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For Professional Use Only

AmpliSens® *Brucella* spp.-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® *Brucella* spp.-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of DNA of *Brucella* species *(B.melitensis, B.abortus, B.suis, B.ovis, B.canis,* and *B.neotomae)* in human (whole blood, synovial fluid, and lymph node aspirate) and animal (blood, milk, placenta, lymph nodes, spleen, aborted fetal liver, hygroma, and parenchymal organs) biological materials and bacterial culture 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

Brucella spp. DNA detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific *Brucella* spp*.* 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 the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

AmpliSens® *Brucella* spp.-FRT PCR kit is a qualitative test that contains the Internal Control (Internal Control STI-704 (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 ® *Brucella* spp.-FRT PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase using a wax layer. Wax melts and reaction components mix only at 95 °C.

3. CONTENT

AmpliSens® *Brucella* spp.-FRT PCR kit is produced in 1 form:

AmpliSens® *Brucella* spp.-FRT of amplified products PCR kit variant FRT, **REF** R-B10(RG)-CE.

AmpliSens® *Brucella* spp.-FRT PCR kit variant FRT includes:

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FEP/FRT *Brucella* spp. ready-to-use single-dose test tubes (*under wax)*	colorless clear liquid	0.008	55 tubes of 0.2 ml
PCR-mix-2-FL	colorless clear liquid	0.77	1 tube
Positive Control DNA *Brucella* ($C+_{Brucella}$)	colorless clear liquid	0.1	1 tube
Positive Control STI-88 (CS+)	colorless clear liquid	0.1	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-)*	colorless (or straw colored) clear liquid	1.2	1 tube
Internal Control STI-704 (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 during the DNA extraction procedure directly to the sample/lysis mixture (see DNA-sorb-B, **REF** K1-2-50-CE protocols).

AmpliSens® *Brucella* spp.-FRT PCR kit is intended for 55 reactions, including controls.

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol filters (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia).
- Disposable polypropylene tubes for PCR (0.1- or 0.2-ml; for example, Axygen, USA).
- 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 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.
- 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

Obtaining samples of biological materials for PCR-analysis, transportation and storage are described in manufacturer's handbook [2]. It is recommended that this handbook is read before starting work.

AmpliSens® *Brucella* spp.-FRT PCR kit is intended for analysis of DNA extracted with DNA extraction kits from human (whole blood, synovial fluid, and lymph node aspirate) and animal (blood, milk, placenta, lymph nodes, spleen, aborted fetal liver, hygroma, and parenchymal organs) biological materials and bacterial culture:

6.1 Sampling

Human material:

- *Whole peripheral blood* is collected into the tubes with 3 % EDTA solution (50 µl of EDTA solution per 1 ml of blood).
- *Lymph node aspirate* is collected into sterile tubes with 100 µl of sterile 0.9 % NaCl solution or transport medium (manufactured by FBIS CRIE).
- *Synovial fluid* is collected to a sterile disposable tube.

Animal material:

- *Blood* is collected to tubes with 6 % EDTA solution (50 µl of EDTA solution per 1 ml of blood).
- *Milk* (10-20 ml) is collected to sterile vessel.
- *Abdominal and stomach fluids, spleen and liver of aborted fetus.*
- *Placenta and fetal membranes of aborted animals*.
- *Fluid of bursa, hydroma*.
- in case of animal slaughter, whole pair lymph nodes (para-aortic, supramammary, inguinal and pelvic) from both sides of the carcass, parts of parenchymatous organs (liver, spleen), testicles with epididymes obtained from males with signs of orchitis or epididymitis are collected for analysis.

Bacterial cultures:

- *Liquid cultures* are used without pretreatment.
- *Bacterial colonies suspicious for Brucell*a spp should be resuspended in 0.5 ml of sterile saline.

The material can be stored at 2–8 °C for 1 day and at the temperature below minus 16 °C for 1 month. Only one freeze-thawing cycle is acceptable

6.2 Pretreatment

Samples of whole blood preserved in EDTA, synovial fluid, lymphatic node aspirate, fluid of bursa and hydroma, and microorganism cultures are used for DNA extraction without pretreatment after disinfection procedure.

Homogenize the samples of parenchymal organs, testicles, placenta, and fetal membranes (separately) by size 1x1x1 cm and whole lymph nodes by trituration using sterile porcelain mortar and mallet. Then add equal volume of sterile saline and mix carefully. Incubate at 20–25 °C for 5 min. Transfer 0.4-0.5 ml of the upper phase to a 1.5-ml tube with a pipette using a tip with aerosol barrier, disinfect it and use 0.1 ml for DNA extraction. Utilize the tube with a hypophase.

Centrifuge 10 ml of milk after disinfection procedure at 3000 rpm for 10–15 min. If the

pellet is practically invisible, add another 10 ml of milk to the same tube and repeat the centrifugation. Discard the supernatant leaving about 200 µl of liquid above the pellet. Resuspend the pellet in this liquid and use 0.1 ml of the suspension for DNA extraction.

6.3 Disinfection:

1. Add 0.1 % sodium merthiolate (1 : 1000 dilution) to a final concentration of 0.01 % (1 : 10000 dilution) to biological material samples and bacterial cultures (if it is required after preliminary treatment) and warm up at the temperature (56 ± 1) °C for 30 min. Use 100 µl of the prepared samples for further tests.
2. Transfer 1 ml of suspect bacterial colonies treated with sodium merthiolate to 1.5-ml tubes and centrifuge at 12000 rpm for 15 min. Discard the supernatant, resuspend the pellet in 100 µl of 0.9 % NaCl and use it in further work.
3. **Lysis Solution** from DNA-sorb-B kit, REF K1-2-50-CE (if it has been stored at 2–8 °C) should be heated at 65 °C until complete crystal dissolution.
4. Add 300 µl of Lysis Solution to each tube with disinfected material (100 µl) and incubate at 65 °C for 15 min.

Further analysis is performed according to the DNA-sorb-B, REF K1-2-50-CE Protocol.

7. WORKING CONDITIONS

AmpliSens® *Brucella* spp.-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:

- DNA-sorb-B, REF K1-2-50-CE.

The DNA extraction for each sample is carried out in the presence of **Internal Control STI-704 (IC).**

Extract the DNA according to the manufacturer's protocol taking into account next additions and improvements:

- **Lysis Solution** has been already added to the tubes with test samples (see ***6.3 Disinfection***)
- To prepare the Negative Control of Extraction, add **300 µl** of **Lysis Solution** and **100 µl** of **Negative Control (C–) reagent** to a tube labeled **C–** (Negative control of Extraction).
- Add **10 µl of Internal Control STI-704 (IC)** to each tube with the **test samples and Lysis Solution**, including Negative Control of Extraction (C-)
- After adding **Universal Sorbent** and **Washing Solution 1**, centrifuge samples at 8000–10000 rpm (10000–13000 rpm in case of using rotor with the 70 mm radius)

8.2. Preparing PCR

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

8.2.1. Preparing tubes for PCR

1. Prepare the required number of tubes with **PCR-mix-1-FEP/FRT *Brucella* spp.** and wax for amplification of DNA from clinical and control samples.
2. Add **7 µl** of **PCR-mix-2-FL** to the surface of the wax layer into each tube ensuring that it does not fall under the wax and mix with **PCR-mix-1-FEP/FRT *Brucella* spp.**
3. Using tips with aerosol filter add **10 µl** of **DNA samples** obtained at the DNA extraction stage.
4. Carry out the control amplification reactions:

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

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

CS+ – Add **10 µl** of **Positive Control STI-88** to the tube labeled **CS+** (Positive Control of Amplification)**.**

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

8.2.2. Amplification

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

Table 1

Amplification program

Step	***Temperature, °C***	***Time***	***Cycles***
Hold	95	5 min	1
Cycling 1	95	10 s	10
	65	25 s	
	72	10 s	
Cycling 2	95	10 s	35
	56	25 s Fluorescence acquiring	
	72	10 s	

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

2. Adjust the fluorescence channel sensitivity according to the Guidelines [2].
3. Insert tubes into the reaction module of the device.
4. Run the amplification program with fluorescence detection.
5. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

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

– The signal of the IC DNA amplification product is detected in the channel for the FAM fluorophore.
– The signal of the *Brucella* DNA 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 cDNA sample in the corresponding column of the results grid.

Principle of interpretation is the following:

1. *Brucella* spp. 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 Guidelines.
2. *Brucella* spp. DNA is **not detected** in a sample if the *Ct* value is not determined (absent) in the channel for the JOE fluorophore, whereas the *Ct* value determined in the channel for the FAM fluorophore is less than the boundary Ct value specified in the Guidelines.

Boundary *Ct* values are specified in the Guidelines [2]

The result of the analysis is considered reliable only if the results obtained for the Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see Table 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+*Brucella*	PCR	Absent	**<**boundary value
CS+	PCR	**<**boundary value	Absent

10. TROUBLESHOOTING

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

1. If the *Ct* value is absent for the Positive Controls of Amplification (C+), this indicates incorrectly chosen amplification program or other errors of amplification stage. PCR should be repeated.
2. If the *Ct* value determined in the channel for the JOE fluorophore is greater than the boundary value, whereas the *Ct* value determined for the FAM fluorophore is less than

the boundary *Ct* value specified in the Guidelines, PCR should be repeated. The result of analysis is **positive** if the same result has been obtained or if the *Ct* value determined in the channel for the JOE fluorophore is less than the specified boundary *Ct* value.

3. If the *Ct* value determined in the channel for the JOE fluorophore is absent, whereas the *Ct* value determined in the channel for the FAM fluorophore is greater than the boundary value or absent, PCR and detection should be repeated. If the same result has been obtained, it is necessary to repeat the analysis of the sample beginning with the DNA extraction stage.
4. If any *Ct* value is determined for the Negative Control of Extraction (C–) in the channel for the JOE fluorophore and for the Negative Control of amplification (NCA) (DNA-buffer) in any channel, it indicates contamination of reagents or samples. In this case, the results of analysis for all samples are **invalid**. The analysis for all samples should be repeated and measures for detecting and elimination of contamination source must be taken.

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

11. TRANSPORTATION

AmpliSens® *Brucella* spp.-FRT PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Brucella* spp.-FRT** PCR kit are to be stored at 2–8 °C when not in use. All components of the **AmpliSens® *Brucella* spp.-FRT** PCR kit are stable until the expiry date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

PCR-mix-1-FEP/FRT *Brucella* spp. is to be stored away from light.

13. SPECIFICATIONS

13.1. Sensitivity

Analytical sensitivity of **AmpliSens® *Brucella* spp.-FRT** PCR kit is no less than $1x10^3$ bacterial cells per 1 ml of sample.

The claimed analytical features of **AmpliSens® *Brucella* spp.-FRT** PCR kit are guaranteed only when additional reagents kit DNA-sorb-B (manufactured by FBIS CRIE) is used.

13.2. Specificity

The analytical specificity of **AmpliSens® *Brucella* spp.-FRT** PCR kit is ensured by selection of specific primers and probes, as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The clinical specificity of **AmpliSens® *Brucella* spp.-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® *Brucella* spp.-FRT** PCR kit for qualitative detection of DNA of *Brucella* species *(B.melitensis, B.abortus, B.suis, B.ovis, B.canis, B.neotomae)* in human and animal biological materials and bacterial culture 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 **AmpliSens® *Brucella* spp.-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
CS+	Positive control STI	**IC**	Internal control
FBIS CRIE	Federal Budget Institute of Science “Central Research Institute for Epidemiology”	**C+*Brucella***	Positive Control of Amplification

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.12.10	Cover page	The phrase “For Professional Use Only” was added
	Content	New sections “Working Conditions” and “Transportation” were added
		The “Explanation of Symbols” section was renamed to “Key to Symbols Used”
	Stability and Storage	The information about the shelf life of reagents before and after the first use was added
		Information that PCR-mix-1-FEP/FRT *Brucella* spp. is to be kept away from light was added
	Key to Symbols Used	The explanation of symbols was corrected
	Text	Positive Control STI (CS+) was changed to Positive Control STI-88 (CS+)
		Positive Control DNA *Brucella* (C+) was changed to Positive Control DNA *Brucella* (C+$_{Brucella}$)
21.06.11 LA	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
15.12.15 PM	Through the text	Corrections according to the template
	8.1. DNA extraction	Information about controls of extraction was added
	8.2.1. Preparing tubes for PCR	
	8.2. 2. Amplification	The sections were rewritten
	9. Data analysis	
	10. Troubleshooting	
	14. References	The reference to the Guidelines was added