspecific responses were not observed while testing the DNA samples of the above mentioned microorganisms, as well as mosquito DNA and whole blood DNA from people who had not been to the malaria-endemic regions.

The clinical specificity of **eSens Plasmodium sp./falciparum/vivax QL PCR kit** was confirmed in laboratory clinical trials.

The information about interfering substances is specified in the Interfering substances and limitations of using test material samples.

13.3 Repeatability, reproducibility

Repeatability and reproducibility were determined by testing positive and negative model samples. Positive samples were a mixture of quality control samples (QCS) containing *Plasmodium* spp., *P. falciparum* and *P. vivax* DNA with concentration of 1x10⁴ GE/ml each, Negative Control (C-) was used as a negative sample.

Repeatability conditions included testing in the same laboratory, by the same operator, using the same equipment within a short period of time. Reproducibility conditions included testing different lots of

PCR kit in different laboratories, by different operators, on different days, using different equipment. The results are presented in Table 7.

Table 7

Sample type	Repeatability		Reproducibility	
	Number of samples	Agreement of results, %	Number of samples	Agreement of results, %
Positive	10	100	40	100
Negative	10	100	40	100

13.4 Diagnostic characteristics

The following samples were used to determine the diagnostic characteristics of the PCR kit (see Table 8):

Table 8

Test material	Number of samples
Blood from patients with suspected malaria	489
Blood from patients with a clinical diagnosis of malaria confirmed by microscopy of a thin blood smear or a thick blood film	88
Total	577

SD Bioline Malaria Ag P.f/Pan (Standart Diagnostics, Inc., Korea) was used as reference assay. The results are presented in Tables 9 and 10.

Table 9

The results of testing eSens Plasmodium spp. / P.falciparum / P.vivax QL PCR kit in comparison with the reference assay

Test material	The results of application of eSens Plasmodium sp./falciparum/vivax QL PCR kit	Results of using the reference assay	
		Positive	Negative
Whole blood	577 samples were tested	Positive	311
		Negative	1
			3
			262

Table 10

Diagnostic characteristics of eSens Plasmodium spp. / P.falciparum / P.vivax QL PCR kit

Test material	Diagnostic sensitivity* (with a confidence level of 95 %)	Diagnostic specificity** (with a confidence level of 95 %)
Whole blood	99.7 (98.2 – 99.9) %	98.9 (97.6 – 99.8) %















* Relative sensitivity in comparison with applied reference method.

** Relative specificity in comparison with applied reference method.

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		Contains sufficient for <n> tests
 LOT	Batch code		Use-by Date
 IVD	<i>In vitro</i> diagnostic medical device		Consult instructions for use
 VER	Version		Keep away from sunlight
	Temperature limit		Keep dry
	Manufacturer	NCA	Negative control of amplification
	Date of manufacture	C-	Negative control of extraction
 EC REP	Authorized representative in the European Community	C+	Positive control of amplification
	Caution	IC	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
01_04/2022		

Ecoli Dx, s.r.o. , Purkyňova 74/2



110 00 Praha 1, Česká republika
Tel: +420 325 209 912

Mobil: +420 739 802 523