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For Professional Use Only

AmpliSens® *Mycoplasma pneumoniae*-EPh

PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Mycoplasma pneumoniae* DNA in the clinical material (sputum, bronchoalveolar lavage, nasopharyngeal swabs, throat swabs, and whole blood) by using electrophoretic detection of the amplified products in agarose gel.

The results of PCR analysis are taken into account in complex diagnostics of disease.

2. PRINCIPLE OF PCR DETECTION

Mycoplasma pneumoniae detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen DNA specific region using special *Mycoplasma pneumonia* primers. After PCR the amplified product is detected in agarose gel. **AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit** uses principle of endogenous internal control: amplification of human prothrombine gene fragment. DNA-target selected as endogenous internal control is the fragment of human genome and must be present in a sample in sufficient quantity equivalent to that of cells in the sample (no less than 10^3 genomes) taking into account that tested infectious agent is an intracellular pathogen. Hereby, endogenous internal control allows not only controlling PCR analyses stages (DNA extraction and PCR conducting) but also to evaluate the adequacy of clinical material sampling and storage. If there is not sufficient quantity of cells in the sample, then the amplified fragment of prothrombine gene will be indistinct or absent at all. **AmpliSens® *Mycoplasma pneumoniae*-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 melting and reaction mix components occur only at 95 ºC.

3. CONTENT

AmpliSens® Mycoplasma *pneumoniae*-EPh PCR kit is produced in 2 forms:

AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit variant 50 R (0.5-ml tubes), **REF** B40-50-R0,5-CE,

AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit variant 50 R (0.2-ml tubes) **REF** B40-50-R0,2-CE.

AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit variant 50 R includes:

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-R *Mycoplasma pneumoniae* 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 red	red clear liquid	0.6	1 tube
Mineral oil for PCR	colorless viscous liquid	2.0	1 dropper bottle
Positive Control DNA *Mycoplasma pneumoniae* ($C+_{M.p.}$)	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.

AmpliSens® *Mycoplasma pneumoniae*-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)
- Vortex mixer
- Tube racks
- PCR box
- Personal thermocycler (for example, Gradient Palm Cycler (Corbett Research, Australia), MaxyGene (Axygen, USA) or equivalent)
- 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 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 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 to read this handbook before starting work

AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit is intended for analysis of DNA extracted by DNA extraction kits from the clinical material:

- *Bronchoalveolar lavage*
- *Sputum*
- *Nasopharyngeal, throat swabs*
- *Whole blood (with EDTA or sodium citrate)*

The following reagents should be used additionally (don't included into this kit):

1. Mucolysin, **REF** 180-CE;
2. Transport medium for swabs, **REF** 956-CE;
3. PBS-buffer, **REF** 62-CE.

6.1. *Nasopharyngeal, throat swabs* and *the whole blood (with EDTA or sodium citrate)* samples do not require special pretreatment.

6.2. *Bronchoalveolar lavage* is to be shaken in source container. Transfer 1 ml of sample into marked screwing tube of 1.5 ml volume with using of the tip with aerosol barrier. Spin the tube for 10 min at 10.000 r/min then carefully remove supernatant with using of vacuum aspirator. Keep 100 µl of supernatant over the residue. Resuspend the residue and use for DNA extraction.

6.3. *Sputum* should be placed into a container with Mucolysin to get dilution 1:5 (5 volumes of Mucolysin per 1 volume of sputum). Mix and incubate at room temperature for about 20-30 min. Shake from time to time. Transfer 1 ml of sample into marked screwing tube of 1.5 ml volume with using of the tip with aerosol barrier. Spin the tube for 10 min at 8,000 - 10,000 r/min. carefully remove the supernatant using vacuum aspirator. Add 100 µl of PBS-buffer (or saline solution), resuspend the pellet and use for DNA extraction.

Only one freeze-thaw cycle of clinical material is allowed.

7. WORKING CONDITIONS

AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. DNA Extraction

It's recommended to use the following nucleic acid extraction kit:

- DNA-sorb-B **REF** K1-2-100-CE.

Carry out the DNA isolation according to the manufacturer instruction.

Internal Control is not used.

8.2. Preparing the PCR

Total reaction volume - 25 µl, volume of DNA sample - 10 µl.

8.2.1 Preparing tubes for PCR

1. Prepare required quantity of the PCR tubes with **PCR-mix-1-R *Mycoplasma pneumoniae*** and wax for amplification of DNA from clinical or control samples.
2. Add **10 µl of PCR-mix-2 red** to the surface of wax layer, ensuring that it doesn't fall under the wax and mix with **PCR-mix-1-R *Mycoplasma pneumoniae.***
3. Add above 1 drop of **mineral oil for PCR** (about 25 µl).

8.2.2 Amplification

1. Use prepared tubes for PCR. Add **10 µl** of **DNA samples**, obtained from clinical or control samples at the stage of DNA extraction, under or directly above the level of oil by tips with aerosol barrier.
2. Carry out the **control amplification reactions:**

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

$C+_{M.p.}$ - Add 10 µl of **Positive Control DNA *Mycoplasma pneumoniae*** to the tube for Positive Control ofAmplification.

$C+_{h}$ - Add 10 µl of **Positive Control DNA human** into the tube for Internal Control

3. Run the following program on the thermocycler (see table 1). When the temperature reaches 95ºC (pause regimen), insert tubes to cells of amplifier and press button to continue. It is recommended to sediment drops from walls of tubes by short vortex (1–3 s) before their insertion in thermocycler.

Table 1

Programming thermocyclers at DNA amplification of *Mycoplasma pneumoniae*

	Thermocyclers with active temperature adjustment:						Thermocyclers with block temperature adjustment: **Biometra, MiniCycler, PTC-100 (MJ Research)**		
	GeneAmp PCR System 2400 (Perkin Elmer)			**GeneAmp PCR System 2700 (Applied Biosystems), Gradient Palm Cycler (Corbett Research)**					
Step	Tempe rature	Time	Cycles	Tempe rature	Time	Cycles	Tempe rature	Time	Cycles
0	**95°C**	pause		**93°C**	pause		**95°C**	pause	
1	**95°C**	5 min	1	**93°C**	5 min	1	**95°C**	5 min	1
2	**95°C**	10 s	42	**95°C**	10 s	42	**95°C**	30 s	42
	65°C	10 s		**65°C**	25 s		**65°C**	30 s	
	72°C	10 s		**72°C**	25 s		**72°C**	30 s	
3	**72°C**	1 min	1	**72°C**	1 min	1	**72°C**	30 s	1
4	**4°C**	storage		**4°C**	storage		**10°C**	storage	

4. Amplification in thermocycler with block temperature adjustment lasts 2 h 30 min, in thermocycler with active temperature adjustment — 1 h 50 min.
5. After the reaction is finished PCR tubes must be collected and sent to the room for PCR products analysis.

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

Analysis of amplification products is performed by separation of DNA fragments in agarose gel.

9. DATA ANALYSIS

It's recommended to use the following detection agarose kit:

- 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 length of specific amplified DNA fragments is:

- *Mycoplasma pneumoniae* - 325 bp
- Human prothrombine gene (Internal Control) - 565 bp

Put the protective mask or use the glass filter while watching and photographing the gel

Results interpretation

Table 2

Results for controls

Control	Controlled step	Specific bands in the agarose gel		Interpretation
		325 bp	565 bp	
C-	DNA extraction	No	No	OK
NCA	Amplification	No	No	OK
$C+_{M.p.}$	Amplification	Yes	No	OK
$C+_{h}$	Amplification	No	Yes	OK

- The sample is considered to be positive for *Mycoplasma pneumoniae* DNA if the band of 325 bp is present in agarose gel. The band of IC (565 bp) could be absent in the samples with high concentration of *Mycoplasma pneumoniae* DNA.
- The sample is considered to be negative for *Mycoplasma pneumoniae* DNA if the band of 325 bp is absent and the band of 565 bp is present.

Besides specific bands the indistinct washed-out bands of primer-dimers may be seen in lanes, they are situated lower than level of 100 bp of nucleotide pairs.

10. TROUBLESHOOTING

Results of analysis are not being registered in the following cases:

- If results of control points analysis do not correspond to the listed above (Table 2), then the tests are to be repeated. Remove any reagents that may be suspect.
- If in lanes none of bands of 325 and 565 nucleotide pairs is observed, result of analysis for this sample is irrelevant and investigation of this sample must be repeated from the very beginning. It can be caused by mistake in clinical processing that provoked loss of DNA or inhibition of PCR.
- If in lines nonspecific bands at different levels are presented, it may be caused by lack of “hot start” or false temperature regimen in thermocycler.
- If in lanes corresponding to negative control (NCA, C–) specific band of 325 bp appears, it means that reagents or samples contamination has taken place. In such cases results of analysis must be considered as irrelevant. Test analysis must be repeated and measures for detecting contamination source must be undertaken.

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

11. TRANSPORTATION

AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Mycoplasma pneumoniae*-EPh** PCR kit are to be stored at 2-8 ºC when not in use. All components of the PCR kit are to be stable until 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 AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit is no less than $5x10^3$ genome equivalents per 1 ml of sample (GE/ml).

Claimed analytical features of AmpliSens® *Mycoplasma pneumoniae*-EPh PCR kit are guaranteed only when additional of reagents kits DNA-sorb-B and EPh manufactured by Federal Budget Institute of Science “Central Research Institute for Epidemiology”) are used.

13.2. Specificity

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

14. REFERENCES

1. Murdoch DR. Molecular genetics methods in the diagnosis of lower respiratory tract infections.APMIS. 2004 Nov-Dec; 112(11-12):713-27.

2. Handbook “Sampling, transportation, storage of clinical material for PCR diagnostics”, developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology”, 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® *Mycoplasma pneumoniae*-EPh PCR kit is tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Sufficient for
LOT	Batch code		Expiration Date
IVD	*In vitro* diagnostic medical device		Consult instructions for use
VER	Version	**NCA**	Negative control of amplification
	Temperature limitation	**C–**	Negative control of extraction
	Manufacturer	**C+$_{M.p.}$**	Positive Control DNA *Mycoplasma pneumoniae*
	Date of manufacture	**C+$_{h}$**	Positive Control DNA human
EC REP	Authorised representative in the European Community	**IC**	Internal control
	Caution		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
23.12.10 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 open reagents was added
	Key to Symbols Used	The explanation of symbols was corrected
24.06.11 VV	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”