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For Professional Use Only

BC-EPh

PCR kit

Instruction Manual

AmpliSens®

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1. INTENDED USE

BC-EPh PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Brucella* microorganisms DNA in the biological material by using electrophoretic detection of the amplified products in agarose gel.

This kit is used only for scientific research.

2. PRINCIPLE OF PCR DETECTION

Brucella detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen DNA specific region using special *Brucella* primers. After PCR the amplified product is detected in agarose gel. **BC-EPh** PCR kit is a qualitative test, which contains the phage λ DNA fragment as Internal Control (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. **BC-EPh** PCR kit uses "hot-start", which greatly reduces frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by separation of nucleotides and Taq-polymerase by using wax layer. Wax melts and reaction components mix only at 95 ºC.

3. CONTENT

BC-EPh PCR kit is produced in 2 forms:

BC-EPh PCR kit variant 50 R (tubes 0.2 ml) **REF** B68-50-R0,2-CE;

BC-EPh PCR kit variant 50 R (tubes 0.5 ml) **REF** B68-50-R0,5-CE.

BC-EPh PCR kit variant 50 R includes:

Reagent	***Description***	***Volume (ml)***	***Quantity***
PCR-mix-1-R *Brucella* spp. ready-to-use single-dose test tubes (*under wax)*	colorless clear liquid	0.005	55 tubes of 0.5 or 0.2 ml
PCR-mix-2 blue	blue clear liquid	0.6	1 tube
Mineral oil for PCR	colorless viscous liquid	2.0	1 dropper bottle
Positive Control DNA *Brucella* (C+$_{Brucella}$)	colorless clear liquid	0.1	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-)*	colorless clear liquid	1.2	1 tube
Internal Control *Brucella* spp. (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 protocol).

BC-EPh PCR kit variant 50 R is intended for 55 reactions, including controls.

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Agarose gel detection kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- PCR box.
- Tube racks.
- Personal thermocyclers (for example, Terzik (DNA-Technology, Russia)).
- Disposable polypropylene microtubes for PCR with 0.5 ml (0.2) capacity (for example, Axygen, USA).
- Refrigerator with temperature 2-8 °C.
- Deep-freezer with temperature no more than minus 16 °C.
- Waste bin for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, 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 compliance with local authorities requirements.

- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid contact with the skin, eyes and mucosa. If skin, eyes and mucosa contact immediately flush with water, seek medical attention.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one directional, it should begin in the Extraction Area, move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.
- Observe the rules and instructions to prevent semination of the environment objects.

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

6. SAMPLING AND HANDLING

BC-EPh PCR kit is intended for analysis of *Brucella (B*. *melitensis*, *B*. *abortus*, *B*. *suis*, *B*. *ovis*, *B*. *canis)* DNA extracted with DNA extraction kits from the biological material:

- *stomach, spleen, liver contents* of abortus*;*
- *placenta and fetal membranes;*
- *hygroma and bursa contents;*
- *blood and other fluids* or agglutinating serum or complement-fixing antibodies serum.

6.1. Material sampling

- 5-10 ml of blood with 3 % EDTA (10:1).
- Other fluids – 10-15 ml.
- Parts of tissues and organs – about 1x1x1 (cm) or less.
- Whole lymph nodes.

In the case of killing animal use whole lymph nodes matched samples (paraaortic, over-udder, inguinal, pelvic) and parts of parenchymatous organs. Use testicles with epididymis of males with epididymitis or orchitis symptoms.

Materials are to be analyzed the next day after sampling if they are stored at the temperature 2-8 °C.

Materials can be stored at the temperature not more than minus 16 °C for one month.

6.2. Preparation of the samples

Whole blood samples with EDTA, synovial fluid, lymph node puncture samples, hygroma and bursa contents and *microorganism cell cultures* are used for DNA extraction without treatment.

Other fluid samples are centrifuged at 3000 rpm for 10-15 min. Carefully remove and discard the supernatant using a tip with aerosol barrier and leaving about 200 µl of the liquid on the sediment. If the pellet is too small, add more material (10 ml) and repeat centrifugation. Suspend the pellet in supernatant and use 100 µl for DNA extraction.

If the volume of fluids is not enough add saline to 10 ml.

Parenchymatous organs, placenta, fetal membranes, testicles and *whole lymph nodes* should be ground in a sterile porcelain mortars or glass homogenizer. Add equal volume of saline (1 ml) and thoroughly homogenize. Incubate at room temperature for 30 min. Transfer 100 µl of the upper part of prepared suspension in a sterile tube for DNA extraction.

7. WORKING CONDITIONS

BC-EPh 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

Carry out the amplification according to the manufacturer instruction.
The volume of clinical sample is 100 µl.

8.2. Preparing the PCR

Total reaction volume - **25 µl**, volume of cDNA sample - **10 µl**.

8.2.1. Detection of *Brucella* DNA

1. Prepare the required number of PCR tubes with **PCR-mix-1-R *Brucella* spp.** and wax for amplification of DNA from clinical and control samples.

2. Add **10 µl** of **PCR-mix-2 blue** to the surface and wax layer of each tube ensuring that it does not fall under the wax and mix with **PCR-mix-1-R *Brucella* spp.**
3. Add above **1 drop** of **mineral oil for PCR** (about **25 µl**).
4. Using tips with aerosol barrier add **10 µl** of **DNA samples** obtained from clinical or control samples.
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 *Brucella* (C+$_{Brucella}$)** to the tube labeled C+ (Positive control of Amplification).

IC+ -Add **10 µl** of **Internal Control *Brucella* spp.** diluted with **DNA-buffer** in 10 times (1:9, respectively) to the tube labeled IC+ (Positive control of IC Amplification).

8.2.2. Amplification of *Brucella* DNA

Run the following program on the thermocycler (see Table 1, Table 2). When the temperature reaches 95 ºC (pause regimen), insert tubes into the cells of amplifier and press the button to continue.

It is recommended to precipitate drops from walls of tubes by short vortexing (1–3 sec) before their insertion in a thermocycler.

Table 1

Programming thermocyclers with active temperature adjustment for *Brucella* DNA amplification

	Terzik (DNA-Technology), Omn-E (Hybaid)			**GeneAmp PCR System 2700 (Applied Biosystems), Gradient Palm Cycler (Corbett Research)**			**Maxygene (Axygen)**		
Step	Temperature, °C	Time	Cycles	Temperature, °C	Time	Cycles	Temperature, °C	Time	Cycles
0	**95**	pause		**95**	pause		**95**	pause	
1	**95**	5 min	1	**95**	5 min	1	**95**	5 min	1
2	**95**	10 sec	42	**95**	10 sec	42	**95**	30 sec	42
	65	10 sec		**63**	25 sec		**63**	45 sec	
	72	10 sec		**72**	25 sec		**72**	45 sec	
3	**72**	1 min	1	**72**	1 min	1	**72**	1 min	1
4	**10**	storage		**4**	storage		**4**	storage	

Table 2

Programming thermocyclers with block temperature adjustment for *Brucella* DNA amplification

	Ampli-3 (Biocom); Biometra, MiniCycler, PTC-100 (MJ Research)		
Step	Temperature, °C	Time	Cycles
0	**95**	pause	
1	**95**	5 min	1
2	**95**	1 min	42
	65	1 min	
	72	1 min	
3	**72**	1 min	1
4	**10**	storage	

After the reaction is finished, PCR tubes must be collected and sent to the room for PCR products analysis.

Amplification products are analyzed by separation of DNA fragments in agarose gel.

The amplified samples can be stored for 16 h at room temperature and for 1 week at 2-8 °C (be sure to warm the samples to room temperature before running electrophoresis).

9. DATA ANALYSIS

It is recommended to use the following agarose kit for electrophoretic detection:

- "EPh" variant 200, **REF** K5-200-CE.

Analysis of results is based on the presence or absence of specific bands of amplified DNA in agarose gel (1.7%). The lengths of specific amplified DNA fragments are:

- *Brucella* – 460 bp (base pairs)
- Internal Control *Brucella spp.* – 770 bp

Put on a protective mask or use the a glass barrier while watching and photographing the gel.

Start analysis from the results for controls (see Table 3).

Table 3

Results for controls

Control	**Controlled step**	**Specific bands in the agarose gel**		**Interpretation**
		460 bp	**770 bp**	
C-	DNA extraction	No	**Yes**	OK
IC+	Amplification	No	**Yes**	OK
NCA	Amplification	No	No	OK
C+	Amplification	**Yes**	No	OK

- The sample is considered to be positive if the 460-bp band is present in agarose gel. The band of IC (770 bp) could be absent in the samples with high concentration of *Brucella* DNA.
- The sample is considered to be negative if the 460-bp band is absent and the 770-bp band is present.

Besides the specific bands, the fuzzy bands of primer dimers can be seen in lanes, they are situated lower than the level of 100 bp.

10. TROUBLESHOOTING

Analysis results are not taken into account in the following cases:

1. If the results of control samples do not correspond to the listed above (Table 3), then the tests should be repeated. Discard any reagents that may be suspect.
2. If in lanes corresponding to any sample both specific bands (460 bp, 770 bp) are absent, it may be mistake of preparation of reagents that provoked loss of DNA or inhibition of PCR.
3. If specific 460-bp band appears in lanes corresponding to negative control (NCA, C–) or Positive control of IC Amplification (IC+), it means that reagents or samples are contaminated. In such cases analysis results must be considered as irrelevant. Test analysis should be repeated and measures for detecting contamination source must be taken.
4. If nonspecific bands appear in lanes at different levels, it may be caused by the lack of “hot start” or a false temperature regime in thermocycler.

11. TRANSPORTATION

BC-EPh PCR kit should be transported at 2–8 ºC for no longer than 5 days. Once received, the PCR kit should be dekitted according to the indicated storage conditions.

12. STABILITY AND STORAGE

All components of **BC-EPh** PCR kit are to be stored at the temperature 2–8 ºC when not in use. All components of the **BC-EPh** PCR kit are stable until the labeled expiration date. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

13. SPECIFICATIONS

13.1. Sensitivity

Analytical Sensitivity of **BC-EPh** PCR kit is no less than $1x10^3$ copies per 1 ml of a sample (cop/ml).

The claimed analytical features of BC-EPh PCR kit are guaranteed only when additional kits of reagents, “DNA-sorb-B” and “EPh” (manufactured by Federal Budget Institute of Science Central Research Institute for Epidemiology) are used.

13.2. Specificity

Specificity of **BC-EPh** PCR kit is ensured by selection of specific primers and strict reaction conditions as well as laboratory and clinical trials.

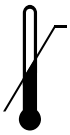
14. REFERENCES

1. Detection of *Brucella* species DNA in the stomach content of aborted sheep fetuses by PCR. B. Çetinkaya, H. Öngör, A. Muz, H. B. Ertas, H. Kalender, and H. M. Erdogan. Vet Rec., Vol. 144, Issue 9, 239-240, February 27, 1999.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science Central Research Institute for Epidemiology ISO 13485 – certified Total Quality Management System, each lot of **BC-EPh** PCR kit is tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code		Sufficient for
RUO	Research Use Only		Expiration Date
VER	Version		Consult instructions for use
	Temperature limitation		Keep away from sunlight
	Upper limit of temperature	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**C+**	Positive control of amplification
FBIS CRIE	Federal Budget Institute of Science “Central Research Institute for Epidemiology”	**IC**	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
25.01.12 LA	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
	Key to Symbols Used	The explanation of symbols was corrected
	Cover page, text	The name of Institution was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
27.05.13 PE	Text	The name of the reagent “Positive Control DNA *Brucella*” was changed to “Positive Control DNA *Brucella* ($C+_{Brucella}$)”
14.06.13 PE	Cover page	Symbol IVD was changed to RUO
	Key to Symbols Used	