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For Professional Use Only

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-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® ***Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT** 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 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

Mycoplasma pneumoniae and *Chlamydophila pneumoniae* detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific *Mycoplasma pneumoniae and Chlamydophila pneumoniae* primers. In the real-time 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. 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® ***Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT** PCR kit is a qualitative test that uses the principle of endogenous control – amplification of human prothrombin gene fragment. The DNA target 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 (not less than 10^3 genome equivalents). Therefore, an endogenous internal control makes it possible not only to monitor the stages of the test (DNA extraction and amplification) but also to assess the adequacy of clinical material collection and storage. If the number of cells in the specimen insufficient, signal of amplification of prothrombin gene will be too low.

AmpliSens® ***Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT** PCR kit variant FRT 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 components mix only at 95 °C

3. CONTENT

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

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT PCR kit variant FRT **REF** R-B42-4x(RG)-CE, **REF** R-B42-4x(iQ)-CE.

AmpliSens®*Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT PCR kit variant FRT in bulk[1] (for use with RG), **REF** R-B42-4x(RG)-CE-B

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT PCR kit variant FRT 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 ml
PCR-mix-2-FL	colorless clear liquid	0.77	1 tube
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*-FRT PCR kit variant FRT 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 a laboratory coat.

[1] In bulk form contains unlabeled tubes. Tubes with identical reagent are packed in one bag with label.

- 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.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia), iCycler iQ or iCycler iQ5 (Bio-Rad, USA).
- Refrigerator at 2 to 8 °C.
- Deep-freezer at minus 24 to minus 16 °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*****-FRT** PCR kit is intended for the analysis of the DNA extracted with 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 a dry sterile probes with a 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 pneumonia*-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 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

Variant FRT

1. Prepare the required number of the tubes with **PCR-mix-1-FEP/FRT *Mycoplasma pneumonia / Chlamydophila pneumoniae*** and wax for amplification of DNA from clinical and control samples.
2. Add **7 µl** of **PCR-mix-2-FL** onto the surface of the wax layer of each tube so that it does not fall under the wax and mix with **PCR-mix-1-FEP/FRT *Mycoplasma pneumonia / Chlamydophila pneumoniae.***
3. Using tips with aerosol filter, add **10 µl** of **DNA samples** obtained from clinical or control samples at the DNA extraction stage to the prepared tubes.
4. Carry out 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 pneumonia* (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. Create a temperature profile on your instrument as follows:

Table 1

Amplification program

	***Rotor-type Instruments*[2]**			***Plate-type Instruments*[3]**		
Step	***Temperature, °C***	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
1	**95**	**5 min**	1	**95**	**5 min**	1
2	**95**	10 s	10	**95**	15 s	10
	63	30 s		**63**	45 s	
	72	10 s		**72**	15 s	
3	**95**	10 s	35	**95**	15 s	35
	60	30 s Fluorescence acquiring		**60**	45 s Fluorescence acquiring	
	72	10 s		**72**	15 s	

[2] For example, Rotor-Gene 3000, Rotor-Gene 6000, or equivalent.
[3] For example, iCycler iQ, iQ5 or equivalent.

Fluorescent signal is detected in the channels for the FAM, JOE, and ROX fluorophores.

2. Adjust the fluorescence channel sensitivity according to Guidelines [2].
3. Insert tubes into the reaction module of the device.

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

4. Run the amplification program with fluorescence detection.
5. Analyse results after the amplification program is completed.

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in three channels:

– The signal of the *Mycoplasma pneumoniae* DNA amplification product is detected in the channel for the FAM fluorophore.
– The signal of the *Chlamydophila pneumoniae* DNA amplification product is detected in the channel for the ROX fluorophore.
– The signal of the human DNA amplification product is detected in the channel for the JOE fluorophore.

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the DNA sample in the corresponding column of the results grid.

Principle of interpretation is the following:

– The sample is considered positive if the *Ct* value determined in the results grid in the channel for the FAM or ROX fluorophore is less than the boundary *Ct* value specified in the Guidelines [2].
– The sample is considered negative if the *Ct* value is not determined (absent) in the channels for FAM or ROX fluorophores, whereas the *Ct* value determined in the channel for the JOE fluorophore is less than the boundary *Ct* value specified in the Guidelines [2].

Boundary *Ct* values are specified in the Guidelines [2]

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct (seeTable 2).

Table 2

Results for controls

Control	Stage for control	*Ct* value in channel for fluorophore		
		FAM	**ROX**	**JOE**
C–	DNA extraction	Absent	Absent	Absent
NCA	PCR	Absent	Absent	Absent
C+$_{M.p.}$	PCR	**<**boundary value	Absent	Absent
C+$_{C.p.}$	PCR	Absent	**<**boundary value	Absent
C+$_{h}$	PCR	Absent	Absent	**<**boundary value

10. TROUBLESHOOTING

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

1. If no signal is detected for any positive controls of amplification (C+$_{M.p.}$, C+$_{C.p.}$, C+$_{h}$) may indicate incorrect selection of amplification program and other mistakes of preparing PCR. Repeat the PCR once again.
2. If the *Ct* value is determined for the Negative Control of extraction (C–) and Negative Control of amplification (NCA) in any channel, it indicates contamination of reagents or samples. In this case, the results of analysis for all samples are considered invalid. It is necessary to repeat the analysis of all tests and to take measures to detect and eliminate the source of contamination.
3. If the Ct value for human DNA in the results grid in the channel for the JOE fluorophore is greater than the boundary *Ct* value, the PCR analysis should be repeated starting from the DNA extraction stage. If the same result is obtained, it may suggest incorrect sampling or storage of the clinical material. Sampling should be repeated.

11. TRANSPORTATION

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

13. SPECIFICATIONS

13.1. Sensitivity

The analytical sensitivity of **AmpliSens®** ***Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT** 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*-FRT** PCR kit are guaranteed only when an 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®** ***Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT** 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*-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, 2010.
2. Guidelines to the AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT PCR kit for qualitative detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* DNA in clinical materials by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection, developed by Federal Budget Institute of Science "Central Research Institute for Epidemiology".

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* / *Chlamydophila pneumoniae*-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	Σ	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
23.10.13 GA	Footer	Catalogue number **REF** R-B42-4x(RG)-CE-B was added
	Content	One more release form was added AmpliSens® *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT PCR kit variant FRT in bulk (for use with RG), **REF** R-B42-4x(RG)-CE-B
25.11.13 GA	Footer	Catalogue number **REF** R-B42-100-F-CE was deleted
	Content	One release form was deleted AmpliSens® *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT PCR kit variant FRT-100 F REF R-B42-100-F-CE.
09.01.14 GA	Sampling and handling	Sections “Sampling” and “Pretreatment” were added
	Protocol Preparing tubes for PCR for the PCR kit variant FRT-100 F	Section was deleted
	Stability and storage	Section was rewritten
	Text	The designation “x”, “y, ”z” was change to “boundary value”
		Misprints were corrected
		Rewritten in accordance with the pattern