Sacace BIOTECHNOLOGIES

CE 1023

IVD For *in Vitro* Diagnostic Use

Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM

Handbook

Real Time PCR kit for qualitative detection of Mycoplasma pneumoniae and Chlamydophila pneumoniae

REF B42-4-100FRT

Σ 100

NAME

Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM

INTRODUCTION

Mycoplasma pneumoniae is spread through respiratory droplet transmission. Once attached to the mucosa of a host organism, *M. pneumoniae* extracts nutrients, grows and reproduces. Attachment sites include the upper and lower respiratory tract, causing pharyngitis, bronchitis, and pneumonia. The infection caused by this bacterium is called atypical pneumonia because of its protracted course and lack of sputum production and wealth of extra-pulmonary symptoms.

Chlamydophila (formerly Chlamydia) **pneumoniae** causes mild pneumonia or bronchitis in adolescents and young adults. Older adults may experience more severe disease and repeated infections. Approximately 50% of young adults and 75% of elderly persons have serological evidence of previous infection. The pathogen is estimated to cause 10-20% of community-acquired pneumonia cases among adults. The estimated number of cases of *C. pneumoniae* pneumonia is 300,000 cases per year.

INTENDED USE

Kit **Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM** is a “Real-Time Amplification” test for the qualitative detection of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* DNA in the biological materials (sputum or tracheal aspirate, bronchial washing fluid, bronchoalveolar lavage, nasopharyngeal and oropharyngeal swabs, and autopsy material).

PRINCIPLE OF ASSAY

Kit **Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM** is based on two major processes**:** DNA is extracted from samples and amplified using real time amplification with fluorescent reporter dye probes specific for Mycoplasma pneumoniae, Chlamydia pneumoniae and Internal Control IC. Test detects an endogenous IC of a human genome DNA fragment which is extracted from the sample and serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition. IC is detected in a channel other than the *M.pneumoniae* or *C. pneumonia.*

MATERIALS PROVIDED

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FRT (Mycoplasma pneumoniae/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/ Chlamydia pneumoniae/IC human DNA)	colorless clear liquid	0.1	2 tubes
DNA-buffer	colorless clear liquid	0.5	2 tubes
Negative Control C–*	colorless clear liquid	1.2	1 tube

Contains reagents for 100 tests.
* *must be used in the extraction procedure as Negative Control of Extraction.*

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit.
- Transport medium.
- Disposable powder-free gloves.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers.
- Disposable polypropylene 1,5/2,0 ml tubes.
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes.
- PCR Workstation.
- Real Time Thermal cycler.
- Disposable polypropylene microtubes for PCR.
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

WARNINGS AND PRECAUTIONS

IVD ***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Dispose of all specimens and unused reagents in accordance with local regulations.
6. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
7. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
8. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
9. Material Safety Data Sheets (MSDS) are available on request.
10. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
11. PCR reactions are sensitive to contamination. Measures to reduce the risk of contamination in the laboratory include physically separating the activities involved in performing PCR in compliance with good laboratory practice.

12. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the PCR and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

Sampling of biological materials for PCR-analysis, transportation, and storage are described in details in the handbook of the manufacturer. It is recommended that this handbook is read before beginning of the work.

STORAGE INSTRUCTIONS

Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM kit must be stored at -16°C or below when not in use. The kit can be shipped at 2-8°C no longer than 5 days but should be stored at -16°C or below immediately on receipt.

STABILITY

Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

The shelf life of reagents before and after the first use is the same, unless otherwise stated.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM can analyze DNA extracted from:

- *Whole blood* collected in EDTA tubes;
- *Autopsy material (tissue)* homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile. Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;
- *bronchial lavage:* centrifuge 10 mL at 3000 g/min for 10-15 min. Remove and discard the supernatant. If the pellet isn't visible add 10 ml of liquid and repeat centrifugation remove and discard the supernatant. Resuspend the pellet in 100 µl of saline water.
- *Tracheal sputum or aspirate.*
- *swabs:* insert the swab into the nuclease-free 1,5 ml tube and add 0,2 mL of Transport medium. Vigorously agitate swabs in medium for 15-20 sec.

Specimens can be stored at +2-8°C for no longer than 12 hours, or freeze at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.

Sacace Biotechnologies recommends to use the following kit:

- ⇨ **Ribo-Sorb** (Sacace, REF K-2-1/100);
- ⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);

Please carry out DNA extraction according to the manufacturer's instruction.

PROTOCOL (REACTION VOLUME 25 µl):

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

Unfreeze reagents before mixing.

1. Prepare the required number of the tubes for amplification of DNA from clinical and control samples.
2. Prepare in a new sterile tube the **Reaction Mix.** For each sample mix **10*(N+1) µl** of **PCR-mix-1-FRT (Mycoplasma pneumoniae/Chlamydophila pneumoniae), 5,0*(N+1) µl** of **PCR-mix-2-FRT, 0,5*(N+1) µl** of **Polymerase (TaqF)**. Vortex briefly;
3. Add **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. Mix by pipetting.
4. Prepare for each panel the following controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **Positive Control DNA (Mycoplasma pneumoniae/ Chlamydia pneumoniae/IC human DNA)** to the tube labeled *C+* $_{Mp/Cp/IC}$;
   - add **10 µl** of **Negative Control C-** to the tube labeled Extraction Negative Control.

Amplification

1. Create a temperature profile on your instrument as follows:

	Rotor-type Instruments[1]			Plate- or modular type Instruments[2]		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
1	**95**	15 min	1	**95**	15 min	1
2	**95**	10 sec	10	**95**	10 sec	10
	60	20 sec		**60**	25 sec	
	72	10 sec		**72**	25 sec	
3	**95**	10 sec	35	**95**	10 sec	35
	60	20 sec *fluorescent signal detection*		**60**	25 sec *fluorescent signal detection*	
	72	10 sec		**72**	25 sec	

[1] For example Rotor-Gene™ 3000/6000/Q (Qiagen) or equivalent
[2] For example, CFX96™/ iCycler iQ™/ iCycler iQ5™ (BioRad) or equivalent

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in Fam (Green), Rox (Orange) and Joe (Yellow), fluorescence channels.

Mycoplasma pneumoniae is detected on the FAM (Green) channel, *Chlamydophila pneumoniae* on ROX (Orange) and IC DNA on the JOE(Yellow)/HEX/Cy3 channel.

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.05*	*20 %*	*Off*
JOE/Yellow	*from 5 Fl to 10 Fl*	*0.1*	*10 %*	*On*
ROX/Orange	*from 5 Fl to 10 Fl*	*0.1*	*5%*	*Off*

Plate- or modular type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

RESULTS INTERPRETATION

The results are interpreted by the real-time PCR instrument software and the evaluation of the crossing or not crossing of the threshold line by the fluorescence curve.

- *Mycoplasma pneumoniae* and/or *Chlamydophila pneumoniae* DNA **is detected** in a sample if the Ct value is present in the results grid in the channel FAM and/or ROX and are less than the boundary Ct value;
- *Mycoplasma pneumoniae* and/or *Chlamydophila pneumoniae* DNA **is not detected** in a sample if the Ct value is not determined (absent) in the channel FAM and/or ROX whereas the Ct value in the channel JOE is less than the boundary value;
- The result **is invalid** if the Ct value is not determined (absent) in the channel FAM and/or ROX whereas the Ct in the JOE channel is not determined or greater than the specified boundary Ct value.

Results for controls

Control	Stage for control	Ct channel Fam/Green	Ct channel Joe/Yellow	Ct channel Rox/Orange	Interpretation
NCE	DNA isolation	Neg	Neg	Neg	Valid result
NCA	Amplification	Neg	Neg	Neg	Valid result
Pos C+	Amplification	Pos	Pos	Pos	Valid result

Boundary values

	Rotor Type Instrument			Plate Type Instrument		
Samples	**Ct Fam/ Green**	**Ct Joe/ Yellow**	**Ct Rox/ Orange**	**Ct Fam/ Green**	**Ct Joe/ Yellow**	**Ct Rox/ Orange**
NCE	/	/	/	/	/	/
NCA	/	/	/	/	/	/
Pos C+	<22	<22	<22	<23	<23	<23
Clinical samples	≤33	≤33	≤33	≤33	≤33	≤33

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of **Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM** 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 specificity of **Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM** PCR 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 absent also in the strains: *Chlamydophila arginini, Chlamydophila pecorum, Chlamydia trachomatis, Chlamydia muridarum, Chlamydia suis, Chlamydophila abortus, Chlamydophila psittaci, Mycoplasma arginini, Mycoplasma mycoides (subspecies capri), Mycoplasma hyorinis, Mycoplasma bovigenitalium, Mycoplasma bovine, Mycoplasma salivarium, Mycoplasma faucium, Mycoplasma gallisepticum, Mycoplasma sinoviae, Mycoplasma genitalium, Mycoplasma hominis.*

Analytical sensitivity

Biological material	Pathogen agent	Material volume, µl	Nucleic acid extraction kit	Sensitivity, GE/ml*
Nasopharyngeal and oropharyngeal mucosa and sputum	*Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*	100	**Ribo-Sorb**	$1x10^3$
			DNA/RNA Prep	$5x10^2$

** Genome equivalents (GE) of the pathogen agent per 1 ml of the sample*

Target region

Channel for fluorophore	FAM	JOE	ROX
DNA-target	Mycoplasma pneumoniae	Internal Control – human DNA	Chlamydophila pneumoniae
Target gene	Putative lipoprotein	Human protrombin gene V	ompA

TROUBLESHOOTING

1. Weak or no signal of the IC.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
2. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. FAM (Green) or Rox (Orange) signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All sample results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All sample results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number	Σ	Contains sufficient for <n> tests
	Store at	VER	Version
	Manufacturer	**NCA**	Negative Control of Amplification
	Consult instructions for use	**NCE**	Negative control of Extraction
	Expiration Date	**C+**	Positive Control of Amplification
IVD	For *in Vitro* Diagnostic Use	**IC**	Internal Control

* *CFX96™/ iCycler iQ™ / iCycler iQ5™* are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen

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