# AmpliSens® *Rickettsia conorii*-FRT PCR kit

RUO

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 |
| RUO | Research Use Only | | 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 |
| | Caution | C+ | Positive control of amplification |
| | Caution | IC | Internal control |

## 1. INTENDED USE

**AmpliSens® *Rickettsia conorii*-FRT** PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Rickettsia conorii* DNA in the biological material (blood, tissue (autopsy, biopsy) material, eschar swabs, cerebrospinal fluid, ticks) using real-time hybridization-fluorescence detection of amplified products.

**Indications and contra-indications for use of the reagent kit**

The reagent kit is used for the analysis of biological material, taken from the persons suspected of tick spotted fever (tick-borne rickettsiosis) without distinction of form and presence of disease manifestation, and ticks.

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

**NOTE:** For research use only. Not for diagnostic procedures

## 2. PRINCIPLE OF PCR DETECTION

*Rickettsia conorii* detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific *Rickettsia conorii* 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.

**AmpliSens® *Rickettsia conorii*-FRT** PCR kit is a qualitative test that contains the Internal Control (Internal Control-FL (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.

**AmpliSens® *Rickettsia conorii*-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 enzyme UDG recognizes and catalyzes the destruction of the DNA containing deoxyuridine, but has no effect on DNA containing deoxythymidine. Deoxyuridine is absent in the authentic DNA, but is always present in amplicons, because dUTP is a part of dNTP mixture in the reagents for the amplification. Due to the deoxyuridine containing contaminating amplicons are sensitive to the destruction by UDG before the DNA-target amplification. So the amplicons cannot be amplified.

The enzyme UDG is thermolabile. It is inactivated by heating at temperature above 50 °C. Therefore, UDG does not destroy the target amplicons which are accumulated during PCR.

At the amplification stage 2 reactions are carried out in one tube simultaneously: amplification of *Rickettsia conorii* DNA as well as amplification of Internal Control-FL (IC) DNA. The results of amplification of *Rickettsia conorii* DNA and Internal Control-FL (IC) DNA are registered in 2 different fluorescence channels.

Table 1

| Channel for fluorophore | FAM | JOE |
|---|---|---|
| DNA-target | IC DNA | *Rickettsia conorii* DNA |
| Target gene | Artificial nucleotide sequence | ompA gene |

## 3. CONTENT

**AmpliSens® *Rickettsia conorii*-FRT** PCR kit is produced in 2 forms:

**variant FRT-50 FN**, REF H-2741-1-CE

**variant FRT-L**, REF H-2742-1-4-CE

**Variant FRT-50 FN** includes:

| Reagent | Description | Volume, ml | Quantity |
|---|---|---|---|
| **PCR-mix-FL *Rickettsia conorii*** | clear liquid from colorless to light lilac colour | 0.6 | 1 tube |
| **PCR-buffer-H** | colorless clear liquid | 0.3 | 1 tube |
| **C+ *Rickettsia conorii*** | 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**, **MAGNO-sorb** protocol).

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

**Variant FRT-L** includes:

| Reagent | Description | Volume, ml | Quantity |
|---|---|---|---|
| **PCR-mix *Rickettsia conorii*-Lyo** | white powder | – | 48 tubes of 0.2 ml |
| **C+ *Rickettsia conorii*** | 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**, **MAGNO-sorb** protocol).

**Variant FRT-L** is intended for 48 reactions (including controls).

## 4. ADDITIONAL REQUIREMENTS

- 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).
- 96 % ethanol for ticks pretreatment.
- Glycerin for the storage of pretreated ticks.
- Vacuum blood collection system.
- Puncture needles.
- Sterile plastic container (50-60 ml) for sampling, storage and transportation of biological samples.
- Swab for collection, transportation and storage of biological samples.
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and mallet) or homogenizer for pretreatment of tissue material and ticks.
- Vacuum aspirator with flask for removing supernatant.
- DNA extraction kit or the automated station for DNA extraction based on magnetic beads with MAGNO-sorb Nucleic Acid Extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol filters (up to 100, 200, 1,000 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 (Bio-Rad, USA)).
- Disposable polypropylene tubes:
  a) tightly closed 2.0-ml tubes for sampling.
  b) tightly closed 1.5 and 2.0-ml tubes for pretreatment
  c) screwed or tightly closed 1.5-ml tubes for reaction mixture preparation.
  d) 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;
  e) 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.
- 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 positive material (specimens, controls) away from all other reagents and add it to the reaction mix in a distantly separated facility. Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge them briefly.
- Use disposable protective gloves, laboratory coats, 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 samples and unused reagents in compliance with the local regulations.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance 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 to 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® *Rickettsia conorii*-FRT** PCR kit is intended for analysis of the DNA extracted with DNA extraction kits from the biological material (blood, tissue (autopsy, biopsy) material, cerebrospinal fluid, eschar swabs, ticks).

*Sampling*

*Blood.* To obtain the bacterial pellet of blood, 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 6 % EDTA (the final concentration after blood sampling is 0.3 %) or 3.2 % sodium citrate solution 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.

Blood samples can be stored before obtaining and preparing leukocytes for DNA extraction:
- at the temperature from 20 to 25 °C – for 2 hours,
- at the temperature from 2 to 8 °C – for 12 hours.

Freezing of whole blood samples is unallowable!

The samples are to be prepared no later than the specified time.

*Tissue (autopsy, biopsy) material.* The material is taken from the area of probable location of infection agent, from the lesional tissue or the area surrounding lesional tissue by a sterile tool (for example, tweezers) into a sterile plastic 50-ml container with tightly closed cap or 2 ml tube. The tube is to be closed tightly.

The tissue (autopsy, biopsy) material samples can be stored:
- at room temperature – for 6 hours,
- at the temperature from 2 to 8 °C – for 3 days,
- at the temperature from minus 24 to minus 16 °C – for 1 week,
- at the temperature not more than minus 68 °C – for a long time.

*Cerebrospinal fluid* is collected in an amount no less than 1 ml by disposable sterile needles into disposable 2.0-ml tubes.

The cerebrospinal fluid samples can be stored before the PCR analysis:
- at the temperature from 2 to 8 °C – for 1 day,
- at the temperature from minus 24 to minus 16 °C – for 1 week,
- at the temperature not more than minus 68 °C – for a long time.

Only one freeze-thawing cycle is required.

*Eschar swabs.* The material is taken by a non-sterile swab on a plastic shaft. The swab is pre-wetted in saline solution and carried with a gentle pressure over the skin area with primary affect. The working part of the probe with a swab is placed in a disposable tube with a snap or screw cap containing 300 ml of saline solution and gently break the plastic shaft no more than 0.5 cm from the working part of the probe so that the tampon stays in the tube together with the material in saline solution. The tube is to be closed tightly.

The material samples can be stored before the PCR analysis:
- at room temperature – for 6 hours;
- at the temperature from 2 to 8 °C – for 3 days;
- at the temperature from minus 24 to minus 16 °C – for 1 week;
- at the temperature not more than minus 68 °C – for a long time.

*Ticks.* The collected material is sorted into species, sex, places and dates of collection and placed into the dry sterile 2.0-ml tube. Number of ticks in pool for analysis should not exceed 10.

The material samples can be stored after sorting and samples formation:
- at the temperature from minus 24 to minus 16 °C – for 1 month,
- at the temperature not more than minus 68 °C or in the Dewar flask with liquid nitrogen – for a long time.

Only one freeze-thawing cycle is required.

*Pretreatment*

To obtain the *bacterial pellet of blood* the pretreatment of blood samples is required.

Using a filter tip transfer 1.5 ml of blood with EDTA into the sterile disposable 2.0-ml tube. Centrifuge at 40 g (for example, 800 rpm for the MiniSpin Eppendorf microcentrifuge) for 10 min. Using a new one filter tip transfer 500-600 µl of supernatant (plasma with leucocytes) into sterile disposable 1.5-ml tube(do not take the pellet with erythrocytes). Centrifuge at 10,000 g (for example, 12,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 10 min. Use the pellet and 100 µl of supernatant for the DNA extraction.

The bacterial pellet of blood can be stored before the PCR analysis:
- at the temperature from minus 24 to minus 16 °C – for 1 week,
- at the temperature not more than minus 68 °C – for a long time.

*Tissue (autopsy, biopsy) material* is to be pretreated. For DNA extraction take 30-50 mg (µl) of the material and homogenize it by trituration using precooled sterile porcelain mortar and mallet or homogenizer. Prepare 10 % suspension using grinded tissue and precooled 0.9 % sodium chloride solution (sterile saline solution) or phosphate buffer (PBS). For this, add 9 volumes of saline solution or phosphate buffer to 1 volume of grinded tissue. Use 100 µl of obtained suspension for DNA extraction.

The pretreated biopsy material samples can be stored before the PCR analysis:
- at the temperature from minus 24 to minus 16 °C – for 1 week,
- at the temperature not more than minus 68 °C – for a long time.

*Cerebrospinal fluid* is to be pretreated.

Centrifuge 1-1.5 ml of cerebrospinal fluid at 10,000 g (for example, 12,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 10 min. Discard the supernatant into the reservoir for the waste dispose. Use the cells pellet in 100 µl of supernatant for DNA extraction.

The cerebrospinal fluid samples can be stored before the PCR analysis:
- at the temperature from minus 24 to minus 16 °C – for 1 week,
- at the temperature not more than minus 68 °C – for a long time.

The material freeze-thawing of is not allowed without the DNA extraction procedure.

Pretreatment of *eschar swabs* is not required.

*Ticks* are to be pretreated. If pools of hungry ticks of the *Ixodidae* family are used for the analysis, number of ticks in one pool should not exceed 3. The engorged ticks are to be analyzed individually. Place the ticks in 1.5-ml tubes, add **500 µl** of 96 % ethanol. Mix and sediment on vortex. Discard ethanol from each tube by a separate tip without filter using vacuum aspirator. Add **500 µl** of 0.9 % sodium chloride solution (sterile saline solution) or phosphate buffer (PBS). Mix and sediment on vortex. Discard supernatant by a separate tip without filter using vacuum aspirator. Transfer the ticks into the sterile porcelain mortar (or homogenizer), add **600 µl** (if a hungry tick is analyzed) or **1 ml** (if a engorged tick or pool of ticks is homogenized) of 0.9 % sodium chloride solution (sterile saline solution) or phosphate buffer (PBS). Homogenize the sample.

Using a separate filter tip transfer the sample into 1.5-ml tube and centrifuge at 2,000 g (for example, 5,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 2 min for the clarification of the sample. Remove **100 µl** of supernatant. Add glycerin into the rest of suspension (10 % from the volume of the rest of suspension). Mix the sample and freeze it at the temperature from minus 24 to minus 16 °C for the subsequent PCR analysis.

The pretreated ticks can be stored before the PCR analysis:
- at the temperature from minus 24 to minus 16 °C – for 1 week,
- at the temperature not more than minus 68 °C or in the Dewar flask with liquid nitrogen – for a long time.

Only one freeze-thawing cycle is required.

*Interfering substances and limitations of using test material samples*

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.

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

## 7. WORKING CONDITIONS

**AmpliSens® *Rickettsia conorii*-FRT** PCR kit should be used at the temperature from 20 to 28° C and relative humidity is 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 blood leukocytes, cerebrospinal fluid, ticks, tissue (autopsy and biopsy) material and eschar swabs;
- **MAGNO-sorb** – for DNA extraction from ticks.

**NOTE:** **If using the RIBO-prep kit** extract the DNA according to the manufacturer’s protocol.

**NOTE:** 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 is **100 µl.** The pellet and **100 µl** of supernatant are used for the analysis of leukocytic fraction of blood and cerebrospinal fluid. **100 µl** of suspension is used for the analysis of tissue (autopsy, biopsy) material, suspension of Ixodid ticks.
Do not add the **Negative Control (C–)** reagent into the tube labeled C– (Negative Control of Extraction).
The volume of elution is **50 µl.**
In case of using variant FRT-L for the amplification carry out the DNA elution in **100 µl** of Buffer for elution.

**NOTE:** **If using the MAGNO-sorb 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** suspension of Ixodid ticks.
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 tick suspension using MAGNO-sorb nucleic acid extraction kit.

1. Warm up **Lysis Solution MAGNO-sorb** and **Washing Solution 5** at 60 ºC 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** (IC) (if it is provided for analysis of given infectious agent), **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 to a magnetic rack 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**. 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 using in PCR without getting the tubes out form the magnetic rack

Obtained samples 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 ≤ 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

**Variant FRT-50 FN**

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

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.

1. Calculate the required quantity of each reagent for reaction mixture preparation. For one reaction:
   - **10 µl** of **PCR-mix-FL *Rickettsia conorii*,**
   - **5 µl** of **PCR-buffer-H**.

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

**NOTE:** Prepare the reaction mixture just before use.

2. Thaw the tube with **PCR-mix-FL *Rickettsia conorii*.** 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 *Rickettsia conorii*** and **PCR-buffer-H**. Sediment the drops by vortex.
4. Take the required number of the tubes or strips taking into account the number of test samples 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** extracted from test samples at the DNA extraction stage using tips with filter.

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

7. Carry out the control reactions:

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

**C+** – Add **10 µl** of **C+ *Rickettsia conorii*** to the tube labeled **C+** (Positive Control of Amplification).

**C–** – Add **10 µl** of **the sample extracted from the C– sample** to the tube labeled **C–** (Negative Control of Extraction).

**NOTE:** Mix the tubes thoroughly by pipetting avoiding foaming.

**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 *Rickettsia conorii*-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** extracted from test samples into the prepared tubes.

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

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+ *Rickettsia conorii*** to the tube labeled **C+** (Positive Control of Amplification).

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

**NOTE:** Mix the tubes thoroughly by pipetting avoiding foaming.

**NOTE:** Carry out the PCR just after the mix of reaction mixture and DNA-samples and controls. Time of the addition of samples to the reaction mixture and the reaction run on the instrument cannot be more than 10-15 min.

### 8.3.2. Amplification

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

Table 2

**Unified amplification and detection program for rotor-type[2] and plate-type[3] 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 | |

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

**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 in one instrument only the tests for the DNA detection are carried out simultaneously, the first step of reverse transcription (50 °C – 15 min) can be omitted for time saving, but the iteration at 60 °C should be increased up to 30 s in the third step.

Table 3

**Amplification and detection program for rotor-type[1] and plate-type[2] instruments**

| Step | Temperature, °C | Time | Fluorescent signal detection | Cycles |
|---|---|---|---|---|
| 1 | 95 | 15 min | – | 1 |
| 2 | 95 | 10 s | – | 45 |
| | 60 | 30 s | FAM, JOE | |

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 (1–3 s) 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 RT-PCR instrument used by measuring fluorescence signal accumulation in two channels:

Table 4

| Channel for the fluorophore | FAM | JOE |
|---|---|---|
| Amplification product | Internal Control-FL (IC) DNA | *Rickettsia conorii* 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** | |
| < boundary value | absent | *Rickettsia conorii* DNA **is not detected** |
| determined or absent | < boundary value | *Rickettsia conorii* DNA **is detected** |
| absent or > boundary value | absent or > boundary value | **Invalid** result* |
| < boundary value | > boundary value | **Equivocal** result** |

* 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, the PCR analysis should be repeated for the corresponding test sample starting from the DNA extraction stage. If the same result is obtained, the sample is considered positive. If the negative result is obtained in the second run, 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 controls of amplification and extraction are correct (see Table 6).**

Table 6

**Results for controls**

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

**AmpliSens® *Rickettsia conorii*-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.

[1] It is preferable to use the amplification program in Table 3, if there is no need to use the unified amplification program.
[2] For example, Rotor-Gene Q (QIAGEN, Germany).
[3] For example, CFX 96 (Bio-Rad, USA).

## 12. STABILITY AND STORAGE

All components of the **AmpliSens® *Rickettsia conorii*-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-FL *Rickettsia conorii* and PCR-buffer-H). All components of the **AmpliSens® *Rickettsia conorii*-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 *Rickettsia conorii* and PCR-buffer-H are to be stored at the temperature from minus 24 to minus 16 °C

**NOTE:** PCR-mix-FL *Rickettsia conorii* is to be kept away from light

**NOTE:** PCR-mix *Rickettsia conorii*-Lyo is to be kept in packages with a desiccant away from light

## 13. SPECIFICATIONS

### 13.1. Analytical sensitivity (limit of detection)

Table 7

| Biological material | The volume of sample for extraction, µl | Nucleic acid extraction kit | PCR kit | Analytical sensitivity (limit of detection), copies/ml |
|---|---|---|---|---|
| Blood | Pellet + 100 | RIBO-prep | variant FRT-50 FN, FRT-L | $10^3$ |
| Tissue (autopsy, biopsy) material | 100 | RIBO-prep | variant FRT-50 FN, FRT-L | $10^3$ |
| Cerebrospinal fluid | Pellet + 100 | RIBO-prep | variant FRT-50 FN, FRT-L | $10^3$ |
| Eschar swabs | 100 | RIBO-prep | variant FRT-50 FN, FRT-L | $10^3$ |
| Ticks | 100 | RIBO-prep | variant FRT-50 FN, FRT-L | $10^3$ |
| | | MAGNO-sorb | variant FRT-50 FN, FRT-L | $10^3$ |

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

Table 8

**The results of AmpliSens® *Rickettsia conorii*-FRT PCR kit analytical sensitivity validation with using the material from healthy people and patients with another causation of disease and samples, contaminated by strain of *Rickettsia conorii* M-1**

| Type of sample | DNA concentration (copies per ml of the sample) | Number of replicates | Number of positives | Hit Rate, % |
|---|---|---|---|---|
| Blood (DNA extraction from blood leukocyte-bacterial fraction and 100 µl plasma) | $1x10^3$ | 20 | 20 | 100 |
| Cerebrospinal fluid | $1x10^3$ | 20 | 20 | 100 |
| Tissue (autopsy, biopsy) material | $1x10^3$ | 20 | 20 | 100 |
| Ticks | $1x10^3$ | 20 | 20 | 100 |
| Eschar swabs | $1x10^3$ | 20 | 20 | 100 |

### 13.2. Analytical specificity

The analytical specificity of **AmpliSens® *Rickettsia conorii*-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 specificity was proved on the follows strains of microorganisms and biological material obtained from healthy people or patients with another causation of disease:

Table 9

**The results of AmpliSens® *Rickettsia conorii*-FRT PCR kit analytical and specificity validation**

| Organisms | The channel for the FAM fluorophore (Internal control) | The channel for the JOE fluorophore (*Rickettsia conorii*) |
|---|---|---|
| *West Nile virus* | Valid | Negative |
| *Japanese encephalitis virus* | Valid | Negative |
| *Omsk hemorrhagic fever virus* | Valid | Negative |
| *Tick-borne encephalitis virus* | Valid | Negative |
| *Anaplasma phagocytophillum* | Valid | Negative |
| *Bartonella henselae* | Valid | Negative |
| *Babesia microti* | Valid | Negative |
| *Leptospira kirschneri* | Valid | Negative |
| *L. borgpetersenii* | Valid | Negative |
| *Shigella sonne* | Valid | Negative |
| *S. flexneri* | Valid | Negative |
| *Salmonella typhi* | Valid | Negative |
| *S. enteritidis* | Valid | Negative |
| *Klebsiella pneumonia* | Valid | Negative |
| *Escherichia coli* NCTC 9001 | Valid | Negative |
| *Enterococcus faecalis* | Valid | Negative |
| *Staphylococcus aureus* | Valid | Negative |
| *S. saprophyticus* | Valid | Negative |
| *Pseudomonas aeruginosa* | Valid | Negative |
| *Proteus mirabilis* | Valid | Negative |
| *Enterobacter cloacae* | Valid | Negative |
| *Yersinia pestis* | Valid | Negative |
| 25 samples of eschar swabs | Valid | Negative |
| 25 blood samples from the patients with another causation of disease | Valid | Negative |
| 25 biopsy material samples from the patients with another causation of disease | Valid | Negative |
| 25 cerebrospinal fluid samples from the patients with another causation of disease | Valid | Negative |
| 25 samples of *Dermacentor reticulatus* ticks | Valid | Negative |

## 14. REFERENCES


1. Mouffok N., Socolovschi C., Benabdellah A. et al. Diagnosis of rickettsioses from eschar swab samples, Algeria//Emerg Infect Dis.- 2011. – Vol. 17. – P.1968-1969.


## 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® *Rickettsia conorii*-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.


**AmpliSens®**

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