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For Professional Use Only

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP
PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of the DNA of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in the clinical material (sputum, nasopharyngeal and oropharyngeal swabs, bronchial washing fluid or bronchoalveolar lavage, whole blood, and autopsy material) by 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

Mycoplasma pneumoniae and Chlamydophila pneumoniae detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using special *Mycoplasma pneumoniae and Chlamydophila pneumoniae* primers. In Fluorescent End-Point 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. A multichannel rotor-type fluorometer is specially designed to detect fluorescence emission from the fluorophores in the reaction mixture after the PCR. It allows detection of the accumulating product without re-opening the reaction tubes after the PCR run.

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP PCR kit is a qualitative test that uses the principle of endogenous control (amplification of a human prothrombin gene fragment). The target DNA selected as an endogenous internal control is a human genome fragment. It must be present in a sample in a sufficient quantity equivalent to the cell content in the sample.

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP 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 a wax layer. Wax melts and reaction component mix only at 95 ºC.

3. CONTENT

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP PCR kit is produced in 2 forms:

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP PCR kit variant FEP (0.5-ml tubes), **REF** B42-50-R0,5-FEP-CE.

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP PCR kit variant FEP (0.2-ml tubes), **REF** B42-50-R0,2- FEP-CE.

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP PCR kit variant FEP includes:

Reagent	***Description***	***Volume, ml***	***Quantity***
PCR-mix-1-FEP/FRT *Mycoplasma pneumoniae / Chlamydophila pneumoniae* ready-to-use single-dose test-tubes *(under wax)*	colorless clear liquid	0.008	55 tubes of 0.2 or 0.5 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 vial
Positive Control DNA *Mycoplasma pneumoniae* (C+$_{M.p.}$)	colorless clear liquid	0.1	1 tube
Positive Control DNA *Chlamydophila pneumoniae* (C+$_{C.p.}$)	colorless clear liquid	0.1	1 tube
Positive Control DNA human (C+$_{h}$)	colorless clear liquid	0.2	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	1 tube

* must be used in the extraction procedure as Negative Control of Extraction (see DNA-sorb-B, **REF** K1-2-50-CE protocols).

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP PCR kit is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- Transport medium for storage and transportation of respiratory swabs.
- Reagent for pretreatment of viscous fluids (sputum).
- Probe with cotton swab for sampling.
- 0.9 % saline solution or 0.01 M potassium-phosphate buffer (pH 7.0) for pretreatment of autopsy material.
- 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 rotor for 2-ml reaction tubes.
- PCR box.
- Personal thermocyclers (for example, Gradient Palm Cycler (Corbett Research, Australia), MaxyGene (Axygen, USA).
- Fluorometer ALA-1/4 (Biosan, Latvia) or equivalent instrument.
- Refrigerator at 2 to 8 °C.
- Deep-freezer at minus 16 to minus 24 °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 the 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® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP PCR kit is intended for analysis of the DNA extracted with the use of DNA extraction kits from:

– *sputum* (in a disposable vial; after pretreatment),
– *bronchial washing fluid or bronchoalveolar lavage* (in a disposable vial; after pretreatment),
– *nasopharyngeal and oropharyngeal swabs* (in a vial with Transport Medium for Storage and Transportation of Respiratory Swabs; pretreatment is not required),
– *whole blood* (in a tube with EDTA or sodium citrate; pretreatment is not required*),*

Whole blood is not to be used for acute respiratory infection diagnostics.

– *autopsy material: injured lung tissue* (after pretreatment).

Sampling

6.1 *Nasopharyngeal swabs.* Use dry sterile probes with cotton swabs. Insert the probe along the external nasal wall to a depth of 2–3 cm towards the inferior nasal conch. 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 Transport Medium for Storage and Transportation of Respiratory Swabs, **REF** 957-CE. 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.

6.2. *Oropharyngeal swabs*. Use dry probes with cotton swabs. Take swabs by rotating the probe over the surface of tonsils, palatine arches, and the posterior wall of the pharynx. Then place the swab (working part of the probe with cotton swab in a sterile disposable tube with 500 µl of Transport Medium for Storage and Transportation of Respiratory Swabs, **REF** 957-CE. 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.

It is recommended to combine the nasopharyngeal and oropharyngeal swabs in one tube. For this working ends of the probes after sampling should be placed in a tube with 0.5 ml of Transport Medium for Storage and Transportation of Respiratory Swabs, **REF** 957-CE and studied as one sample.

Pretreatment

6.3 *Bronchoalveolar lavage and bronchial washing fluid.* Vortex samples in the initial container. Using tips with aerosol filters transfer 1 ml of the sample in the 1.5 ml tube for the centrifugation at 10,000 rpm for 10 min. The supernatant is removed carefully using tip with a filter, reserving 100 µl over sediment, in which the sediment should be resuspended. Use 50 µl of obtained suspension for extraction.

6.4. *Sputum.* Use reagent Mucolysin, REF 180-CE. See the instruction manual to Mucolysin for a proper use. The pretreated sputum (50 µl) is used for DNA extraction. If it is necessary to repeat the test, the rest of sputum can be frozen.

6.5 *Autopsy material* is homogenized using sterile porcelain mortars and pestles. Then, prepare a 10 % suspension in a sterile saline or phosphate buffer. Transfer the suspension to a 1.5-ml tube and incubate for 1-3 min . The supernatant (50 µl) is used for DNA extraction. If it is necessary to repeat the test, the remaining sputum can be frozen.

The samples can be stored at 2 to 8 °C for 1 day, at minus 24 to minus 16 °C for 1 week and at no more than 68 °C for 1 year. It is allowed to freeze-thaw the samples one time.

7. WORKING CONDITIONS

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-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 protocol.

8.2. Preparing the PCR

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 *Mycoplasma pneumoniae / Chlamydophila pneumoniae*** and wax for the 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 *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*.**
3. Add above **1** drop of **mineral oil for PCR** (about **25 µl**).
4. Prepare 2 tubes with **PCR-mix-1-FEP/FRT *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*** 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 *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae***. Add above **1** drop of **mineral oil for PCR.**
5. Using tips with aerosol filters, add **10 µl** of **DNA samples** obtained from clinical or control samples at the DNA extraction stage.
6. Carry the control amplification reactions:

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

C+$_{M.\ p.}$ - Add **10 µl** of **Positive Control DNA *Mycoplasma pneumonia* (C+$_{M.p.}$)** to the tube labeled C+$_{M.p.}$ (Positive Control of Amplification).

C+$_{C.\ p.}$ - Add **10 µl** of **Positive Control DNA *Chlamydophila pneumoniae* (C+$_{C.p.}$)** to the tube labeled C+$_{C.p.}$ (Positive Control of Amplification).

C+ $_{h}$ - Add **10 µl** of **Positive Control DNA human (C+$_{h}$)** to the tube labeled C+$_{h}$ (Positive Control of Amplification).

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

8.2.2 Amplification

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

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

Table 1

Programming thermocyclers at DNA amplification of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*

	Thermocyclers with active temperature adjustment: **GeneAmp PCR System 2700, Gradient Palm Cycler, MyCycler, MaxyGene**			Thermocyclers with block temperature adjustment: **Uno-2**		
Step	Temperature	Time	Cycles	Temperature	Time	Cycles
0	**95 °C**	pause		**95 °C**	pause	
1	**95 °C**	5 min	1	**95 °C**	5 min	1
2	**95 °C**	10 s	42	**95 °C**	25 s	42
	63 °C	25 s		**63 °C**	40 s	
	72 °C	25 s		**72 °C**	25 s	
3	**72 °C**	1 min	1	**72 °C**	1 min	1
4	**4 °C**	storage		**10 °C**	storage	

3. Proceed to fluorescence detection after the amplification program is completed.

9. DATA ANALYSIS

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

Before the detection run, the required settings of the detector software should be adjusted according to the Guidelines [2].

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 at the channel for detection of specific cDNA exceed threshold value for negative samples, but does not exceed threshold value for positive samples (signal is in grey zone);

nd – invalid result (specific signal and IC signal does not detect (does not exceed threshold value) in the sample).

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).

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	Result of automatic interpretation			Interpretation
		FAM channel (*Mycoplasma pneumoniae*)	**ROX channel** (*Chlamydophila pneumoniae*)	**HEX channel** (IC)	
C–	DNA extraction	«Myc. pn. – nd»	«Chl. pn. – nd»	-	OK
NCA	PCR	«Myc. pn. – nd»	«Chl. pn. – nd»	-	OK
C+$_{M.\,p.}$	PCR	**«Myc. pn. – pos»**	«Chl. pn. – neg»	-	OK
C+$_{C.p.}$	PCR	«Myc. pn.- neg»	**«Chl. pn. – pos»**	-	OK
C+$_{h}$	PCR	«Myc. pn.- neg»	«Chl. pn. – neg»	**+**	OK

10. TROUBLESHOOTING

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

1. Preparing the PCR and detection should be repeated for samples with result **nd** (except NCA and C–). If the same result is obtained, it is necessary to repeat the sample analysis starting from the extraction stage. For the NCA and C– samples, the result **nd** is normal.
2. Preparing the PCR and detection should be repeated for samples with the result **eq**. If the same result is obtained, the samples are considered to be positive.
3. No positive signal in C+ may indicate incorrect selection of amplification program and another mistakes of preparing PCR. Repeat the PCR once again.
4. Positive signal in C– and NCA indicates reagent or sample contamination. In this case, the results of analysis must be considered as invalid. The analyses must be repeated and measures for detecting and eliminating the contamination source must be taken.

11. TRANSPORTATION

AmpliSens® ***Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP** PCR kit should be transported at 2–8 °C for no longer than 5 days.

12. STABILITY AND STORAGE

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

13. SPECIFICATIONS

13.1. Sensitivity

The analytical sensitivity of **AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP** PCR kit is not less than $1x10^3$ genome equivalents per 1 ml of sample (GE/ml).

The claimed analytical features of **AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP** PCR kit are guaranteed only when an 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® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP** PCR kit is ensured by the 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® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP** PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook "Sampling, Transportation, 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® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FEP** PCR kit for qualitative detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* DNA in the clinical materials by using end-point hybridization-fluorescence detection, developed by Federal Budget Institute of Science "Central Research Institute for Epidemiology".

15. QUALITY CONTROL

In compliance with the Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of **AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-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
RUO	Research Use Only		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
		$C+_{h}$, $C+_{C.p.}$, $C+_{M.p.}$	Positive control of amplification

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
09.01.14 GA	Sampling and handling	Sections “Sampling” and “Pretreatment” were added
	Stability and storage	Section was rewritten
	Text	Misprints were corrected
		Rewritten in accordance with the pattern