


Mycoplasma pneumoniae / Chlamydophila pneumoniae Real-TM

Handbook

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

REF B42-4-50FRT

REF TB42-4-50FRT

 **50**

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 contains an (IC) which 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.1: Real Time PCR kit (B42-4-50FRT)

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

- **PCR-mix-1**, 55 ready-to-use single-dose test tubes;
- **PCR-mix-2-Flu**, 0,77 ml;
- **Positive Control Chlamydia pneumoniae C+**, 0,1 ml;
- **Positive Control Mycoplasma pneumoniae C+**, 0,1 ml;
- **Negative Control C-**, 1,2 ml;
- **Internal Control IC** (human DNA), 0,2 ml;
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TB42-4-50FRT)

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

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

Contains reagents for 50 extractions

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

- **PCR-mix-1**, 55 ready-to-use single-dose test tubes;
- **PCR-mix-2-Flu**, 0,77 ml;
- **Positive Control Chlamydia pneumoniae C+**, 0,1 ml;
- **Positive Control Mycoplasma pneumoniae C+**, 0,1 ml;
- **Negative Control C-**, 1,2 ml;
- **Internal Control IC** (human DNA), 0,2 ml;
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- 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 / Chlamydophila pneumoniae Real-TM must be stored at 2-8°C. **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 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.

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 (EN375). 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 with **DNA-Sorb-B** 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 kit is recommended:

⇒ **DNA-Sorb-B** (Sacace, REF K-1-1/B)

Please carry out DNA extraction according to the manufacture's instruction. Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION

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 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 required quantity of **PCR-mix-1** tubes for samples and controls.
2. Add **7 µl** of **PCR-mix-2 Flu** into each tube.
3. Add **10 µl** of **extracted DNA** sample to appropriate tube.
4. Prepare for each panel 4 controls:
 - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
 - add **10 µl** of **Chlamydia pneumoniae C+** to the tube labeled *Chl. Pneum. C+*;
 - add **10 µl** of **Mycoplasma pneumoniae C+** to the tube labeled *Myc. Pneum. C+*;
 - add **10 µl** of **Internal Control** to the tube labeled *IC*.

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	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	20 s	
3	95	10 s	35	95	15 s	35
	60	30 s <i>fluorescent signal detection</i>		60	45 s <i>fluorescent signal detection</i>	
	72	10 s		72	15 s	

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

² For example, SaCycler-96™ (Sacace), 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, *Chlamydia pneumoniae* on ROX (Orange) and IC DNA on the JOE (Yellow)/HEX/Cy3 channel.

INSTRUMENT SETTINGS

Rotor-type instruments

Important: For the Rotor-Gene 6000 must be used software 1.7 Build 67 or updated version (for software information contact info@sacace.com).

Channel	Calibrate/Gain Optimisation...	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	<i>from 5 FI to 10 FI</i>	0.05	5 %	Off
ROX/Orange	<i>from 5 FI to 10 FI</i>	0.1	5%	Off
JOE/Yellow	<i>from 5 FI to 10 FI</i>	0.1	0 %	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	Pos	Neg	Valid result
NCA	Amplification	Neg	Neg	Neg	Valid result
Chl. pneum C+	Amplification	Neg	Neg	Pos (< 33)	Valid result
Myc. pneum C+	Amplification	Pos (<33)	Neg	Neg	Valid result
IC DNA	Amplification	Neg	Pos (<31)	Neg	Valid result

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* / *Chlamydophila pneumoniae* Real-TM** was 100%. The potential cross-reactivity of the kit ***Mycoplasma pneumoniae* / *Chlamydophila 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* / *Chlamydophila 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; *Chlamydophila*- ompA

TROUBLESHOOTING

1. Weak or no signal of the IC for the Negative Control of extraction.

- 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.
- The IC was not added to the sample during the pipetting of reagents.
 - ⇒ Make attention during the DNA extraction procedure.

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

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* Rotor-Gene™ is a registered trademark of Qiagen



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