CE

IVD

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

TABLE OF CONTENTS

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 biological 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 PCR kit is also used for studying the role of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* role in the pathogenesis of noninfectious chronic diseases, such as cardiovascular system diseases, by the *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* DNA detection in the whole blood by using nucleic acid extraction kit RIBO-sorb.

The results of PCR analysis are taken into account in the 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 to genes regions *putative lipoprotein* of *Mycoplasma pneumoniae* and *ompA* of *Chlamydophila pneumoniae* primers. In real-time 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. 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. 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.

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. In variant FRT, “hot-start” is guaranteed by the separation of nucleotides and Taq-polymerase by using a wax layer. Wax melts and reaction components mix only at 95 °C.

In variant FRT-100 F, “hot-start” is guaranteed by the separation of nucleotides and Taq-polymerase by using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT PCR kit is produced in 1 form:

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT PCR kit variant FRT-100 F, **REF** R-B42-100-F-CE

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT PCR kit variant FRT-100 F includes:

Reagent	***Description***	***Volume, ml***	***Quantity***
PCR-mix-1-FRT (F) *Mycoplasma pneumonia / Chlamydophila pneumoniae*	colorless clear liquid	0.2	5 tubes
PCR-mix-2-FRT	colorless clear liquid	0.6	1 tube
Polymerase (TaqF)	colorless clear liquid	0.06	1 tube
Positive Control DNA *Mycoplasma pneumoniae / Chlamydophila pneumoniae / Prothrombin* (C+$_{M.p. / C.p / P}$)	colorless clear liquid	0.1	2 tubes
TE-buffer	colorless clear liquid	0.5	2 tubes
Negative Control (C–)*	colorless clear liquid	1.2	1 tube

* must be used in the extraction procedure as Negative Control of Extraction (see RIBO-sorb, **REF** K2-1-Et-50-CE or RIBO-prep **REF** K2-9-Et-50-CE protocols).

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT PCR kit variant FRT-100 F is intended for 100 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- Transport Medium for Storage and Transportation of Respiratory Swabs
- Pediatric or flexible nasopharyngeal velor swab on a plastic applicator probe for the swabs from inferior nasal meatus for children and adults.
- Probe (polystyrene with viscose swabs) for oropharyngeal swabs for children and adults.
- Reagent for pretreatment of viscous fluids (sputum).
- Sterile saline or phosphate buffer for preconditioning autopsy material.
- Porcelain mortar and pestle for homogenize the autopsy material.
- Microcentrifuge (12,000 g).

- DNA extraction kit or the DNA extraction automatic station.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with filters (up to100 and 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); Rotor-Gene Q (QIAGEN, Germany) iCycler iQ or iCycler iQ5 (Bio-Rad, USA); CFX 96 (Bio-Rad, USA)).
- Disposable polypropylene PCR tubes (0.1- or 0.2-ml) when working with PCR kit variant FRT-100 F:
    a) 0.2-ml PCR tubes with optical transparent domed or flat caps if a plate-type instrument is used;
    b) 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used.
- 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 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 analysis of DNA extracted by using DNA extraction kits from sputum, nasopharyngeal and oropharyngeal swabs, bronchial washing fluid or bronchoalveolar lavage, whole blood, and autopsy material.

Sampling

6.1. *Nasopharyngeal swabs* .Use nasopharyngitis dry cotton swabs. If the nasal cavity is filled by mucus it is necessary to blow one's nose. 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. 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 viscose 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 viscose swab in a sterile disposable tube with 500 µl of transport medium. 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. Store the samples at 2–8 °C for 3 days or at minus 24 to minus 16 °C for 1 week.

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.

6.3. *Tracheal sputum or aspirate.* Sputum is taken in sterile hermitic disposable plastic containers after gargling the oral cavity with water. Trachea aspirates are get traditional method and placed in sterile hermitic disposable plastic containers.

6.4. The samples of *bronchoalveolar lavage or bronchial washing fluid* are taken in disposable tight closing polypropylene 5 ml volume cups (in order to avoid cells adhesion on the internal cup surface). Store the samples at 2–8 °C for 1 day or at minus 24 to minus 16 °C for 1 week.

6.5. *Autopsy material* is placed immediately in sterile disposable containers and either frozen after taking or studied within an hour. Store the samples at minus 68 °C for 1 year. Only one material freezing – defrosting is allowed.

6.6. *Whole blood* is taken in the type Vacuette® tubes with solution or EDTA evaporation. Closed tube with material is turned several times to remix preservative. Use whole blood, gathered on an empty stomach in the morning for analysis. Storage for 3 days at 2–8 °C is allowed.

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

<u>*Pretreatment*</u>

6.7 *Nasal and oropharyngeal swabs.* Vortex the tube, then centrifuge it at 5,000 rpm for 5 s to sediment drops from the interior wall of the tube lid. Use 100 µl of material sample for extraction.

6.8 *Nasopharyngeal sputum or aspirate; tracheal sputum or aspirate*. Use reagent Mucolysin (REF 180-CE) for sputum and aspirate pretreatment. See the instruction manual to Mucolysin for a proper use. The pretreated sputum (100 µl) is used for DNA extraction. If it is necessary to repeat the test, the rest of sputum can be frozen.

6.9 *Bronchoalveolar lavage and bronchial washing fluid.* Vortex samples in the initial cup. Add 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, reserving 200 µl over sediment, which should be resuspended. Use 100 µl of material sample for extraction. If it is necessary to repeat the test, the remaining material can be frozen.

6.10 *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 centrifuge at 10,000 rpm for 5 min. The supernatant (100 µl) is used for DNA extraction. If it is necessary to repeat the test, the remaining sputum can be frozen.

7. WORKING CONDITIONS

AmpliSens® ***Mycoplasma pneumoniae / Chlamydophila pneumoniae*-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 kits:

- RIBO-sorb, **REF** K2-1-Et-50-CE
- RIBO-prep, **REF** K2-9-Et-50-CE
- NucliSENS easyMAG automated system (for details see Guidelines [2]).

Extract the DNA according to the manufacturer's protocol.

8.2. Preparing the PCR

The Positive Control of Amplification (C+) and Negative Control Amplification (NCA) are used every time when amplification reactions are carried out. In addition Negative Control of Extraction (C-) is studied.

8.2.1 Preparing tubes for PCR for the PCR kit variant FRT-100 F

The total reaction volume is **25 µl**, the volume of the **DNA** sample is **10 µl**.

1. Thaw the required number of tubes with **PCR-mix-1-FRT (F)** ***Mycoplasma pneumoniae / Chlamydophila pneumoniae,*** **PCR-mix-2-FRT,** and **polymerase (TaqF)** and then centrifuge briefly.

   Take the required number of tubes/strips for amplification of the DNA obtained from clinical and control samples.
2. For N reactions, add to a new tube:

   10*(N+1) µl of PCR-mix-1-FRT (F) ***Mycoplasma pneumoniae / Chlamydophila***

pneumoniae,

5.0*(N+1) µl of PCR-mix-2-FRT

0.5*(N+1) µl of polymerase (TaqF).

Vortex the tube, then centrifuge it briefly. Transfer **15 µl** of the prepared mixture to each tube.

3. Using tips with aerosol filter, add **10 µl** of **DNA samples** obtained at the RNA reverse transcription stage.
4. Carry out the control amplification reactions:

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

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

C– -Add **10 µl** of the sample extracted from **Negative Control (C–)**

8.3 Amplification

1. Create a temperature profile on your instrument as follows(see Table 1):

Table 1

Amplification program for DNA of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*

	***Rotor-type Instruments*[1]**			***Plate-type Instruments*[2]**		
Step	***Temperature, °C***	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
Hold	**95**	**15 min**	1	**95**	**15 min**	1
Cycling 1	**95**	10 s	10	**95**	10 s	10
	60	20 s		**60**	25 s	
	72	10 s		**72**	25 s	
Cycling 2	**95**	10 s	35	**95**	10 s	35
	60	20 s Fluorescence acquiring		**60**	25 s Fluorescence acquiring	
	72	10 s		**72**	25 s	

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

Amplification program for some other models of instruments is specified in Guidelines [2].

2. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin* and Guidelines [2].

3. Insert tubes into the reaction module of the device.

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

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

The results are interpreted by the software of used instrument by the crossing (or not-crossing) of the fluorescence curve with the threshold line. (see Table 2)

Table 2

Target compliance with detection channels

Detection channel		
FAM	**JOE**	**ROX**
Mycoplasma pneumoniae	Human DNA	*Chlamydophila pneumoniae*

Principle of interpretation:

- *Mycoplasma pneumonia* and/or *Chlamydophila pneumoniae* DNA is **detected** if the *Ct* value determined in the results grid in the channel for the FAM and/or ROX fluorophore are less than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
- *Mycoplasma pneumonia* and/or *Chlamydophila pneumoniae* DNA **not detected** in a sample if the *Ct* value is not determined (absent) in the channels for FAM and/or ROX fluorophores, whereas the *Ct* value determined in the channel for the JOE (Human DNA) fluorophore is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.
- The result is **invalid** if the *Ct* value is not determined (absent) in the channel for FAM or ROX fluorophores, whereas the *Ct* value in the channel for the JOE fluorophore is not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage. If the same result is obtained in the second run, re-sampling of material is recommended.

Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit. See also Guidelines [2]

The result of the analysis is considered reliable only if the results obtained for both Positive and Negative Controls of Amplification as well as for the Negative Control

of Extraction are correct (seeTable 3).

Table 3

Results for controls

Control	Stage for control	*Ct* value in the channel for fluorophore		
		FAM (*Mycoplasma pneumoniae*)	**ROX** (*Chlamydophila pneumoniae*)	**JOE** (Human DNA)
C–	DNA extraction	Absent	Absent	Absent
NCA	PCR	Absent	Absent	Absent
C+	PCR	**<**boundary value	**<**boundary value	**<**boundary value

10. TROUBLESHOOTING

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

1. If the *Ct* value determined for the Positive Control of Amplification (C+) in the channels for the **FAM or JOE** fluorophores is greater than the boundary *Ct* value or absent, the amplification and detection should be repeated for all samples was not detected in the respective channel.
2. If the *Ct* value is determined for the Negative Control of Amplification (NCA) and/or Negative Control of Extraction (C–) in the channels for the **FAM or JOE** fluorophores, the PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which DNA-target was detected.

11. TRANSPORTATION

AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT 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*-FRT** PCR kit are to be stored at 2–8 °C (except for PCR-mix-1-FRT (F) *Mycoplasma pneumonia / Chlamydophila pneumonia*, PCR-mix-2-FRT and polymerase (TaqF)) when not in use. All components of the **AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT** PCR kit are stable until the expiry 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-FRT (F) *Mycoplasma pneumonia / Chlamydophila pneumonia*, PCR-mix-2-FRT, and polymerase (TaqF) are to be stored at the temperature from minus 24 to minus 16 °C

PCR-mix-1-FRT (F) *Mycoplasma pneumonia / Chlamydophila pneumonia* is to be kept away from light

13. SPECIFICATIONS

13.1. Sensitivity

Clinical material	Volume, ml	Nucleic acid extraction kit	Microorganism	Sensitivity, GE/ml[3]
Nasopharyngeal and oropharyngeal mucosa and sputum treated with mucolisin	100	RIBO-sorb	*Mycoplasma pneumoniae, Chlamydophila pneumoniae*	$1x10^3$
		RIBO-prep		$5x10^2$
		NucliSENS easyMAG		$5x10^2$

13.2. Specificity

The analytical specificity of **AmpliSens® *Mycoplasma pneumoniae / Chlamydophila pneumoniae*-FRT** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes have been 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 makes it possible to detect DNA of the specific fragments of the claimed pathogens. The specificity of this kit was confirmed by investigation of the following reference strains: *Streptococcus* spp., *Moraxella catarrhalis, Staphilococcus aureus, Staphilococcus saprophiticus, Haemophilus influenzae, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Mycobacteria tuberculosis* 27294 105, *Neisseria flava, Neisseria sicca, Neisseria mucosa, E. coli ATCC, NCTC, Enterococcus faecalis, Legionella pneumophila, Shigella flexneri, Shigella sonnei, Salmonella enteritidis, Yersinia enterocollitica, Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica* as well as human genomic DNA. Activity of the components of PCR kit is absence in point of strains *Chlamydophila arginini, Chlamydophila pecorum, Chlamydia trachomatis, Chlamydia muridarum, Chlamydia suis, Chlamydophila abortus, Chlamydophila psittaci, Mycoplasma arginini, Mycoplasma mycoides (*подвид *capri), Mycoplasma hyorinis, Mycoplasma bovigenitalium, Mycoplasma bovine, Mycoplasma salivarium, Mycoplasma faucium, Mycoplasma gallisepticum, Mycoplasma sinoviae, Mycoplasma genitalium, Mycoplasma hominis.*

[3] The quantity of genome equivalents of microorganism per 1 ml of the sample placed in a transport medium.

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 the biological material by real-time hybridization-fluorescence detection of amplified products 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
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
		C+	Positive control of amplification

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
25.11.13 GA	Footer	Catalogue number **REF** R-B42-100-F-CE was added Catalogue numbers **REF** R-B42-50-Mod(iQ,Dt)-CE; **REF** R-B42-50-Mod(RG)-CE; **REF** R-B42-100-F-Mod(RG,iQ,Dt)-CE was deleted
	Content	Three release form was deleted: AmpliSens® *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT PCR kit variant FRT (for use with iQ, Dt), REF R-B42-50-Mod(iQ,Dt)-CE AmpliSens® *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT PCR kit variant FRT (for use with RG), REF R-B42-50-Mod(RG)-CE. AmpliSens® *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT PCR kit variant FRT-100 F (for use with RG, iQ, Dt), REF R-B42-100-F-Mod(RG,iQ,Dt)-CE One more release form was added AmpliSens®*Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT PCR kit variant FRT-100 F REF R-B42-100-F-CE
09.01.14 GA	Protocol Preparing tubes for PCR for the PCR kit variant FRT	Section was deleted
	Stability and storage	Section was rewritten
	Text	Misprints were corrected
		Rewritten in accordance with the pattern