

AmpliSens® *Rickettsia conorii*-FRT PCR kit



For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

	Catalogue number		Contains sufficient for <n> tests
	Batch code		Use-by Date
	In vitro diagnostic medical device		Consult instructions for use
	Version		Keep away from sunlight
	Temperature limit		Keep dry
	Manufacturer		Negative control of amplification
	Date of manufacture		Negative control of extraction
	Authorized representative in the European Community		Positive control of amplification
	Caution		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: The results of PCR analysis are taken into account in complex diagnostics of disease.

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.

The PCR kit 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	<i>Rickettsia conorii</i> 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, H-2741-1-CE

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

Variant FRT-50 FN includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL <i>Rickettsia conorii</i>	clear liquid from colorless to light lilac colour	0.6	1 tube
PCR-buffer-H	colorless clear liquid	0.3	1 tube
C+ <i>Rickettsia conorii</i>	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, K2-9-Et-50-CE, MAGNO-sorb, K2-16-200-CE; K2-16-1000-CE protocol).

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

Variant FRT-L includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix <i>Rickettsia conorii</i> -Lyo	white powder	–	48 tubes of 0.2 ml
C+ <i>Rickettsia conorii</i>	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, K2-9-Et-50-CE, MAGNO-sorb, K2-16-200-CE; K2-16-1000-CE 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 leukocytes) 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**, **REF** K2-9-Et-50-CE – for DNA extraction from blood leukocytes, cerebrospinal fluid, ticks, tissue (autopsy and biopsy) material and eschar swabs;
- **MAGNO-sorb**, **REF** K2-16-200-CE, **REF** K2-16-1000-CE – 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 PCR kit 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 °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 (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 from 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 follows¹:

Table 2

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.

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.

NOTE:

Table 3

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.

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

(1–3 s) before placing them into the instrument.

NOTE: 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
Signal registration, indicating the amplification product accumulation	Internal Control-FL (IC) DNA	<i>Rickettsia conorii</i> 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:

Results interpretation

Table 5

Ct value in the channel for the fluorophore		Result
FAM	JOE	
< boundary value	absent	<i>Rickettsia conorii</i> DNA is not detected
determined or absent	< boundary value	<i>Rickettsia conorii</i> 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

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

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).

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.

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

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.

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 diagnostic specificity validation

Organisms	The channel for the FAM fluorophore (Internal control)	The channel for the JOE fluorophore (<i>Rickettsia conorii</i>)
<i>West Nile virus</i>	Valid	Negative
<i>Japanese encephalitis virus</i>	Valid	Negative
<i>Omsk hemorrhagic fever virus</i>	Valid	Negative
<i>Tick-borne encephalitis virus</i>	Valid	Negative
<i>Anaplasma phagocytophilum</i>	Valid	Negative
<i>Bartonella henselae</i>	Valid	Negative
<i>Babesia microti</i>	Valid	Negative
<i>Leptospira kirschneri</i>	Valid	Negative
<i>L. borgpetersenii</i>	Valid	Negative
<i>Shigella sonnei</i>	Valid	Negative
<i>S. flexneri</i>	Valid	Negative
<i>Salmonella typhi</i>	Valid	Negative
<i>S. enteritidis</i>	Valid	Negative
<i>Klebsiella pneumoniae</i>	Valid	Negative
<i>Escherichia coli</i> NCTC 9001	Valid	Negative
<i>Enterococcus faecalis</i>	Valid	Negative
<i>Staphylococcus aureus</i>	Valid	Negative
<i>S. saprophyticus</i>	Valid	Negative
<i>Pseudomonas aeruginosa</i>	Valid	Negative
<i>Proteus mirabilis</i>	Valid	Negative
<i>Enterobacter cloacae</i>	Valid	Negative
<i>Yersinia pestis</i>	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 <i>Dermacentor reticulatus</i> ticks	Valid	Negative

13.3. Diagnostic characteristics

Table 10

Results of AmpliSens® *Rickettsia conorii*-FRT PCR kit testing in comparison with the reference method

Sample type	Results of using AmpliSens® <i>Rickettsia conorii</i> -FRT PCR kit	Results of using reference method ⁴	
		positive	negative
Blood	198 samples were analyzed	132	0
		66	0
Eschar swabs	52 samples were analyzed	44	0
		8	0

In accordance with the submitted data the diagnostic sensitivity of the AmpliSens® *Rickettsia conorii*-FRT PCR kit is 59.6-73.2 % for the samples of blood and 71.9-93.1% for eschar swabs with a confidence coefficient of 95 %.

The diagnostic specificity of the AmpliSens® *Rickettsia conorii*-FRT PCR kit is 86.3-100 % for all types of biological material with a confidence coefficient of 95 %.

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.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.04.18 PM	13. Specifications	The values of diagnostic characteristics were specified
19.12.18 DV	Throughout the text	Corrections according to the template
	8. Protocol	In subsection DNA extraction eschar swab for nucleic acid extraction kit RIBO-prep was added
	11. Transportation	The section was supplemented
10.09.19 EM	Through the text	The text formatting was changed
	1. Intend use	The subsection Indications and contra-indications for use of the reagent kit was added
	4. Additional requirements	Automated station for DNA extraction was added, "flocked swab" was changed to "swab"
	6. Sampling and handling	"Cotton swab" was changed to "swab" for eschar swabs sampling
	14. References	The section was actualized
03.06.20 KK	Footer	The phrase "Not for use in the Russian Federation" was added
11.03.21 MM	—	The name, address and contact information for Authorized representative in the European Community was changed

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⁴ («IgG/IgM-antibodies *Rickettsia conorii*» («Vircell», Spain) was used as a reference method.