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For Professional Use Only

AmpliSens® *Coxiella burnetii*-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

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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.

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). 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.

3. CONTENT

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

AmpliSens® *Coxiella burnetii*-FRT PCR kit variant FRT-50 F, **REF** R-B85-50-F(RG,iQ,Mx,Dt)-CE

AmpliSens® *Coxiella burnetii*-FRT PCR kit variant FRT-50 F includes:

Reagent	***Description***	***Volume, ml***	***Quantity***
PCR-mix-1-FRT *Coxiella burnetii*	colorless clear liquid	0.6	1 tubes
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 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 (see 8.1. DNA Extraction).

AmpliSens® *Coxiella burnetii*-FRT PCR kit 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 ticks and internal organs tissue homogenization.
- 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) when working with PCR kit variant FRT-100 F:
    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 with the range from 2 to 8 °C.
- Deep-freezer with the range 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 (samples, 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 afterward.
- 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 samples and unused reagents in accordance with 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 samples and reagents contact with the skin, eyes and mucous membranes. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the 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

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.

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

Only one freeze-thawing cycle is allowed.

Pretreatment

6.1 *Ticks*

For ticks analysis of individual specimen is preferably. If ticks were oil treated place they in tubes like Eppendorf. Add 500 µl of 96 % ethanol and stir by vortex. Centrifuge the tube 3-5 sec at 5,000 rpm to delete drops from internal surface of the tube cap. Remove liquid carefully by vacuum aspirator. Add in this tube with ticks 500 µl of 0.9 % sodium chloride solution or PBS stir on vortex and centrifuge 3-5 sec at microcentrifuge to delete drops from internal surface of the tube cap. Remove liquid carefully by vacuum aspirator. Use sterile porcelain mortars and sterile pestles for ticks suspension preparation. If there is an automatic homogenizer TissueLyser LT the following homogenization parameters should be used: 1) for ticks of *Rhipicephalus, Haemaphysalis, Dermacentor* genera, 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 ticks of *Ixodes* genera, 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 homogenization fed ticks in mortar, ticks should be pre-pierce sterile disposable needle in several places to enter the blood.

Grind the ticks in 700 µl (if sample consist of 1 starve tick of *Rhipicephalus, Haemaphysalis, Dermacentor* genera), or in 300 µl (if sample consist of 1 tick of *Ixodes* genera) in 1-1.5 ml (if sample consist of pool of ticks or fed tick of *Rhipicephalus, Haemaphysalis, Dermacentor* genera) or in 1 ml (if sample consist of pool of ticks or fed tick of *Ixodes* genera) of 0.15 M sodium chloride solution Mix solution with ticks by small portions. Centrifuge obtained suspension 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 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 proportion 1:20. Closed tube with the whole peripheral blood should be overturned several times. Add to Eppendorf tube 1.5 ml of whole blood collected with EDTA and centrifuged at 800 rpm (380 g for 50 mm diameter rotor) for 10 min and Transfer the upper layer of plasma (500-600 µl) with white blood cell into the second Eppendorf tube and centrifuged at 9,000 g for 5 min. The supernatant (except 200 µl supernatant of cells pellet) were transferred 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 least 500 µl sterile 0.9 % saline solution or PBS and mix thoroughly. In case of preparing of placenta using homogenizers is not recommended. A ready 10 % suspension to assert at room temperature for 2-3 minutes, then the upper phase was transfer into 1.5 ml tubes Take 50 µl of the suspension for DNA extraction.

6.4 *Sputum*

Pretreatment of material to perform according to instructions to the reagent Mucolysin REF 180-CE. 50 µl of sample used for DNA extraction.

6.5 *Liquor and bronchial washing fluid*

Transfer 1 ml of sample to the Eppendorf tube and centrifuged at 9,000 g for 5 min. The supernatant (except 200 µl supernatant of cells pellet) were transferred 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 extraction of DNA can be stored at a temperature

not higher than minus 20 °C for 1 month or long at a 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;

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 and 300 µl of Solution for Lysis into each tube.
- Add 50 µl suspension of ticks, of 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 into each tube.

for Negative Control of Extraction

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

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**.

Variant FRT-50 F

1. Prepare the reaction mixture for the required number of reactions. PCR run should include amplification reactions for three controls: Negative Control of Extraction (C–), Positive Control (C+) and Negative Control of Amplification (NCA). In addition, it is necessary to take reserve of reagents: count on one extra reaction.
2. Mix in the separate tube **PCR-mix-1-FRT *Coxiella burnetii,* RT-PCR-mix-2-FEP/FRT and polymerase (TaqF),** based on the volume of each reaction:
   10 µl of PCR-mix-1-FRT *Coxiella burnetii*;
   5.0 µl of RT-PCR-mix-2-FEP/FRT;
   0.5 µl of polymerase (TaqF).
3. Take the required number of tubes for amplification of the DNA obtained from clinical and control samples.
4. Add **15 µl** of **reaction mix** into each prepared tube.

Do not store prepared mix!

5. Using tips with aerosol barrier add **10 µl** of **DNA samples** obtained from test or control samples at the DNA extraction stage into tubes with reaction mix. Mix it carefully.
6. 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+

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

Perform the amplification reaction immediately after DNA samples and controls are added to the reaction mixture.

8.2.2. Amplification

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

Table 1

Amplification program

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

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

– 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 channels for JOE fluorophores, 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.

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 (seeTable 2).

Table 2

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. If the *Ct* value determined for the Positive Control of Amplification (C+) in the channels for the **JOE** fluorophores 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 channels for the **JOE** fluorophores, 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 in triplicate.

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.

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

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

13. SPECIFICATIONS

13.1. Sensitivity

Biological material (specimen volume)	Nucleic acid extraction kit	PCR kit	Sensitivity, GE/ml[1)]	Material pretreatment

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

- ticks of *Dermacentor* genera (50 µl ticks suspension); - blood (white blood cell fraction of blood, 200 µl); - 10 % suspension of tissue of spleen and liver (50 µl)	RIBO-prep	PCR kit variant FRT-50 F	$5x10^3$	Indicated sensitivity can be reached only if the specified pretreatment instructions are followed and the specified specimen volume is used

13.2. 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, Moscow, 2010.
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 the 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.

16. KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code		Sufficient for
IVD	*In vitro* diagnostic medical device		Expiration Date
VER	Version		Consult instructions for use
	Temperature limitation		Keep away from sunlight
	Manufacturer	**NCA**	Negative control of amplification
	Date of manufacture	**C–**	Negative control of extraction
EC REP	Authorised representative in the European Community	**C+**	Positive control of amplification
		IC	Internal control