AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT PCR kit

IVD

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Contains sufficient for <n> tests
LOT	Batch code		Use-by Date
IVD	*In vitro* 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

1. INTENDED USE

AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT 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.

AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT 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.

Variant FRT-50 FN 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	*Plasmodium* spp. DNA	*P.falciparum* DNA	*P.vivax* DNA	IC DNA
Target gene	Non-transcribed area between cox3 and cox1	Cox1	Cox1	Artificially synthesized sequence

3. CONTENT

AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT PCR kit is produced in 2 forms:

variant FRT-50 FN, REF H-3981-1-CE

variant FRT-L, REF H-3982-1-4-CE

Variant FRT-50 FN includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *Plasmodium* spp.	clear liquid from colorless to light lilac colour	0.6	1 tube
PCR-buffer-H	colorless clear liquid	0.3	1 tube
C+ *Plasmodium* 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 (see **RIBO-prep** or **MAGNO-sorb** protocols).

Variant FRT-50 FN is intended for 55 reactions (including controls).

Variant FRT-L includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix *Plasmodium* spp.-Lyo	white powder	–	48 tubes of 0.2 ml
C+ *Plasmodium* 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 (see **RIBO-prep** or **MAGNO-sorb** protocols).

Variant FRT-L is intended for 48 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 based on magnetic beads with MAGNO-sorb Nucleic Acid Extraction kit.
- 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 (Bio-Rad, USA)).
- Disposable polypropylene tubes for variant FRT-50 FN:
  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

AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT PCR kit is intended for analysis of the DNA extracted with DNA extraction kits from the biological material (whole blood, mosquitoes).

Sampling

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.

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

Pretreatment of *whole blood* is not required.

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 $1x10^4$ 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	Detected
	Potassium EDTA	2.0 µg/ml	Not detected

7. WORKING CONDITIONS

AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT 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

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

- **RIBO-prep** – for DNA extraction from whole blood and mosquitoes;
- **MAGNO-sorb** – for DNA extraction from whole blood.

If using the RIBO-prep reagent kit extract the DNA according to the manufacturer's protocol.

The volumes of reagents and samples when the DNA is extracted by the RIBO-prep reagent kit:

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

Add **10 µl** of **Internal Control-FL (IC)** to each tube.

The volume of the test sample:

- **50 µl** for whole blood;
- **100 µl** for suspension of mosquitoes.

Add **100 µl** of **Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).

The volume of elution:

- **50 µl** (in case of using variant FRT-50 FN for the amplification);
- **100 µl** (in case of using variant FRT-L for the amplification).

If using the MAGNO-sorb reagent kit extract the DNA according to the Section 8.1.1.

The volumes of reagents and samples when the DNA is extracted by the MAGNO-sorb reagent kit:

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

Add **10 µl** of **Internal Control-FL (IC)** to each tube.

The volume of the test sample is **100 µl** of whole blood.

Add **100 µl** of **Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).

The volume of elution is **100 µl.**

8.1.1 DNA extraction from 100 µl of whole blood using MAGNO-sorb nucleic acid extraction kit

1. Warm up **Lysis Solution MAGNO-sorb** and **Washing Solution 5** at 60 °C until crystals disappear.
2. Prepare the required number of 1.5-ml tubes including the tube for Negative Control of Extraction. Mark the tubes.
3. Mix in a disposable 1.5-ml tube **Internal Control-FL (IC)**, **Component A** and **Magnetized silica** in the following proportion calculated per one sample: **10 µl** of **IC**, **10 µl** of **Component A** and **20 µl** of **Magnetized Silica**. Do not forget to add extra volumes for one more reaction. For example:

Number of samples for DNA extraction	Internal Control-FL (IC), µl	Component A, µl	Magnetized silica, µl
6	70	70	140
12	130	130	260
18	190	190	380
24	250	250	500

4. Add **40 µl** of the prepared mixture of **Internal Control-FL (IC)**, **Component A** and **Magnetized silica** into each tube.
5. Add **900 µl** of **Lysis Solution MAGNO-sorb** into the tubes.
6. Add **100 µl** of **test sample** into each prepared tube.
7. Add **100 µl** of **Negative Control (C–)** into the tube for the Negative Control of extraction (C–) (for each panel).
8. Tightly close the tubes. Vortex. Incubate the tubes at **60 °C** for **10 min** in a thermostat.
9. Sediment the drops on a vortex. Transfer the tubes to a magnetic rack and incubate for **2 min**.
10. Carefully remove the supernatant inserting the tip near the internal tube wall and using vacuum aspirator. Take a new tip for each sample. Transfer the tubes to a regular rack.
11. Add **700 µl** of **Washing Solution 5** to the tubes.
12. Wash the magnetized silica mixing on vortex. Then sediment the drops on vortex.
13. Transfer the tubes to a regular rack, open the caps and transfer them to a magnetic rack. Incubate for **2 min**.
14. Remove the supernatant and transfer the tubes to a regular rack.
15. Repeat washing procedure with **Washing Solution 5** (steps 11-14).
16. Carry out washing procedure with **700 µl** of **Washing Solution 6** as described above.
17. Add **200 µl of Washing Solution 7**, mix, and vortex shortly to sediment drops. Place the tubes to a regular rack and open the tubes.
18. Transfer the tubes to the magnetic rack for **1 min** and then remove the supernatant.
19. Dry the sorbent. To do this, open the tubes and incubate them in the magnetic rack for **10 min**.
20. Add **100 µl** of **Buffer for elution** to each tube and vortex.
21. Incubate the tubes at **60 °C** for **5 min** in a thermostat. Vortex the tubes **2 min** later.
22. Vortex the tubes shortly and transfer them to the magnetic rack. Incubate for **2 min**. Supernatant contains purified DNA.

NOTE: Remove the purified DNA for subsequent PCR without getting the tubes out from the magnetic rack.

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.

Variant FRT-50 FN

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.

NOTE: Avoid transferring the sorbent together with the DNA samples extracted by magnetic separation.

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+ *Plasmodium* 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.

Variant FRT-L

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

Use disposable filter tips for adding reagents, DNA and control samples into tubes.

1. Take the required number of the tubes with ready-to-use lyophilized reaction mixture **PCR-mix *Plasmodium* spp.-Lyo** for amplification of DNA from test and control samples (see the number of control samples in point 3).
2. Add **25 µl** of **DNA samples** obtained by extraction of the test samples.

NOTE: Avoid transferring the sorbent together with the DNA samples extracted by magnetic separation.

3. Carry out the control reactions:

NCA – Add **25 µl** of **TE-buffer** to the tube labeled NCA (Negative Control of Amplification)

C+ – Add **25 µl** of **C+ *Plasmodium* spp.** to the tube labeled C+ (Positive Control of Amplification)

C– – Add **25 µl** of **the sample extracted from the Negative Control (C–) reagent** to the tube labeled C– (Negative control of Extraction).

8.2.2. Amplification

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

Table 3

AmpliSens unified amplification program for rotor-type[1] and plate-type[2] instruments

Step	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.

2. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin*.
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	*Plasmodium* spp. DNA	*P.falciparum* DNA	*P.vivax* 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.

Principle of interpretation is the following:

Table 5

Results interpretation

***Ct* value in the channel for the fluorophore**				**Result**
FAM	**JOE**	**ROX**	**Cy5**	
< boundary value	absent	absent	determined or absent	*Plasmodium* spp. DNA **is detected**
< boundary value	< boundary value	absent	determined or absent	*P.falciparum* DNA **is detected**
< boundary value	absent	< boundary value	determined or absent	*P.vivax* DNA **is detected**
< boundary value	< boundary value	< boundary value	determined or absent	*P.falciparum* and *P.vivax* DNA **are detected**
absent	absent	absent	< boundary value	*Plasmodium* spp., *P.falciparum*, *P.vivax* DNA **are NOT detected**
absent or > boundary value	absent or > boundary value	absent or > boundary value	absent or > boundary value	**Invalid*** result
absent	> boundary value	> boundary value	determined or absent	**Equivocal****
absent	> boundary value	absent	determined or absent	**Equivocal****
absent	absent	> boundary value	determined or absent	**Equivocal****
> boundary value	absent	absent	determined or absent	**Equivocal****
< boundary value	> boundary value	> boundary value	determined or absent	**Equivocal****

* In case of **invalid** result, the PCR analysis should be repeated for the corresponding test sample starting from the DNA extraction stage.

** In case of **equivocal result** it is necessary to repeat PCR-analysis of the corresponding test sample, starting from the DNA extraction stage. If the same result was obtained once again, the sample is considered positive. If the negative result was obtained, the sample is considered equivocal and re-sampling of the material for analysis is recommended.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

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 6).

Table 6

Results for controls

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

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 greater than the boundary *Ct* value or 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.

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

11. TRANSPORTATION

AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT 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 **AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT** 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 included in variant FRT-50 FN).

All components of the **AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT** PCR kit are stable until the expiry date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

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

NOTE: PCR-mix *Plasmodium* spp.-Lyo is to be kept in packages with a desiccant away from light

[1] For example, Rotor-Gene Q (QIAGEN, Germany).
[2] For example, CFX 96 (Bio-Rad, USA).

13. SPECIFICATIONS

13.1. Analytical sensitivity (limit of detection)

Table 7

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	variant FRT-50 FN, FRT-L	$2x10^3$
Whole blood	100	MAGNO-sorb	variant FRT-50 FN, FRT-L	10^3
Mosquitoes	100	RIBO-prep	variant FRT-50 FN, FRT-L	10^3

The claimed features are achieved while respecting the rules specified in the section "*Sampling and Handling".*

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT** 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 8

Pathogen	Strain	Concentration
Japanese encephalitis virus (*JEV*)	Pekin-1	Titre 10^6 $TCID_{50}$/ml
Rickettsia heilongjiangensis	Primorye-25/81	No less than 10^5 copies/ml
Dengue virus (*DENV*)	DENV-1/8/Taiand/01/2013	No less than 10^5 GE/ml
Leptospira spp. (*Icterohaemorrhagiae* serogroup)	M20 (Copenhageni)	No less than 10^7 microorganisms/ml
Leptospira spp. (*Javanica* serogroup)	Poi	
Rickettsia prowazekii	Madrid-E	
Rickettsia raoultii	DnS-14-Shaiman	
Chikungunya virus (*CHIKV*)	Ross late	
Tick-borne encephalitis virus (*TBEV*)	Komarovo	
Zika virus (*ZIKV*)	MRS-Opy Martinique-PaPi 2015	10^7 U/ml
Rickettsia sibirica subsp. *sibirica*	Baevo-105-87	No less than 10^8 copies/ml
Rickettsia conorii subsp. *conorii*	M1	
Borrelia miyamotoi	Izh-4	
Yellow fever virus (*YFV*)	17D	
West Nile virus (*WNV*)	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 **AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT** 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 $1x10^4$ 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 9.

Table 9

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 10):

Table 10

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 11 and 12.

Table 11

The results of testing AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT PCR kit in comparison with the reference assay

Test material	The results of application of AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT PCR kit		Results of using the reference assay	
			Positive	Negative
Whole blood	577 samples were tested	**Positive**	311	3
		Negative	1	262

Table 12

Diagnostic characteristics of AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT PCR kit

Test material	Diagnostic sensitivity[3] (with a confidence level of 95 %)	Diagnostic specificity[4] (with a confidence level of 95 %)
Whole blood	99.7 (98.2 – 99.9) %	98.9 (97.6 – 99.8) %

[3] Relative sensitivity in comparison with applied reference method.
[4] Relative specificity in comparison with applied reference method.

14. REFERENCES

1. Daniel Getacher Feleke, Yonas Alemu, and Nebiyou Yemanebirhane Performance of rapid diagnostic tests, microscopy, loop-mediated isothermal amplification (LAMP) and PCR for malaria diagnosis in Ethiopia: a systematic review and meta-analysis Malaria journal. 2021; 20: 384. Published online 2021 Sep 27.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of the **AmpliSens® *Plasmodium* spp. / *P.falciparum* / *P.vivax*-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

AmpliSens®

EC REP
Ecoli Dx, s.r.o., Purkyňova 74/2
110 00 Praha 1, Czech Republic
Tel.: +420 325 209 912
Cell: +420 739 802 523

Federal Budget Institute of Science "Central Research Institute for Epidemiology"
3A Novogireevskaya Street
Moscow 111123 Russia