AmpliSens® *Coxiella burnetii*-FRT PCR kit

IVD

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code		Sufficient for
IVD	*In vitro* diagnostic medical device		Use-by Date
VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	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
		IC	Internal control

1. INTENDED USE

AmpliSens® *Coxiella burnetii*-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of the DNA of *Coxiella burnetii* in the ticks, biological human material (blood, sputum, bronchial washing fluid, liquor, autopsy material) and animal material (blood, autopsy material, placenta and abortive material) using real-time hybridization-fluorescence detection of amplified products.

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

2. PRINCIPLE OF PCR DETECTION

Coxiella burnetii detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific primers and TaqF-polymerase. 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® *Coxiella burnetii* -FRT PCR kit is a qualitative test that contains the Internal Control **(Internal Control STI-87 (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® *Coxiella burnetii* -FRT PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by the separation of nucleotides and Taq-polymerase by using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

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

Table 1

Channel for fluorophore	FAM	JOE
DNA-target	Internal Control STI-87 (IC) DNA	*Coxiella burnetii* DNA
Target gene	Artificially synthesized sequence	IS1111

3. CONTENT

AmpliSens® *Coxiella burnetii*-FRT PCR kit is produced in 1 form:

variant FRT-50 F, REF R-B85-50-F(RG,iQ,Mx,Dt)-CE

Variant FRT-50 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FRT *Coxiella burnetii*	clear liquid from colorless to light lilac colour	0.6	1 tube
RT-PCR-mix-2-FEP/FRT	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
Positive Control DNA *Coxiella burnetii* / STI (C+$_{Coxiella\ burnetii\ /\ STI}$)	colorless clear liquid	0.2	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Internal Control STI-87 (IC)*	colorless clear liquid	0.6	1 tube

* add **10 µl** of **Internal Control STI-87 (IC)** during the DNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep**, REF K2-9-Et-50-CE protocol). It also must be used in the extraction procedure as Negative Control of Extraction (C-) (see 8.1. DNA Extraction).

Variant FRT-50 F is intended for 60 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- 0.15 M NaCl (saline solution) or phosphate buffer saline (PBS) (NaCl, 137 mM; KCl, 2.7 mM, NaH_2PO_4, 10 mM; $K_4P_2O_7$, 2 mM; pH 7.5±0.2) for pretreatment of ticks, internal organs tissue and autopsy material.
- 96 % ethanol for pretreatment of oil treated ticks.
- Glycerol for pretreatment of ticks.
- Homogenizer TissueLyser LT (QIAGEN, Germany) and stainless steel balls with 5 mm and 7 mm diameter. Is recommended to use for homogenization of ticks and internal organ tissues.
- Sterile porcelain mortars and pestle for pretreatment of internal organs and autopsy material.
- Reagent for pretreatment of viscous fluids (sputum).
- DNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Disposable pipette tips with aerosol filters (up to 100 µl).
- Tube racks.
- Vortex mixer.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia); Rotor-Gene Q (QIAGEN, Germany) iCycler iQ5 (Bio-Rad, USA))
- Disposable polypropylene PCR tubes (0.1- or 0.2-ml):
  a) 0.2-ml PCR tubes with optical transparent domed or flat caps if a plate-type instrument is used;
  b) 0.2-ml PCR tubes with flat caps 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 extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid inhalation of vapors, samples and reagents contact with the skin, eyes, and mucous membranes. Harmful if swallowed. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice if necessary.
- Safety Data Sheets (SDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING

NOTE: Obtaining samples of biological materials for PCR-analysis, transportation, and storage are described in the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® *Coxiella burnetii*-FRT PCR kit is intended for analysis of the DNA extracted with DNA extraction kits from:

- Ixodic ticks: *Rhipicephalus, Haemaphysalis, Dermacentor, Ixodes*.

Human material

- Whole peripheral blood, sputum, bronchial washing fluid, liquor, autopsy material (tissue of brain, heart, lung, spleen)

Animal material

- Blood, placenta, abortive material, autopsy material (spleen).

Blood, sputum, bronchial washing fluid, liquor, autopsy material transported to the laboratory in a container with ice for 1 day.

When arrived to the laboratory blood, sputum, bronchial washing fluid, liquor should be pretreated with obtaining bacterial pellet. Then immediately start nucleic acid extraction or frozen the sample for long storage. Ticks stored alive (up to 1 month) or for one week at the temperature from minus 24 to minus 16 °C, subsequent storage should be at the temperature not more than minus 68 °C. Autopsy and abortive material, as well as the placenta, is to be stored for 1 week at the temperature from minus 24 to minus 16 °C, subsequent storage should be at the temperature not more than minus 68 °C.

NOTE: Only one freeze-thawing cycle is allowed.

Pretreatment

6.1 *Ticks*

For ticks it is preferable to analyze individual specimens. Place oil-treated ticks into Eppendorf tubes, add 500 µl of 96 % ethanol, and vortex. Centrifuge the tubes for 3-5 sec to remove drops from the inner surface of the tube caps. Remove liquid carefully using vacuum aspirator. Add 500 µl of 0.9 % sodium chloride solution (sterile saline solution) or phosphate buffer (PBS). Centrifuge the tubes for 3-5 sec to remove drops from the inner surface of the tube caps. Remove liquid carefully using vacuum aspirator. Use sterile porcelain mortars and sterile pestles to prepare tick suspension. In case of using an automatic homogenizer TissueLyser LT the following homogenization parameters should be applied: 1) for *Rhipicephalus, Haemaphysalis, Dermacentor* ticks, balls' diameter – 7 mm, frequency – 50 Hz, time of homogenization – 10-12 min, buffer volume – 700 µl (starve tick) or 1000-1500 µl (fed tick and pool of ticks); 2) for *Ixodes* ticks, balls' diameter – 5 mm, frequency – 50 Hz, time of homogenization – 5-10 min, buffer volume – 300 µl (starve tick) or 700-1000 µl (fed tick and pool of ticks).

In the case of congested tick homogenization in mortar, ticks should be pre-pierced with sterile disposable needle in several places to release the blood.

Grind the ticks in 700 µl (if the sample consists of 1 *Rhipicephalus, Haemaphysalis* or *Dermacentor* tick), or in 300 µl (if the sample consists of 1 *Ixodes* tick) in 1-1.5 ml (if the sample consist of pool of ticks or fed tick of *Rhipicephalus, Haemaphysalis, Dermacentor* genera) or in 1 ml (if the sample consists of tick pool or congested *Ixodes* tick) of 0.15 M sodium chloride solution Mix solution with ticks by small portions. Centrifuge obtained suspension for 2 min at 5,000 rpm. Take 50 µl of supernatant for DNA extraction. Add glycerol (10 % of volume) to residual part of suspension and freeze at the temperature from minus 24 to minus 16 °C for possible subsequent analysis.

6.2 *Blood*

Fasting draw of the whole peripheral blood is carried out in the morning to the tube with 6 % EDTA solution in 1:20 ratio. Closed tube with the whole peripheral blood should be overturned several times. Add 1.5 ml of whole blood collected with EDTA to the Eppendorf tube and centrifuge it at 800 rpm (380 g for 50 mm diameter rotor) for 10 min. Transfer the upper layer of plasma (500-600 µl) with white blood cells into another tube and centrifuge it at 9,000 g for 5 min. Transfer the supernatant (except 200 µl supernatant above the cell pellet) into a container with a disinfectant solution. Use cell pellet and 200 µl of supernatant above it for DNA extraction.

6.3 *Animal internal organs, placenta and abortive material, human autopsy material*

Homogenize pieces of not less than 0.5 cm^3 with a porcelain mortar and a pestle. Add at least 500 µl of sterile 0.9 % sodium chloride solution (saline solution) or phosphate buffer (PBS) and mix thoroughly. In case of placenta pretreatment it is not recommended to use homogenizers. Prepared 10 % suspension should be settled at room temperature for 2-3 minutes, and then transfer the upper phase into 1.5 ml tubes. Use 50 µl of the suspension for DNA extraction.

6.4 *Sputum*

Perform the pretreatment of sputum according to the **Mucolysin,** REF 180-CE, *Instruction Manual*. Use 50 µl of the sample for DNA extraction.

6.5 *Liquor and bronchial washing fluid*

Transfer 1 ml of the sample to the tube and centrifuge it at 9,000 g for 5 min. Transfer the supernatant (except 200 µl supernatant above the cell pellet) into a container with a disinfectant solution. Use cell pellet and 200 µl of supernatant above it for DNA extraction.

Material after pretreatment before the DNA extraction can be stored at the temperature not more than minus 20 °C for 1 month or for a long time at the temperature not more than minus 68 °C.

7. WORKING CONDITIONS

AmpliSens® *Coxiella burnetii*-FRT PCR kit should be used at 18–25 °C.

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;

NOTE:

Extract the DNA according to the manufacturer's protocol taking into account next improvement:

for suspension of ticks, tissue and Mucolysin treated sputum

- Add **10 µl** of **Internal Control STI-87 (IC)** and **300 µl** of **Solution for Lysis** into each tube.
- Add **50 µl** of suspension of ticks, tissue and Mucolysin treated sputum.

for pellets of blood cell, liquor and bronchial washing fluid

- Add **300 µl** of **Solution for Lysis** into the tubes with pellets of blood cell, liquor and bronchial washing fluid. Mix the contents of the tubes thoroughly by vortexing, then centrifuge tubes.
- Add **10 µl** of **Internal Control STI-87 (IC)** into each tube.

for Negative Control of Extraction

- Add only **10 µl** of **Internal Control STI-87 (IC)** and **300 µl** of **Solution for Lysis** to the tube labeled (C–) (Negative Control of Extraction).

8.2. Preparing PCR

8.2.1 Preparing tubes for PCR

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. Prepare the reaction mixture for the required number of reactions.

NOTE: Note that even it is necessary to carry out Negative Control of Extraction (C-), Positive Control of Amplification (C+) and Negative Control of Amplification (NCA). In addition, it is necessary to take reagents for one extra reaction.

2. Take the required number of tubes/strips for amplification of the DNA obtained from clinical and control samples.
3. For N reactions, add to a new tube:
   10*(N+1) µl of PCR-mix-1-FRT *Coxiella burnetii*;
   5.0*(N+1) µl of RT-PCR-mix-2-FEP/FRT;
   0.5*(N+1) µl of polymerase (TaqF).
   Vortex the tube, then centrifuge it briefly. Transfer **15 µl** of the prepared mixture to each tube.

NOTE: Do not store the prepared mix!

4. Using tips with aerosol filter add **10 µl** of **DNA samples** obtained at the DNA extraction stage. Mix it carefully by pipetting.
5. Carry out the control amplification reactions:

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

C+ – Add **10 µl** of **Positive Control DNA *Coxiella burnetii* / STI (C+$_{Coxiella\ burnetii\ /\ STI}$)** to the tube labeled C+ (Positive Control of Amplification)

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

NOTE: Perform the amplification reaction immediately after adding DNA samples and controls to the reaction mixture.

8.2.2. Amplification

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

Table 2

Amplification program

	Rotor-type instruments[1]			Plate-type instruments[2]		
Step	**Temperature, °C**	**Time**	**Cycles**	**Temperature, °C**	**Time**	**Cycles**
1	**95**	15 min	1	**95**	15 min	1
2	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	25 s	
	72	15 s		**72**	15 s	
3	**95**	5 s	40	**95**	5 s	40
	56	20 s Fluorescence acquiring		**56**	25 s Fluorescence acquiring	
	72	15 s		**72**	15 s	

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

2. Insert tubes into the reaction module of the device. **Well 1** must be filled with the test tube.
3. Run the amplification program with fluorescence detection.
4. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

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

– the signal of the Internal Control STI-87 DNA amplification product is detected in the channel for the FAM fluorophore.
– the signal of the *Coxiella burnetii* DNA fragment amplification product is detected in the channel for the JOE fluorophore.

Results are interpreted by the crossing (or not-crossing) the 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:

– *Coxiella burnetii* DNA is **detected** if the *Ct* value determined in the results grid in the channel for the JOE fluorophore is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
– *Coxiella burnetii* DNA is **not detected** in a sample if the *Ct* value is not determined (absent) in the channel for JOE fluorophore, whereas the *Ct* value determined in the channel for the FAM fluorophore is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.
– The result is **invalid** if the *Ct* value is not determined (absent) in the channel for JOE fluorophores, whereas the *Ct* value in the channel for the FAM fluorophore is not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit. See also Guidelines [2]

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see Table 3).

Table 3

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

[1] For example, Rotor-Gene 3000/Rotor-Gene 6000 (Corbett Research, Australia).
[2] For example, iCycler iQ, iQ5 (Bio-Rad, USA), Mx3000P (Stratagene, USA).

10. TROUBLESHOOTING

Results of analysis are not taken into account in the following cases:

1. If the *Ct* value determined for the Positive Control of Amplification (C+) in the channel for the JOE fluorophore is greater than the boundary *Ct* value or absent, the amplification and detection should be repeated for all samples in which specific DNA was not detected.
2. If the *Ct* value is determined for the Negative Control of Extraction (C–) in the channel for the JOE fluorophore, the PCR analysis should be repeated for all samples in which *Coxiella burnetii* DNA was detected.
3. If the *Ct* value is determined for the Negative Control of Amplification (NCA) in the channels for the FAM or/and JOE fluorophores, the PCR analysis should be repeated for all samples in which *Coxiella burnetii* DNA was detected, with carrying out the NCA at least three times.

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

11. TRANSPORTATION

AmpliSens® *Coxiella burnetii*-FRT PCR kit should be transported at 2–8 °C for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Coxiella burnetii*-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-1-FRT *Coxiella burnetii*, RT-PCR-mix-2-FEP/FRT and polymerase (TaqF)). All components of the **AmpliSens® *Coxiella burnetii*-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-1-FRT *Coxiella burnetii,* RT-PCR-mix-2-FEP/FRT, and polymerase (TaqF) are to be stored at the temperature from minus 24 to minus 16 °C

NOTE: PCR-mix-1-FRT *Coxiella burnetii* is to be kept away from light

13. SPECIFICATIONS

13.1. Analytical sensitivity

Biological material (specimen volume)	Nucleic acid extraction kit	Sensitivity, GE/ml[3]	Material pretreatment
- *Dermacentor* ticks (50 µl of tick suspension); - *blood* (200 µl of white blood cell fraction); - 10 % *tissue* suspension of spleen and liver (50 µl)	RIBO-prep	$5x10^3$	Indicated sensitivity can be reached only if the specified pretreatment instructions are followed and the specified specimen volume is used

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *Coxiella burnetii*-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 analytical specificity was studied in bacteria *Rickettsia conorii* ssp. *Caspia, Ehrlichia muris* and *Francisella tularensis*, as well as virus - *West Nile virus*, Crimean-Congo hemorrhagic fever and *Herpesvirus*.

The clinical specificity of **AmpliSens® *Coxiella burnetii*-FRT** PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook “Sampling, Transportation, and Storage of Clinical Material for PCR diagnostics”, developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology” of Federal Service for Surveillance on Consumers’ Rights Protection and Human Well-Being.
2. Guidelines to the **AmpliSens® *Coxiella burnetii*-FRT** PCR kit for qualitative detection of the DNA of *Coxiella burnetii* in the ticks, biological human material (blood, sputum, bronchial washing fluid, liquor, autopsy material) and animal material (blood, autopsy material, placenta and abortive material) by polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology”.

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® *Coxiella burnetii*-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
19.10.17 PM	Through the text	Corrections according to the template
13.03.19 EM	3. Content	The colour of the reagent was specified
24.04.20 MA	Through the text	The text formatting was changed
	Footer	The phrase “Not for use in the Russian Federation” was added
	2. Principle of PCR detection	The table with targets was added
18.03.21 VA	—	The name, address and contact information for Authorized representative in the European Community was changed

AmpliSens®

EC REP

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[3] Genome equivalents (GE) of the pathogen agent per 1 ml of a sample