CE

IVD

For Professional Use Only

AmpliSens® *Legionella pneumophila*-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

Ecoli s.r.o., Studenohorska 12
841 03 Bratislava 47
Slovak Republic
Tel.: +421 2 6478 9336
Fax: +421 2 6478 9040

Federal Budget Institute of
Science “Central Research
Institute for Epidemiology”
3A Novogireevskaya Street
Moscow 111123 Russia

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1. INTENDED USE

AmpliSens® ***Legionella pneumophila*-FRT** PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Legionella pneumophila* DNA in clinical materials (tracheal sputum or aspirate, nasopharyngeal and oropharyngeal swabs, bronchial washes or bronchoalveolar lavage, and autopsy material), microorganism cultures, and environmental samples (water, washes from environmental objects, biofilms, and soil) as well as for quantitation of *Legionella pneumophila* DNA in environmental samples (water, washes from environmental objects, biofilms, and soil) by using real-time hybridization-fluorescence detection.

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 special *Legionella pneumophila* primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that 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®** ***Legionella pneumophila*-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 by using a wax layer. Wax melts and reaction component mix only at 95 ºC.

AmpliSens® ***Legionella pneumophila*-FRT** PCR kit can be used as:

- a qualitative test for *Legionella pneumophila* DNA detection in the clinical materials. During the test, multiplex real-time PCR of *Legionella pneumophila* mip-gene DNA and protrombin gene DNA is performed. Protrombin gene DNA is used as endogenous internal control. *Legionella pneumophila* mip-gene DNA amplification is detected in the JOE/Yellow channel, while the protrombin gene DNA amplification is detected on FAM/Green channel. Protrombin gene DNA is a human genome DNA fragment; it should be present in an adequate amount in the DNA sample (no less than 10^3 genome equivalents). Both improper storage conditions and poor DNA extraction process can lead to DNA degradation and loss. So, the endogenous internal control

allows not only to control analysis steps but also to estimate the adequacy of sampling and storage.

- a qualitative test for *Legionella pneumophila* DNA detection in environmental samples. In this case the Internal Control STI-338 (IC) is used. *Legionella pneumophila* mip-gene DNA amplification is detected in the JOE/Yellow channel, while the Internal Control STI-338 (IC) DNA amplification is detected in the FAM/Green channel.
- a quantitative test for *Legionella pneumophila* DNA calculation in water. In this case, the Internal Control STI-338 (IC) is used. *Legionella pneumophila* mip-gene DNA amplification is detected in the JOE/Yellow channel, while the Internal Control STI-338 (IC) DNA amplification is detected in the FAM/Green channel. To quantify *Legionella pneumophila* and Internal Control DNA copies, quantitative standards are used.

For quantitation of *Legionella pneumophila* DNA in water samples, every sample must be tested two times, starting from the extraction step. The result is given as the average of two results.

The number of *Legionella pneumophila* DNA copies per 1 L of water is calculated according to the following formula:

$$C_{L.pn.\ DNA}\ (cop/L) = K_{L.pn.\ DNA}/K_{IC} \times C_{IC} \times 2$$

$C_{L.pn.\ DNA}$ (cop/L) – number of *Legionella pneumophila* **DNA copies in 1 L of water,**

$K_{L.pn.\ DNA}$ (cop/ml) – calculated number of *Legionella pneumophila* **DNA copies in 1 ml of sample,**

K_{IC} (cop/ml) - calculated number of Internal Control STI-338 (IC) **DNA copies in 1 ml of sample,**

C_{IC} (cop/ml) - number of Internal Control STI-338 (IC) **DNA copies in 1 ml of IC according to *Important Product Information Bulletin,***

2 – recalculation coefficient (adjustment for sample filtration).

Since the degree of water concentration is taken into account in calculations, treat water samples strictly according to this manual.

3. CONTENT

AmpliSens® *Legionella pneumophila*-FRT PCR kit is produced in 1 form:

AmpliSens® *Legionella pneumophila*-FRT PCR kit variant screen-titre-FRT,

REF R-B50(RG)-CE.

AmpliSens® *Legionella pneumophila*-FRT 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	70 tubes of 0.2 ml
PCR-mix-2-FL		colorless clear liquid	0.77	1 tube
DNA calibrators	**LS1**	colorless clear liquid	0.06	1 tube
	LS2	colorless clear liquid	0.06	1 tube
	LS3	colorless clear liquid	0.06	1 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*-FRT PCR kit is intended for 70 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.
- Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia) Instrument.
- Disposable polypropylene microtubes for PCR (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 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 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 accordance 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 mucosa. If skin, eyes, or mucosa 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 and then 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*-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 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 to tubes with environmental samples.

For Positive Control (PC), add 50 µl of Positive Control DNA *Legionella pneumophila* and 50 µl of Negative Control to 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. Using tips with aerosol barrier, add **10 µl** of **DNA samples** obtained from clinical or control samples at the DNA extraction stage.

The tubes with PCR-mix-1-FEP/FRT *Legionella pneumophila* that are not used at the moment should be stored away from light.

4. For the qualitative test, carry out the following 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).

5. For the quantitative test, carry out the following control amplification reactions:

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

LS1 - Add **10 µl** of **DNA calibrator LS1** to the tube labeled LS1.

LS2 - Add **10 µl** of **DNA calibrator LS2** to the tube labeled LS2.

LS3 - Add **10 µl** of **DNA calibrator LS3** to the tube labeled LS3.

8.2.2 Amplification

Program the Rotor-Gene according to manufacturer's manual and Guidelines.

Create a temperature profile on your Rotor-Gene instrument as follows:

Table 1

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*
Hold	95	5 min	–	1
Cycling	95	10 s	–	10
	60	20 s	–	
	72	10 s	–	
Cycling 2	95	10 s	–	35
	56	20 s	FAM/Green, JOE/Yellow	
	72	10 s	–	

Fluorescence is detected on the 2-nd step of stage Cycling 2 **(56 °C)** in FAM/Green and JOE/Yellow fluorescence channels.

Adjust the fluorescence channel sensitivity according to Guidelines.

9. DATA ANALYSIS

Internal Control is detected in the FAM /Green fluorescence channel, *Legionella pneumophila* DNA is detected in the JOE /Yellow fluorescence channel.

For **quantitative test,** set the standard values using *Important Product Information Bulletin* (LS1, LS2, and LS3 values should be set in both JOE /Yellow/HEX and FAM /Green channels).

See Guidelines for data analysis settings for Rotor-Gene 3000 or Rotor-Gene 6000.

9.2. Results interpretation

The results are interpreted by the software of Rotor-Gene 3000 or Rotor-Gene 6000 Instrument by the crossing (or not) of the fluorescence curve with the threshold line.

9.2.1. Qualitative test

The analysis results are considered valid, only if the control samples results comply the following:

Results for controls

Control	Stage for control	Ct channel FAM /Green	Ct channel JOE/Yellow	Interpretation
C–	DNA extraction	Pos (< W*)	Neg	OK
PC	DNA extraction	Pos (< W*)	Pos (< Y*)	OK
NCA	Amplification	Neg	Neg	OK
LS3	Amplification	Pos (< X*)	Pos (< Z*)	OK

*For W, X, Y and Z values see Guidelines.

1. The sample is considered positive for *Legionella pneumophila* if its Ct value is detected in the results grid in the JOE/Yellow channel and is less than **A** (see Guidelines).

2. The sample is considered negative for *Legionella pneumophila* if its Ct value is not detected in the results grid (the fluorescence curve does not cross the threshold line) in JOE/Yellow channel and in the results grid on the FAM/Green channel the Ct value doesn't exceed **B** for clinical samples and **C** for environmental samples.

9.2.2. Quantitative test

The results of analysis are considered reliable only if the results obtained for the control samples are correct (see the table below).

Results for controls

Control	Stage for control	Ct value in FAM/Green channel	Ct value in JOE/Yellow channel	Interpretation
C–	DNA extraction	Pos (< W*)	Neg	OK
PC	DNA extraction	Pos	Pos	OK
NCA	Amplification	Neg	Neg	OK
LS1	Amplification	Pos	Pos	OK
LS2	Amplification	Pos	Pos	OK
LS3	Amplification	Pos	Pos	OK

Calculate the concentration of *Legionella pneumophila* DNA in control and study samples.

1. The PC sample *Legionella pneumophila* DNA concentration must fall in the range specified in *Important Product Information Bulletin.*
2. The correlation coefficient R^2 for the calibration curve must be greater than 0.97.
3. The efficiency value must be in range 0.85–1.15.

10. TROUBLESHOOTING

10.1. Qualitative test

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

1. PCR should be repeated for samples with Ct greater than A in the JOE/Yellow channel and less than B in the FAM/Green channel. If the result is the same or less than A, the sample in considered as **positive**.
2. If Ct value is absent in both JOE/Yellow and FAM/Green channels or the Ct value in FAM/Green channel is greater than **B** for clinical samples and **C** for environmental samples, PCR should be repeated. If the same result is obtained, analysis of the sample should be repeated starting from the extraction stage.
3. If a signal is detected for Negative Control of extraction (C–) in the JOE/Yellow channel and/or for Negative Control of amplification (NCA) in any channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples

are considered invalid. Repeat analysis of all tests and take measures to detect and eliminate the source of contamination.

4. If no signal is detected for Positive Controls of amplification, this may suggest incorrect programming of the temperature profile of the Rotor-Gene 3000 or Rotor-Gene 6000 Instrument, incorrect configuration of the PCR reaction, or noncompliance of storage conditions for kit components with the manufacturer's instruction, or expiration of the reagent kit. Check programming of the Rotor-Gene Instrument (see 8.2.2), storage conditions, and the expiration date of reagents and then repeat PCR.

10.2. Quantitative test

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

1. If the signal is detected for Negative Control of extraction (C-) in the JOE/Yellow channel and/or for Negative Control of amplification (NCA) in any channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered invalid. Repeat analysis of all tests and take measures to detect and eliminate the source of contamination.
2. If the PC concentration does not fall in the range specified in the *Important Product Information Bulletin,* this suggests errors at extraction or amplification stages. The test must be repeated.
3. If the number of Internal Control STI-338 (IC) DNA copies in 1 ml of sample is 5-fold smaller than the concentration of IC specified in the *Important Product Information Bulletin*, this suggests errors at the extraction stage. The test must be repeated.

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*-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® *Legionella pneumophila*-FRT** PCR kit are to be stored at 2–8 °C when not in use. All components of the **AmpliSens® *Legionella pneumophila*-FRT** 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*-FRT** PCR kit is not less than $1x10^3$ genome equivalents per 1 ml of sample (GE/ml).

The claimed analytical features of **AmpliSens® *Legionella pneumophila*-FRT** PCR kit are guaranteed only when additional reagents 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*-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 deposited in gene banks by sequence comparison analysis. The clinical specificity of **AmpliSens® *Legionella pneumophila*-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, 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*-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	**PCE**	Positive control of extraction
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
29.12.10 KM	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
01.07.11 RT	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
26.11.11 LA	Text	Information that concentration values of control samples are specified in the Important Product Information Bulletin is added