



Pneumocystis jirovecii (carinii) Real-TM

Handbook

Real Time PCR Kit for the detection of
Pneumocystis jirovecii (carinii) in
biological materials

 **REF P2-50FRT**

 **50**

NAME

Pneumocystis jirovecii (carinii) Real - TM

INTRODUCTION

Pneumocystis pneumonia (PCP) or pneumocystosis is a form of pneumonia, caused by the yeast-like fungus (which had previously been erroneously classified as a protozoan) *Pneumocystis jirovecii (carinii)*. *P. jirovecii* is now one of several organisms known to cause life-threatening opportunistic infections in patients with advanced HIV infection worldwide. An accurate diagnosis requires access to modern medical care not available worldwide. Currently, the frequency of documented Pneumocystis infection is increasing in Africa, with Pneumocystis organisms found in up to 80% on infants with pneumonia who have HIV infection.

INTENDED USE

The **Pneumocystis jirovecii (carinii) Real-TM** is a Real-Time test for the qualitative detection of *Pneumocystis jirovecii (carinii)* in biological materials.

PRINCIPLE OF ASSAY

Kit **Pneumocystis jirovecii (carinii) Real – TM** is based on two major processes: DNA extracted from samples and amplification using real time amplification with fluorescent reporter dye probes specific for *Pneumocystis jirovecii (carinii)* and Internal Control (IC). Test contains an IC (β -globine gene) which serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (P2-50FRT)

Part N° 2– “**Pneumocystis jirovecii (carinii) Real-TM**”: Real Time Amplification kit

- **PCR-mix-1 Pneumocystis jirovecii (carinii)/Glob**, 0,6 ml;
- **PCR-buffer-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **Positive Control *Pneumocystis jirovecii (carinii)* and Human DNA C+**, 0,1 ml;
- **Negative Control C-**, 0,5 ml;*
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

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

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit
- 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)
- Disposable gloves, powderless
- Biohazard waste container

Zone 2: RT and amplification:

- Desktop microcentrifuge for “eppendorf” type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Reaction tubes or plate
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Tube racks

STORAGE INSTRUCTIONS

Pneumocystis jirovecii (carinii) Real-TM must be stored – 20°C. The kit can be shipped at 2-8°C for 3-4 days but should be immediately stored at -20°C on receipt.

STABILITY

Pneumocystis jirovecii (carinii) 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.

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

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- 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.

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.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Pneumocystis jirovecii (carinii) Real-TM can analyze DNA extracted from:

- *Sputum*: must be treated with the following procedure:
 - Collect sputum into 50 mL single-use PP tubes with a screw cap.
 - In a biological safety cabinet, homogenize samples after mixing with equal volume of 4% NaOH solution. (*N-acetyl-L-cysteine may be added if required in the amount of 50-70 mg per sample*). Mix intensely with a tube rotator for 5-20 minutes (depending on the density of a sample).
 - Centrifuge samples at 3000 rpm (2800-3000 g) for 15 min and carefully discard the supernatant leaving 500-1000 µl in the tube. Resuspend the sediment and transfer it into a 1.5 ml tube.
 - Centrifuge samples at 12000 rpm for 5-10 min, discard the supernatant and resuspend the sediment with 100 µl of PBS (or saline water) in the same 1,5 ml sample tube for DNA isolation.
- *bronchial or tracheal lavage*: centrifuge 10 ml of sample at 7000 rpm for 10-15 min. Remove and discard the supernatant. If the pellet isn't visible add further 10 ml of sample and repeat centrifugation. Discard the supernatant and resuspend the pellet with 100 µl of PBS (or saline water).
- *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 (1 volume of tissue to 1 volumes of saline solution). Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;
- *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.

It is recommended to process samples immediately after collection. Store samples at 2–8 °C for no longer than 24 hours, or freeze at –20/80°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:

⇒ **DNA/RNA Prep** (Sacace, REF K-2-9)

Please carry out DNA extraction according to the manufacture’s instruction.

PROTOCOL (Reaction volume 25 µl):

1. Prepare Mix of PCR-buffer-FRT and TaqF Polymerase by adding **30 µl** of **TaqF Polymerase** in the tube with **PCR-buffer-FRT**. This mix is stable for 3 months at + 2-8°C. If you don’t need to use the all volume in 3 months, it is possible to prepare a lower quantity of Mix of PCR-buffer-FRT and TaqF Polymerase at the rate of 10 to 1, (for example **150 µl** of **PCR-buffer-FRT** and **15 µl** of **TaqF Polymerase**).
2. Prepare required quantity of reaction tubes for samples and controls and add for each tube **10 µl** of **PCR-mix-1** and **5 µl** of **Mix** (PCR-buffer-FRT with TaqF Polymerase).
3. Add **10 µl** of **extracted DNA** sample to appropriate tube.
(Re-centrifuge all the tubes with extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don’t disturb the pellet, sorbent inhibit reaction!).
4. Prepare for each panel 2 controls:
 - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control (NCA);
 - add **10 µl** of **Positive Control *Pneumocystis jirovecii* (carinii)** and **Human DNA C+** to the tube labeled. *C+*;

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

β-globine gene (IC) is detected on the FAM (Green) channel, *Pneumocystis jirovecii* (carinii) on the JOE (Yellow)/HEX/Cy3 channel.

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	5 s	5	95	5 s	5
	60	20 s		60	20 s	
	72	15 s		72	15 s	
3	95	5 s	40	95	5 s	40
	60	20 s <i>fluorescent signal detection</i>		60	30 s <i>fluorescent signal detection</i>	
	72	15 s		72	15 s	

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

² For example, SaCycler-96™ (Sacace), CFX96™/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied), SmartCycler® (Cepheid), LineGeneK® (Bioer)

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 3000/6000, RotorGene Q)

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	0.03	10 %	On
JOE/Yellow	0.03	10 %	On

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.

DATA ANALYSIS

***Pneumocystis carinii* DNA** amplification product is detected in the **JOE/Yellow/HEX** channel, Internal Control - β -globine gene amplification product is detected in the **FAM/Green** channel.

The results are interpreted by the software of the PCR instrument by the crossing (or not crossing) of the fluorescence curve with the threshold line.

The analysis results are considered valid, only if the control samples results comply with the following:

Results for controls

Control	Stage for control	Ct in channel		Interpretation
		FAM/Green	JOE/Yellow/HEX	
NCE	DNA extraction	Neg	Neg	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	≤ 35	≤ 33	OK

1. The sample is considered **positive** if Ct values detected in the FAM/Green and JOE/Yellow/HEX channel are less than the boundary Ct values (≤ 38) for these channels. The fluorescence curve should have a typical sigmoid shape and cross the threshold line in the region of significant fluorescence increase only once.
2. The sample is considered **negative** if its fluorescence curve does not cross the threshold line (Ct value is absent) and does not have the typical shape.

The results of analysis are 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.

QUALITY CONTROL PROCEDURE

An Internal Control (IC) is detected in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity of **Pneumocystis carinii Real-TM** PCR kit is 200 *Pneumocystis carinii* DNA copies/ml.



*The claimed analytical features of **Pneumocystis carinii Real-TM** PCR kit are guaranteed only when additional reagent kit (DNA/RNA-prep) is used.*











Specificity

The analytical specificity of **Pneumocystis carinii Real-TM** PCR kit is ensured by 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 **Pneumocystis carinii Real-TM** PCR kit was confirmed in laboratory clinical tests.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam/Green): retesting of the sample is required.
 - The PCR was inhibited.
 - ⇒ Make sure that you use a recommended DNA extraction method and follow the manufacturer's instructions.
 - ⇒ Re-centrifuge all the tubes before pipetting the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant.
 - 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 for the IC detection select the fluorescence channel reported in the protocol.
 - Improper DNA extraction.
 - ⇒ Repeat analysis starting from the DNA extraction stage
 - ⇒ Make attention during the DNA extraction procedure.
2. Weak (Ct > 38) signal on the Joe (Yellow)/Cy3/HEX channel: the result is considered equivocal. It is necessary to repeat the analysis twice. If a positive Ct value is detected twice, the sample is considered as positive.
3. Joe (Yellow)/Cy3/HEX 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 among tubes.
 - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
 - Contamination during PCR procedure. All samples results are invalid.
 - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
 - ⇒ Pipette the Positive controls at the end.
 - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

	List Number		Caution!
	Lot Number		Contains sufficient for <n> tests
	For <i>in Vitro</i> Diagnostic Use		Version
	Store at	NCA	Negative Control of Amplification
	Manufacturer	C-	Negative control of Extraction
	Consult instructions for use	C+	Positive Control of Amplification
	Expiration Date	IC	Internal Control

- * SaCycler™ is a registered trademark of Sacace Biotechnologies
- * CFX96™, iQ5™ are registered trademarks of Bio-Rad Laboratories
- * Rotor-Gene™ Technology is a registered trademark of Qiagen
- * MX3005P® is a registered trademark of Agilent Technologies
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