CE

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For Professional Use Only

AmpliSens® *Legionella pneumophila*-FEP

PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® ***Legionella pneumophila*****-FEP** PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Legionella pneumophila* DNA in the clinical materials (tracheal sputum or aspirate, nasopharyngeal and oropharyngeal swabs, bronchial washes or bronchoalveolar lavage, and autopsy material), microorganism cultures, environmental samples (water, washes from environmental objects, biofilms, and soil) by using end-point 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

Legionella pneumophila detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *Legionella pneumophila* primers. In end-point PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. A multi-channel rotor-type fluorometer is specially designed to detect fluorescent excitation from the fluorophores in a reaction mix after PCR. It allows detection of the accumulating product without re-opening the reaction tubes after the PCR run **AmpliSens®** ***Legionella pneumophila*****-FEP** PCR kit is a qualitative test that contains the 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. 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 by using a wax layer. Wax melts and reaction component mix only at 95 ºC.

3. CONTENT

AmpliSens® *Legionella pneumophila*-FEP PCR kit is produced in 2 forms:

AmpliSens® *Legionella pneumophila*-FEP PCR kit (0.5-ml tubes), REF B50-50-R0,5-FEP-CE.

AmpliSens® *Legionella pneumophila*-FEP PCR kit (0.2-ml tubes), REF B50-50-R0,2-FEP-CE.

AmpliSens® *Legionella pneumophila*-FEP PCR kit includes:

Reagent	*Description*	*Volume, ml*	*Amount*
PCR-mix-1-FEP/FRT *Legionella pneumophila* ready-to-use single-dose test tubes (*under wax)*	colorless clear liquid	0.008	55 tubes of 0.5 or 0.2 ml
PCR-mix-2-FL	colorless clear liquid	0.77	1 tube
PCR-mix-Background	colorless clear liquid	0.5	1 tube
Mineral oil for PCR	colorless viscous liquid	4.0	1 dropper bottle
DNA calibrator LS3	colorless clear liquid	0.06	2 tube
Positive Control DNA *Legionella pneumophila* *	colorless clear liquid	0.5	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-)**	colorless clear liquid	1.6	2 tubes
Internal Control STI-338 (IC)***	colorless clear liquid	0.5	1 tube

* must be used in the extraction procedure as Positive Control of Extraction (PCE).

** 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 (DNA-sorb-B, REF K1-2-50-CE protocol).

AmpliSens® *Legionella pneumophila*-FEP PCR kit is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Disposable powder-free gloves.
- 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.
- Personal thermocyclers (for example, Gradient Palm Cycler (Corbett Research, Australia) or equivalent instrument.
- Fluorometer ALA-1/4 (Biosan, Latvia) or equivalent instrument.
- Disposable polypropylene microtubes for PCR (0.5- or 0.2-ml; for example, Axygen, USA).
- Refrigerator for 2–8 °C.

- Deep-freezer for ≤ –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 filters and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, 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 samples and unused reagents in compliance with local authorities' requirements.
- Samples should be considered potentially infectious and handled in a biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant, such as 0.5% sodium hypochlorite or another suitable disinfectant.
- Avoid contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes contact, immediately flush with water and seek medical attention.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- The laboratory process must be one directional; it should begin in the Extraction Area move to the Amplification and Detection Area. Do not return samples, equipment and reagents to the area in which 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 [1]. It is recommended that this handbook is read before starting work.

Clinical material:

1. Tracheal sputum or aspirate should be taken to a disposable container after pretreatment.
2. Nasopharyngeal and oropharyngeal swabs (in Transport Medium for Storage and

Transportation of Respiratory Swabs, REF 957-CE). These samples do not require pretreatment.

3. Bronchial washes (bronchoalveolar lavage) in disposable container after pretreatment.
4. Autopsy material (fragments of affected parts of lungs) after pretreatment.

It is recommended to combine nasopharyngeal and oropharyngeal swabs. To do this, working ends of probes are placed in one tube with 500 µl of Medium for Storage and Transportation of Respiratory Swabs (REF 957-CE) and studied as one sample.

Nasopharyngeal swabs are taken with a probe with a dry cotton swab. Insert the probe gently along the external nasal wall to a depth of 2–3 cm towards the inferior nasal concha. Then move the probe slightly lower, insert it in the inferior nasal meatus under the inferior nasal concha, rotate, and remove along the external nasal wall.

When the material is obtained, place the working part of the probe with the cotton swab in a sterile disposable tube with 500 µl of sterile saline or phosphate buffer solution. Break off the terminal part of the probe or cut it off to allow tight closing of the tube cup. Close the tube with the solution and the working part of the probe.

Oropharyngeal swabs are taken with a probe with a dry cotton swab. Take swabs by rotating the probe over the surface of tonsils, palatine arches, and the posterior wall of the pharynx after gargling the oral cavity with water.

When the material is obtained, place the working part of the probe with the cotton swab in a sterile disposable tube with 500 µl of sterile saline or phosphate buffer solution. Break off the terminal part of the probe or cut it off to allow tight closing of the tube cup. Close the tube with the solution and the working part of the probe.

The sample of bronchoalveolar lavage should be mixed by inverting in the original vessel. Using a tip with aerosol barrier, transfer 1.5 ml to a new tube and centrifuge it for 10 min at 10 000 rpm. Decant the supernatant leaving 100 µl of liquid above the pellet. Resuspend the pellet in 100 µl of supernatant and take 50 µl of the suspension for DNA extraction.

The sputum should be treated with Mucolysin reagent REF 180-CE according to Mucolysin manual. Use 50 µl of the pretreated sputum for DNA extraction. If it is necessary to repeat the test, freeze the remaining sputum.

Autopsy material is homogenized with a sterile porcelain mortar and pestle, with subsequent preparation of a 10% suspension in sterile saline or phosphate buffer. Transfer the suspension to a 1.5 ml tube and allow a precipitate to form for 1–3 min. 50 µl of the pretreated supernatant is used for DNA extraction. If it is necessary to repeat the test, store the remaining suspension frozen at ≤ –16 °C.

Nasopharyngeal and throat swabs are used for analysis in case of legionellosis in acute respiratory disease (Pontiac fever). In case of pneumonia, *Legionella pneumophila* DNA can be

detected in oropharyngeal swabs, urine, and blood plasma, though in an insignificant percent of cases. For this reason, these types of clinical material can be analyzed if samples of sputum, tracheal aspirate, or bronchoalveolar lavage cannot be obtained. Negative result of this analysis is not final. If *Legionella pneumophila* DNA is detected in oropharyngeal swabs, urine, and blood plasma, the positive result is final.

Microorganism cultures suspicious for *Legionella* spp.

Resuspend cultures in 0.5 ml of saline or phosphate buffer. Use 50 µl of the suspension for DNA extraction.

Thus obtained material can be stored for 1 day at 2–8 °C, 1 month at ≤ –16 °C and 1 year at ≤ –68 °C.

Only one freeze–thaw cycle is allowed.

Environmental samples

1. Water (wastewater, water from water bodies, and drinking water) (0.5 L) after pretreatment. 0.5 L of water is preliminary filtered through a paper filter using a glass funnel. After preliminary filtration, water is filtered through a membrane filter with a pore diameter not more than 0.45 µm. After filtration, the membrane filter is cut with sterile scissors (to a disposable Petri dish) and placed with sterile pincers to 1.5-ml tubes with 1 ml of saline solution. The tube is incubated at room temperature for 15–20 min under occasional mixing on vortex to ensure the transition of microflora to the solution. 50 µl of thus obtained solution is used for DNA extraction.
2. Washes from environmental objects are obtained with a probe with a cotton swab soaked in a sterile saline solution. The working part of the probe with the swab is placed in a tube with 1.5 ml of saline, the other part of the probe is broken off and discarded. 50 µl of the solution is used for DNA extraction.
3. Biofilm scraped from internal surface of water-supply, industrial, and other types of equipment (for example, from trays in air conditioners). Samples of moist biofilms under water or at the water–air interface are obtained with a dry sterile probe (the working part of the probe with a swab is placed in a 1.5-ml tube with 0.5 ml of saline and the other part of probe is broken off and discarded). 50 µl of the sample is used for DNA extraction. Samples of dry biofilms are obtained with a swab saturated in sterile saline. The working part of probe with the swab is placed in a 1.5-ml tube with 0.5 ml of saline and the other part of the probe is broken off and discarded). 50 µl of the sample is used for DNA extraction.
4. Soil (100 g) is collected at sites of presumable bacterial contamination and used after pretreatment. Transfer the soil (0.4–1.0 g per tube) to 5-ml tubes with tightly closing caps. Add 3 ml of saline to each tube, mix carefully, and decant for 5 min. The supernatant (50 µl)

is used for DNA extraction.

Thus obtained samples can be stored for 1 week at room temperature or longer at ≤ –16 °C. Samples can be transported at any temperature.

Only one freeze–thaw cycle is allowed.

7. WORKING CONDITIONS

AmpliSens® *Legionella pneumophila*-FEP 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 kit:

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

Extract DNA according to the manufacturer's instructions.

Add 10 µl of Internal Control STI-338 into tubes with environmental samples.

For Positive Control of Extraction (PCE) add 50 µl of Positive Control DNA *Legionella pneumophila* and 50 µl of Negative Control into the tube.
The sample volume is 50 µl. Add 50 µl of Negative Control to each tube.

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 *Legionella pneumophila*** 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 of each tube ensuring that it does not fall under the wax and mix with **PCR-mix-1-FEP/FRT *Legionella pneumophila*.**
3. Add above **1** drop of **mineral oil for PCR** (~ **25 µl**).
4. Prepare 2 tubes with **PCR-mix-1-FEP/FRT *Legionella pneumophila*** and mark them as **Background**. Add **17 µl** of **PCR-mix-Background** to the surface of wax layer of each tube, ensuring that it does not fall under the wax and mix with **PCR-mix-1-FEP/FRT *Legionella pneumophila***. Add above **1** drop of **mineral oil for PCR.**
5. Using tips with aerosol barrier, add **10 µl** of **DNA samples** obtained from clinical or control samples at the DNA extraction stage.
6. Carry out the control amplification reactions:

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

LS3 - Add **10 µl** of **DNA calibrator LS3** to the tube labeled LS3 (Positive Control of Amplification).

8.2.2 Amplification

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

It is recommended to sediment drops from walls of tubes by short centrifuging (1–3 s) before placing them in the thermocycler.

Table 1

Programming thermocyclers at DNA amplification of *Legionella pneumophila*

	Thermocyclers with block temperature adjustment: **MiniCycler, PTC-100 (MJ Research), Uno-2 Biometra**			Thermocyclers with active temperature adjustment:					
				GeneAmp PCR System 2400 (Applied Biosystems)			**GeneAmp PCR System 2700 (Applied Biosystems), MaxyGene (Axygen Scientific), Gradient Palm Cycler (Corbett research)**		
Step	Tempe-rature	Time	Cycles	Tempe-rature	Time	Cycles	Tempe-rature	Time	Cycles
0	**95 °C**	pause		**95 °C**	pause		**95 °C**	pause	
1	**95 °C**	5 min	1	**95 °C**	5 min	1	**95 °C**	5 min	1
2	**95 °C**	25 s	10	**95 °C**	10 s	10	**95 °C**	10 s	10
	60 °C	40 s		**60 °C**	20 s		**60 °C**	25 s	
	72 °C	25 s		**72 °C**	10 s		**72 °C**	25 s	
3	**95 °C**	25 s	35	**95 °C**	10 s	35	**95 °C**	10 s	35
	56 °C	40 s		**56 °C**	20 s		**56 °C**	25 s	
	72 °C	25 s		**72 °C**	10 s		**72 °C**	25 s	
4	**72 °C**	1 min	1	**72 °C**	1 min	1	**72 °C**	1 min	1
5	**10 °C**	storage		**10 °C**	storage		**10 °C**	storage	

9. DATA ANALYSIS

Detection is conducted on ALA-1/4 florescence detector.

Please read ALA-1/4 Operating Manual before using this kit.

Program the detector according to the manufacturer's manual and Guidelines.

9.2. Results interpretation

1. When the analysis is complete the results are automatically shown in the table as follows:
   pos – positive result;
   neg – negative result;
   eq – equivocal result (signal is in grey zone);
   nd – invalid result (specific signal and IC signal are absent in the sample).
2. Result of the analysis is considered reliable only if both Positive and Negative Controls of amplification as well as Negative Control of extraction are passed (Table 2).

Table 2

Results for controls

Control	Stage for control	Result of automatic interpretation		Interpretation
		FAM channel (IC)	HEX channel (test samples)	
C–	DNA extraction	+	*Legionella* - neg	OK
PCE	DNA extraction	+	*Legionella* - pos	OK
NCA	Amplification	-	*Legionella* - nd	OK
LS3	Amplification	+	*Legionella* – pos	OK

10. TROUBLESHOOTING

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

- PCR and detection should be repeated for samples with the **nd** result (except for NCA). If the same result is obtained, repeat analysis of the sample once again starting from the extraction stage. For NCA, the **nd** result is normal.
- PCR and detection should be repeated for samples with result "**eq"**. In the case of the same result, the samples are considered to be positive.
- If no signal is detected for the positive controls of amplification, it may suggest that the programming of the temperature profile of the used Instrument was incorrect, or that the configuration of the PCR reaction was incorrect, or that the storage conditions for kit components did not comply with the manufacturer's instruction, or that the reagent kit expired. Programming of the used instrument, storage conditions, and the expiration date of reagents should be checked and then PCR should be repeated.
- Positive signal for negative controls (C–, NCA) indicates contamination of reagents or samples. In this case, the results of the analysis are considered invalid. Repeat the analysis of all samples and take measures to detect and eliminate the source of contamination.

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

11. TRANSPORTATION

AmpliSens® *Legionella pneumophila*-FEP PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Legionella pneumophila*-FEP** PCR kit are to be stored at 2–8 °C when not in use. All components of the **AmpliSens® *Legionella pneumophila*-FEP** PCR kit are stable until the expiration 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 *Legionella pneumophila* is to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

Analytical Sensitivity of **AmpliSens® *Legionella pneumophila*-FEP** PCR kit is not less than $1x10^3$ copies per 1 ml of sample (copies/ml).

The claimed analytical features of **AmpliSens® *Legionella pneumophila*-FEP** PCR kit are guaranteed only when additional reagent kit DNA-sorb-B (manufactured by Federal Budget Institute of Science "Central Research Institute for Epidemiology") is used.

13.2. Specificity

The analytical specificity of **AmpliSens® *Legionella pneumophila*-FEP** 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 deposited in gene banks by sequence comparison analysis. The clinical specificity of **AmpliSens® *Legionella pneumophila*-FEP** 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, 2008.

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® *Legionella pneumophila*-FEP** 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	**LS3**	Positive control of amplification
FBIS CRIE	Federal Budget Institute of Science “Central Research Institute for Epidemiology”	**IC**	Internal control
PCE	Positive control of extraction		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
11.01.11	Cover page	The phrase “For Professional Use Only” was added
	Intended use	The phrase “The results of PCR analysis are taken into account in complex diagnostics of disease” 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 *Legionella pneumophila* is kept away from light was added
	Key to Symbols Used	The explanation of symbols was corrected
	Footer	Reference numbers were changed from B50-R0,5-FEP-CE; B50-R0,2-FEP-CE to B50-50-R0,5-FEP-CE; B50-50-R0,2-FEP-CE
01.07.11 RT	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”