Pneumocystis jirovecii PCR Kit

Multiplex real-time PCR assay for the direct, qualitative, and quantitative detection of *Pneumocystis jirovecii* from human bronchoalveolar lavage fluid.

For Research Use Only. Not for Use in Diagnostic Procedures.

Catalog Number: 86-PMJHU-100
Size: 100 determinations
Version: 2019-07-22 - ALPCO 1.0

INTENDED USE

The *Pneumocystis jirovecii* PCR Kit is a multiplex real-time PCR assay for the direct qualitative and quantitative detection of *P. jirovecii* from human bronchoalveolar lavage fluid. For Research Use Only. Not for use in diagnostic procedures.

PRINCIPLE OF THE TEST

The *P. jirovecii* multiplex real-time PCR assay is for the direct, qualitative, and quantitative detection of *P. jirovecii* from human bronchoalveolar lavage fluid (BAL). After DNA isolation, amplification of the gene fragment (if present) specific for *P. jirovecii* (mt LSU; large subunit) occurs. The amplified targets are detected with hydrolysis probes, which are labeled at one end with a quencher and at the other end with a fluorescent reporter dye (fluorophore). In the presence of a target the probes hybridize to the amplicons. During the extension step, the Taq-polymerase breaks the reporter-quencher proximity. The reporter emits a fluorescent signal, which is detected by the optical unit of a real-time PCR instrument. The fluorescence signal increases with the number of formed amplicons. With the standards, Standard A, Standards B and Standards C, included in the kit, it is possible to quantify the results. The *P. jirovecii* multiplex real-time PCR kit contains an Internal Control DNA (ICD) that detects PCR inhibition, monitors reagent integrity, and confirms that nucleic acid extraction was sufficient.

WARNINGS and PRECAUTIONS

- Good laboratory practices must be used and the instructions for carrying out the test must be strictly followed.
- Do not mix reagents from kits with different lot numbers.
- Do not pipet samples or reagents by mouth. Avoid contact with bruised skin or mucosal membranes.
- During handling reagents or samples, wear appropriate safety clothing (appropriate gloves, lab coat, safety goggles) and wash your hands after finishing the test procedure.
- Do not smoke, eat, or drink in areas where samples or reagents are being used.
- Extraction, PCR preparation and the PCR run should be separated in different rooms to avoid cross-contaminations.
- Samples must be treated as potentially infectious as well as all reagents and materials being exposed to the samples and must be handled according to appropriate safety regulations.
- Do not use the kit after the expiration date.
- All reagents and materials used must be disposed properly after use. Please refer to the relevant regulations for disposal.

REAGENTS PROVIDED

Reagents provided in the kit are sufficient for 100 determinations.

Table 1. Reagents Provided

Kit Code	Reagent	Amount		Lid Color
1	Reaction Mix	2x	1050 uL	yellow
2	Taq-Polymerase	1x	80 uL	red
D	Internal Control DNA	2x	1700 uL	orange
N	No Template Control	1x	450 uL	white
P	Positive Control	1x	200 uL	blue
10^1	Standard A	1x	100 uL	dark blue
10^3	Standard B	1x	100 uL	dark blue
10^5	Standard C	1x	100 uL	dark blue

STORAGE INSTRUCTIONS

- Protect all reagents from light and store at -20°C. All reagents can be used until the expiration date. After expiry the quality guarantee is no longer valid.
- Carefully thaw reagents before use (e.g., in a refrigerator at 2 - 8°C).
- Reagents can be used up to 20 freeze/thaw cycles without influencing the assay performance. After the initial thaw, separate into aliquots and freeze immediately.
- During PCR preparation, all reagents should be stored cold (2 - 8°C).

MATERIALS REQUIRED BUT NOT PROVIDED

- Extraction platform if extraction not performed manually
- Real-time PCR instrument
- Real-time PCR, nuclease-free consumables (plates, tubes, foil)
- Centrifuge with a rotor for the reaction vials
- Vortex mixer
- Pipettes (0.5 - 20 uL, 20 - 200 uL, 100 - 1000 uL)
- Filter tips
- Powder-free disposal gloves
- PCR Grade water (bioscience grade, nuclease-free)
- Color Compensation Kit IV for use with the LightCycler® 480II

The *P. jirovecii* PCR assay has been tested using the following extraction platforms and PCR instruments. If using another extraction platform or PCR instrument, refer to the settings for the tested instruments as a starting point and modify as necessary.

Table 2. Extraction Platforms and PCR Instruments

Extraction platform	
ALPCO	DNA/RNA Extraction Kit
Promega	Maxwell® RSC
Real-time PCR instrument	
Roche	LightCycler® 480II
Agilent Technologies	Mx3005P
Applied Biosystems	ABI 7500
Bio-Rad	CFX96™
QIAGEN	Rotor-Gene Q

Note: Only use 0.1 mL tubes on the Rotor Gene Q (QIAGEN)

If using other extraction platforms or real-time PCR instruments, please contact ALPCO at techsupport@alpco.com

PREPARATION OF SAMPLES

For DNA isolation from bronchoalveolar lavage (BAL), use a commercially available DNA isolation kit (DNA Extraction Kit (ALPCO)) or DNA extraction system (e.g. Maxwell® RSC (Promega)). Extract DNA according to the manufacturer's instructions.

The *P. jirovecii* PCR assay contains an Internal Control DNA that detects PCR inhibition, monitors reagent integrity, and confirms that nucleic acid extraction was sufficient. The Internal Control DNA can either be used as PCR inhibition control or as an extraction control for the sample preparation procedure and a PCR inhibition control.

If the Internal Control DNA is used only as a PCR inhibition control, 1 uL of the Internal Control DNA should be added to the Master Mix (see Table 4). If the Internal Control DNA is used as an extraction control for the sample preparation procedure and as a PCR inhibition control, 20 uL of the Internal Control DNA must be added during extraction procedure. The Internal Control DNA should always be added to the sample-lysis buffer mixture and must not be added directly to the sample. It is also recommended to add 1 uL of the Internal Control DNA to the negative control and positive control PCR Mix.

MASTER MIX PREPARATION

Calculate the total number of PCR reactions (sample and control reactions) needed. One positive control and one negative control must be included in each assay run. It is recommended adding a 10% volume overage to compensate for imprecision in pipetting (see Tables 3 and 4). Thaw,

mix gently, and centrifuge briefly the Reaction Mix, the Taq-Polymerase, the Positive Control, the No Template Control, Internal Control DNA, and Standards A - C, before using. Keep reagents appropriately cold during working step (2 - 8°C).

Table 3. Calculation and pipetting example for 10 reactions of the Master Mix (ICD as extraction and PCR inhibition control)

Kit code	Master Mix Components	Volume per Reaction	10 reactions (+10% overage)
1	Reaction Mix	19.3 uL	212.3 uL
2	Taq-Polymerase	0.7 uL	7.7 uL
	Total Volume	20 uL	220 uL

Table 4. Calculation and pipetting example for 10 reactions of the Master Mix (ICD only as PCR inhibition control)

Kit code	Master Mix Components	Volume per Reaction	10 reactions (+10% overage)
1	Reaction Mix	19.3 uL	212.3 uL
2	Taq-Polymerase	0.7 uL	7.7 uL
D	Internal Control DNA	1.0 uL	11 uL
	Total Volume	21 uL	231 uL

Mix the components of the Master Mix gently and briefly spin down.

PREPARATION OF THE PCR MIX

1. Pipette 20 uL of the Master Mix into all reaction well (tubes or plate wells).

2. Negative Control: Add 5 uL of the No Template Control to the pre-pipetted Master-Mix.

Note: If the Internal Control DNA is used as an extraction control for the sample preparation procedure and as PCR inhibition control, add 1 uL of the Internal Control DNA to the PCR-Mix of the negative control.

3. Sample: Add 5 uL of DNA-Extract to the pre-pipetted Master-Mix.

4. Positive Control: Add 5 uL of Positive Control to the pre-pipetted Master-Mix.

Note: If the Internal Control DNA is used as an extraction control for the sample preparation procedure and as PCR inhibition control, add 1 uL of the Internal Control DNA to the PCR-Mix of the positive control.

5. Standards: Add 5 uL Standards (A-C) to the pre-pipetted Master-Mix.

Note: If the Internal Control DNA is used as an extraction control for the sample preparation procedure and as PCR inhibition control, add 1 uL of the Internal Control DNA to the PCR-Mix of the standards.

Note: Using the following cyclers requires to include a standard curve in each run: ABI 7500 (Applied Biosystems) and CFX96™ (Bio-Rad).
For all other cyclers, only one sample of the standard curve (Standard B) must be included in the experimental set-up as calibrator for each new real-time PCR run. Here, the application of a standard curve is only required to run once per lot number.

Cover tubes or plate. Spin down and place in the real-time PCR instrument. The PCR reaction should be started according to the PCR instrument set-up (see Tab. 5, Tab. 6, Tab. 7, Tab. 8).

PCR INSTRUMENT SET-UP

DNA real-time PCR Profile:

Table 5. DNA real-time PCR profile for LightCycler® series and Rotor-Gene Q

Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	10 sec, 95 °C 15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

Table 6. DNA real-time PCR profile for Mx3005P, ABI7500, CFX96™

Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	10 sec, 95 °C 15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.
Note: The total copy number per reaction of Standard A, Standard B, and Standard C has to be typed in into the setup file of the software program of the respective real-time PCR cycler. A total volume of 5 uL DNA is used resulting in following concentrations:
Standard A: 5×10^1 copies/reaction
Standard B: 5×10^3 copies/reaction
Standard C: 5×10^5 copies/reaction
Note: The standard curve can be saved on the real-time PCR cycler for each parameter. Apart from the ABI 7500 (Applied Biosystems) and the CFX96™ (Bio-Rad) cycler, the standard curve is only required to run once per lot number. Using the ABI 7500 (Applied Biosystems) and the CFX96™ (Bio-Rad) cycler requires to include a standard curve in each run. For all other cyclers, only one sample of the standard curve (Standard B) must be included in the experimental set-up

as calibrator for each new real-time PCR run. The general recommendation is, however, to test always all three standards with every run.

Universal real-time PCR profile:

Note: The universal real-time PCR profile should only be used for DNA assays when combining DNA and RNA real-time PCR assays in one run.

Table 7: Universal real-time PCR profile for LightCycler® series

Reverse Transcription	10 min, 58 °C
Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annelaing/Extension	10 sec, 95 °C 15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

Table 8: Universal real-time PCR profile for Mx3005P, ABI7500, CFX96™ and Rotor-Gene Q

Reverse Transcription	10 min, 58 °C
Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annelaing/Extension	15 sec, 95 °C 30 sec, 95 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.
Note: The total copy number per reaction of Standard A, Standard B, and Standard C must be typed in into the setup file of the software program of the respective real-time PCR cycler. A total volume of 5 uL DNA is used resulting in following concentrations:
Standard A: 5×10^1 copies/reaction
Standard B: 5×10^3 copies/reaction
Standard C: 5×10^5 copies/reaction
Note: The standard curve can be saved on the real-time PCR cycler for each parameter. Apart from the ABI 7500 (Applied Biosystems) and the CFX96™ (Bio-Rad) cycler, the standard curve is only required to run once per lot number. Using the ABI 7500 (Applied Biosystems) and the CFX96™ (Bio-Rad) cycler requires to include a standard curve in each run. For all other cyclers, only one sample of the standard curve (Standard B) must be included in the experimental set-up

as calibrator for each new real-time PCR run. The general recommendation is, however, to test always all three standards with every run.

DETECTION CHANNEL SET-UP

Table 9. Selection of Appropriate Detection Channels

Real-time PCR Instrument	Detection	Detection Channel	Note
Roche LightCycler® 480II	Pneumocystis jirovecii	465/510	Color Compensation Kit IV is required
	ICD	533/580	
Agilent Techn. Mx3005P	Pneumocystis jirovecii	FAM	Check that reference dye is none
	ICD	HEX	
ABI 7500	Pneumocystis jirovecii	FAM	Check that passive reference option ROX is none
	ICD	VIC	
Bio-Rad CFX96™	Pneumocystis jirovecii	FAM	-
	ICD	VIC	
Qiagen Rotor-Gene Q	Pneumocystis jirovecii	Green	The gain settings must be set to 5, according to the default settings
	ICD	Yellow	

QUALITY CONTROL

The analysis of the samples is done by the software of the real-time PCR instrument used, according to the manufacturer's instructions. Negative control and positive control must show correct results (see Table 7, Fig. 1, Fig. 2, Fig. 3) to determine a valid run. The Positive Control has a concentration of 10^3 copies/uL. In each PCR run it is used in a total amount of 5 x 10^3 copies.

Table 10. For a valid run, the following conditions must be met:

Sample	Assay result	ICD Ct	Target Ct
Positive Control	Positive	NA *[1]	See Quality Assurance Certificate
Negative Control	Negative	Ct > 20	0

*[1] *No Ct value is required for the ICD to make a positive call for the Positive Control*

If the positive control is not positive within the specified Ct range but the negative control is valid, prepare all new reactions including the controls.

If the negative control is not negative but the positive control is valid prepare all new reactions including the controls.

If the required criteria are not met, the following items should be checked before repeating the test:

- Expiry of the used reagents
- Functionality of the used instrumentation
- Correct performance of the test procedure

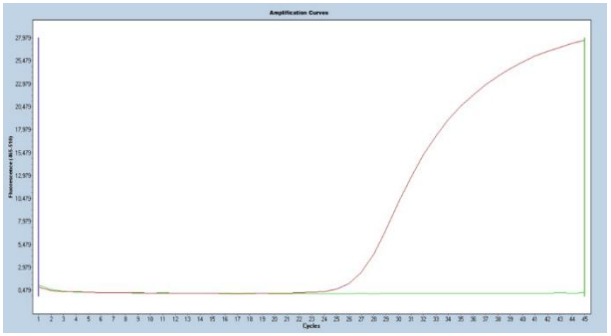

Figure 1: Correct run of the positive control (red) and negative control (green) (*P. jirovecii*) on the LightCycler® 480II

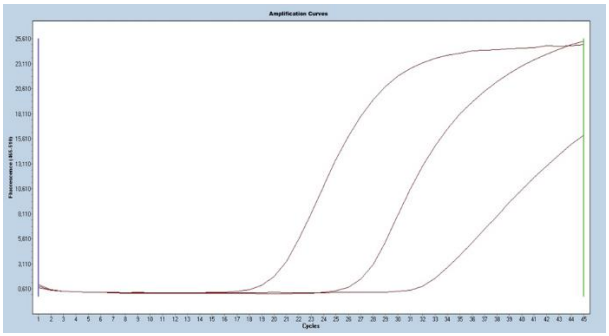

Figure 2: Standard series *P. jirovecii* with Standard A (10^1 DNA copies per µl), Standard B (10^3 DNA copies per µl) and Standard C (10^5 DNA copies per µl) on the LightCycler 480II

RESULTS INTERPRETATION

The result interpretation is done according to Table11.

Table 11: Sample interpretation

Pneumocystis jirovecii	**ICD**	**Result**
positive	positive / negative	*P. jirovecii* detected
negative	positive	Target genes not detected
negative	negative	invalid

A sample is evaluated positive if the sample DNA and the Internal Control DNA show an amplification signal in the detection system. A sample is also evaluated positive if the sample DNA shows an amplification signal but none for the Internal Control DNA in the detection system. The detection of the Internal Control DNA is not necessary because high concentrations of the amplicon can cause a weak or absent signal of the Internal Control DNA.

A sample is evaluated negative if the sample DNA shows no amplification signal, but an amplification signal for the Internal Control DNA in the detection system. An inhibition of the PCR reaction can be excluded by the detection of the Internal Control DNA.

A sample is evaluated invalid if the sample DNA and Internal Control DNA show no amplification signal in the detection system. The sample contains a PCR inhibitor. The extracted sample needs to be further diluted with PCR water (1:10) and reamplified, or the isolation and purification of the sample must be improved.

VALIDITY OF QUANTITATIVE DETECTION

For the validity of a quantitative diagnostic test run, all control conditions of a valid qualitative diagnostic test run must be met. Furthermore, for accurate quantification results a valid standard curve must be generated. For a valid quantitative diagnostic test run, the following control parameter values of the standard curve should be achieved.

	Control parameter	Valid value
Roche LightCycler® 480II	Efficiency	1.9 – 2.1
	Slope	-3.1 – -3.6
Agilent Techn. Mx3005P	Rsq	> 0.98
	Slope	-3.1 – -3.6
ABI 7500	R^2	> 0.98
	Slope	-3.1 – -3.6
Bio-Rad CFX96™	R^2	> 0.98
	Slope	-3.1 – -3.6
Qiagen Rotor-Gene Q	R^2	> 0.98
	M	-3.1 – -3.6

QUANTIFICATION OF SAMPLES

To quantify *P. jirovecii* positive samples, a standard curve with the Standards A-C must be performed separately. The standard curve measurement must be saved separately. However, the same standard curve measurement can be used in all runs with products from the same lot number by importing the saved experiment.

Note: This is not valid for the following cyclers: ABI 7500 (Applied Biosystems) and CFX96™ (Bio-Rad). Here, a standard curve hast to be measured with each run.

For all other cyclers, one sample of the standard curve (Standard B) has to be included in the experimental set-up as calibrator for each new real-time PCR run.

To quantify *P. jirovecii* positive samples, all standard samples (A - C), the positive control and the negative control, as well as the unknown sample, must be selected and analyzed according to the instructions of the cycler manufacturer. Correct quantification results are only reliable if Ct-values of the *P. jirovecii* specific target gene (mt LSU; large subunit) can be detected within the standard Ct-range.

With the quantitative *P. jirovecii* multiplex real-time PCR the amount of DNA in copies/reaction of the parameter is calculated. The conversion in copies/ml is done with a correction factor K and considers the dilutions of the extraction procedure (dependent on the extraction kit used) and the PCR set-up.

The conversion of the result of the quantitative *P. jirovecii* multiplex real-time PCR in copies/ml is calculated with following formula:

C [copies/ml] = c [copies/reaction] x K
C [copies/ml] - concentration of sample in copies/ml sample
c [copies/reaction] - DNA concentration in PCR reaction (result of quantitative PCR)
K - correction factor
For the calculation of the correction factor, following information must be considered:
- Sample dilution
- Starting volume of sample for DNA extraction
- DNA extract from total eluate used for PCR reaction

Table 12: Example of calculation of correction factor K using the Maxwell® RSC (Promega)

Description	Factor
300 uL sample put into extraction*, eluted in 60 uL final volume	No factor
5 uL DNA extract put into PCR (total eluate 60 uL =1/12)	X 12
300 uL sample scaled up to 1 ml*	X 3. $\overline{3}$
Correction factor K for *P. jirovecii*	40

** Result is based on 1 ml BAL starting material*

LIMITATIONS OF THE METHOD

1. This assay is only validated for human bronchoalveolar lavage fluid (BAL).
2. Inappropriate sample collection, transport, storage and processing or a pathogen load in the specimen below the analytical sensitivity can result in false negative results.
3. The presence of PCR inhibitors may cause invalid results.
4. Mutations or polymorphisms in primer or probe binding regions may affect detection of new variants resulting in a false negative result with the *P. jirovecii* assay.
5. As with all PCR based *in vitro* diagnostic tests, extremely low levels of target below the limit of detection (LoD) may be detected, but results may not be reproducible.
6. A positive test result does not necessarily indicate the presence of viable organisms. However, a positive result is indicative for the presence of target genes ((mt LSU; large subunit).

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity

The *P. jirovecii* PCR assay has a detection limit of ≥ 10 RNA copies per reaction.

The following figures 3 show a dilution series of *P. jirovecii* (10^5 - 10^1 DNA copies per uL) on the LightCycler® 480II.

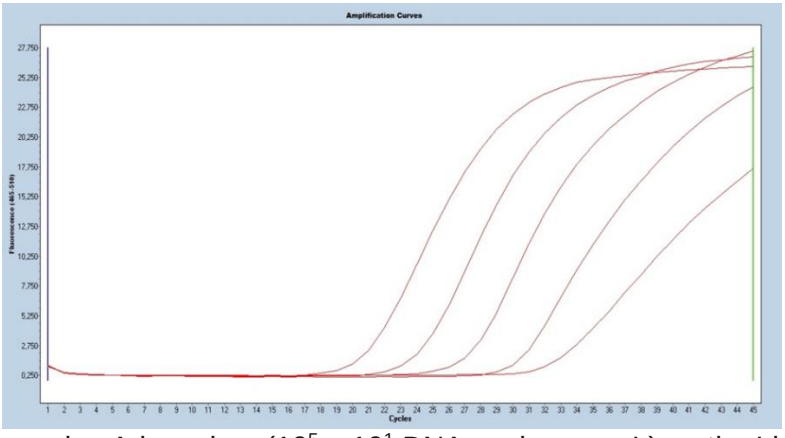

Figure 3. Dilution series Adenovirus (10^5 – 10^1 DNA copies per uL) on the LightCycler® 480II.

The detection limit of the whole procedure depends on the sample matrix, DNA extraction and DNA concentration.

Analytical Specificity

The analytical specificity of the *P. jirovecii* multiplex real-time PCR is specific for *Pneumocystis jirovecii*. No cross-reaction could be detected for the following species (see Tab. 9, * detected with sequence alignment):

Table 11: Cross-reactivity Testing

Acinetobacter baumannii	-	Corynebacterium diphtheriae	-	Klebsiella pneumoniae	-	Rhizomucor pusillus
Alternaria alternata	-	Coxsackie B4, human	-	Lactobacillus plantarum	-	Saccharomyces cerevisiae
Adenovirus 1, human, strain Adenoid 71	-	Cytomegalovirus, human	-	Legionella pneumophila subsp. pneumophila	-	Scedosporium apiospermum
Adenovirus 7, human, strain Gomen	-	Doratomyces microsporus	-	Moraxella catarrhalis	-	Scedosporium proloficans
Aspergillus fumigatus*	-	Epstein-Barr-Virus, strain B95-8	-	Mycoplasma pneumoniae	-	Sporothrix schenckii
Aspergillus terreus	-	Fusarium solani	-	Neisseria meningitidis	-	Staphylococcus aureus
Bordetella parapertussis	-	Haemophilus influenzae Rd	-	Parainfluenza virus 1, human strain C35	-	Staphylococcus epidermidis
Bordetella pertussis	-	Helicobacter felis	-	Parainfluenza virus 2, human, strain Greer	-	Staphylococcus haemolyticus
Candida glabrata	-	Herpes simplex virus 1, strain McIntyre	-	Parainfluenza virus serotype 3	-	Staphylococcus hominis subsp. novobiosepticus R22
Candida parapsilosis	-	Herpes simplex virus 2, strain MS	-	Parainfluenza virus 4b, human, strain CH19503	-	Streptococcus pneumoniae
Candida tropicalis	-	Human Metapneumovirus	-	Respiratory syncitial virus, human, strain 932(	-	Streptococcus pyogenes
Cladosporium spp	-	Influenza virus, infectious A/PR/8/34	-	Respiratory syncitial virus, human, strain Long		Streptococcus salivarius
Coronavirus 229E, human		Klebsiella oxytoca		Rhinovirus, genogroup A, human		Varicella Zoster Virus (Type B)

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