

Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM

Handbook

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

REF TB42-4-100FRT

 **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* in the biological materials (whole blood, tissue, swabs, etc).

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. pneumoniae*.

MATERIALS PROVIDED

Module No.2: Complete Real Time PCR test with DNA purification kit (TB42-4-100FRT)

Part N° 1 – “**DNA-Sorb-B**”: isolation of DNA from clinical specimens;

- **Lysis Solution,** 2 x 15 ml;
- **Washing Solution 1,** 2 x 15 ml;
- **Washing Solution 2,** 2 x 50 ml;
- **Sorbent,** 2 x 1,25 ml;
- **DNA-eluent,** 2 x 5,0 ml.

Contains reagents for 100 extractions.

Part N° 2– “**Mycoplasma pneumoniae / Chlamydomphila pneumoniae Real-TM**”: Real Time Amplification

- **PCR-mix-1-FRT,** 5 x 0,2 ml;
(**Mycoplasma pneumoniae/Chlamydomphila pneumoniae**)
- **PCR-mix-2-FRT,** 0,6 ml;
- **Polymerase (TaqF),** 0,06 ml;
- **Positive Control DNA,** 2 x 0,1 ml;
(**Mycoplasma pneumoniae/ Chlamydia pneumoniae/IC human DNA**)
- **DNA-buffer,** 2 x 0,5 ml;
- **Negative Control C-*,** 1,2 ml

Contains reagents for 100 tests.

* *must be used in the extraction procedure as Negative Control of Extraction*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- 60°C ± 2°C dry heat block
- Vortex mixer
- Pipettors (capacity 5-40 µl; 40-200 µl; 200-1000 µl) with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Biohazard waste container
- Refrigerator
- Freezer

Zone 2: Real Time amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

Mycoplasma pneumoniae / Chlamydomphila pneumoniae Real-TM kit must be stored at -20°C when not in use. **DNA-sorb-B** must be stored at 2-8°C. The kits can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY


Mycoplasma pneumoniae / Chlamydomphila 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.

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.

WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

-  Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- 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 specimens and unused reagents in accordance with local authorities' regulations.
- Specimens 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 sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse 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 techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. 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.

*** Only for Module No.2**

PRODUCT USE LIMITATIONS

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.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

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

- *Whole blood* collected blood in ACD or EDTA tubes;
- *tissue* (≈1,0 gr) 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.
- *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

The following kits are recommended:

- ⇒ **DNA-Sorb-B** (Sacace, REF K-1-1/B);
- ⇒ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006);

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

SPECIMEN AND REAGENT PREPARATION (reagents supplied with module No.2)

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 56°C until disappearance of ice crystals.
2. Prepare required quantity of 1.5 ml polypropylene tubes.
3. Add to each tube **300 µl** of **Lysis Solution**.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
 - add **100 µl** of **C- (Negative Control)** to labeled *Cneg*.
6. Vortex the tubes and incubate for 5 min at 65°C, then centrifuge for 7-10 sec
7. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
8. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature.
9. Centrifuge all tubes for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and centrifuge for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
11. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
12. Repeat step 11.
13. Incubate all tubes with open cap for 5 min at 65°C.
16. Resuspend the pellet in **50 µl** of **DNA-eluent**. Incubate for 10 min at 65°C and vortex periodically.
17. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. The amplification can be performed on the same day of extraction.

PROTOCOL (Reaction volume 25 µl):

1. Prepare **Reaction mix**. For N reactions, add to a new tube **10*(N+1) µl** of **PCR-mix-1-FRT (Mycoplasma pneumoniae/Chlamydomphila pneumoniae)**, **5,0*(N+1) µl** of **PCR-mix-2-FRT**, **0,5*(N+1) µl** of **Polymerase (TaqF)**. Vortex briefly;
2. Add **15 µl** of **Reaction mix** into each tube.
3. Add **10 µl** of **extracted DNA** sample to appropriate tube.
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:

Step	Rotor-type Instruments ¹			Plate- or modular type Instruments ²		
	Temperature, °C	Time	Repeats	Temperature, °C	Time	Repeats
1	95	15 min	1	95	15 min	1
2	95	10 s	10	95	10 s	10
	60	20 s		60	25 s	
	72	10 s		72	25 s	
3	95	10 s	35	95	10 s	35
	60	20 s <i>fluorescent signal detection</i>		60	25 s <i>fluorescent signal detection</i>	
	72	10 s		72	25 s	

¹ For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)

² For example, SaCycler-96™ (Sacace), CFX96™ / iQ iCycler/iQ5™ (BioRad).

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, *Chlamydomphila 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 FI to 10 FI	0.05	20 %	Off
JOE/Yellow	from 5 FI to 10 FI	0.1	10 %	On
ROX/Orange	from 5 FI to 10 FI	0.1	5%	Off

Plate-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 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

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.

Boundary values

Samples	Rotor Type Instrument			Plate Type Instrument		
	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 the primers and probes was validated with negative samples. They did not generate any signal with the specific *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* primers and probes. The specificity of the kit **Mycoplasma pneumoniae / Chlamydomphila pneumoniae Real-TM** was 100%. The potential cross-reactivity of the kit **Mycoplasma pneumoniae / Chlamydomphila pneumoniae Real-TM** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **Mycoplasma pneumoniae / Chlamydomphila pneumoniae Real-TM** allows to detect *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* DNA in 100% of the tests with a sensitivity of not less than 500 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

Target region: *Mycoplasma*: putative lipoprotein; *Chlamydomphila*- 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 samples 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 samples 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

	List Number		Caution!
	Lot Number		Contains sufficient for <n> tests
	Store at		Version
	Manufacturer	NCA	Negative Control of Amplification
	Consult instructions for use	NCE	Negative control of Extraction
	Expiration Date	C+	Positive Control of Amplification
RUO	For Research Use Only	IC	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies

* CFX96™, jCycler™ and iQ5™ are registered trademarks of Bio-Rad Laboratories

* Rotor-Gene™ is a registered trademark of Qiagen



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