*Pneum*ID

REF #: OLM2008

PCR kit for the detection of *Pneumocystis jirovecii* – 50 tests

INTRODUCTION

Real-time quantitative PCR (qPCR) has become the most widely used molecular technology for diagnostic applications designed to detect and quantify pathogens. *Pneumocystis jirovecii* is a common cause of life-threatening pneumonia in individuals with impaired immune systems.

This organism cannot be cultured in vitro and consequently laboratory detection relies heavily on microscopic identification directly from patient specimens using fluorescent stains or antibodies.

The lack of sensitivity of this method has led to *Pneumocystis*-specific real-time quantitative PCR (qPCR) being proposed as an additional tool to aid conventional diagnostic procedures for *Pneumocystis* pneumonia, by providing sensitive and specific detection of *P. jirovecii* DNA in clinical specimens.

*Pneum*ID is a multiplex qPCR test designed to detect genomic DNA of *P. jirovecii* from human respiratory samples, with simultaneous detection of both *Pneumocystis* and a human DNA target from a respiratory sample extract.

*Pneum*ID has been designed, optimised and validated in strict compliance with the **MIQE** guidelines for qPCR assay design and reporting. This kit provides the reagents for amplification of *P. jirovecii* DNA using qPCR in conjunction with hydrolysis probe detection chemistry.

KIT CONTENTS

- ***Pneum*ID Primer/Probe Mix (50 reactions)**
  FAM, HEX and ROX labelled (see table below)

Target	Fluorophore	Absorption (nm)	Emission (nm)
P. jirovecii	FAM	494	518
Human β globin gene (HBG)	HEX	535	556
Internal extraction control	ROX	575	602

Table 1. Primer/probe mix contents and characteristics of fluorescent dyes

- ***Pneum*ID positive control (*P. jirovecii* and HBG)**
- **Internal extraction control DNA (IEC)**
- **qPCR Master Mix**
- **RNase/DNase-free water**

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit
- 1.5 mL microcentrifuge tubes
- Pipettes and tips
- qPCR instrument
- Vortex and centrifuge
- qPCR plate and plate seals

KIT STORAGE

IMMY's *Pneum*ID kit should be stored at -20°C, where it is stable for 18 months from date of manufacture. Components should be kept at 0°C, not be exposed to temperatures above -20°C for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. Please note that exposure to light leads to photo bleaching of the fluorescent reporters and so reduces the sensitivity of the assay. IMMY does not recommend using the kit after the expiry date stated on the pack.

SUITABLE SAMPLE MATERIAL

DNA should be extracted from culture or suitable clinical specimen. Good laboratory practice recommends including at least one positive and negative extraction control per analysis. Extracted samples should be stored between -80°C and -20°C for long term storage. Please be aware that low concentrations of DNA can be unstable if stored for long periods. Repeated freeze/thaw cycles should be avoided. IMMY's *Pneum*ID kit is validated for use with respiratory samples, prepared by a nucleic acid extraction method compatible with PCR amplification. Please ensure that the samples are appropriate in terms of purity, concentration, and DNA integrity. Always run at least one negative (no template) control with the samples. To prepare a negative-control, replace the template DNA sample with RNase/DNase-free water.

DYNAMIC RANGE OF TEST

Under optimal PCR conditions the primers in IMMY's *Pneum*ID kits result in amplification efficiencies of >90%. The test has a broad dynamic range of at least six orders of magnitude and is sensitive to <1 *Pneumocystis* genome copy.

RISK ANALYSIS

There are no hazardous substances included in the manufacture of the *Pneum*ID qPCR kit. The kit reagents present no specific risk to the user. Additional chemicals and materials may be required for the procedures described in this handbook. IMMY advises the careful reading of all warnings, instructions and material safety data sheets provided by the supplier. General safety regulations must be followed when handling chemicals, biohazards and other materials

GENERAL PRECAUTIONS

- The *Pneum*ID qPCR kit is for *in vitro* diagnostic use.
- The test procedure should be performed as outlined in the Instructions for Use provided. Any deviation from the outlined protocols may result in assay failure or the generation of erroneous results.
- Standard precautions and local laboratory/institution guidelines should be followed when handling samples.
- Low concentrations of DNA can be unstable if stored for long periods. IMMY advises that sample storage time should be minimised before testing.
- Do not mix reagents from different kits.
- Do not substitute reagents from different manufacturers.
- Avoid reagent contamination by following good laboratory practice and segregating workflow as appropriate.
- Ensure all additional required consumables are RNase/DNase-free.
- Wear protective clothing and disposable gloves when performing the test.
- Use disposable filter tips for all pipetting.
- Thaw DNA samples on ice and keep on ice.
- Always ensure *Pneum*ID reagents are defrosted thoroughly, mixed, spun down and kept on ice.
- Do not use reagents after their expiry date.

ASSAY LIMITATIONS

- Positive results do not rule out co-infection with other organisms.
- Extraction efficiency can have a negative impact on *Pneum*ID test results. IMMY advises that the internal extraction control provided is used in all sample extractions to monitor for this.

PRINCIPLES OF THE TEST

REAL TIME PCR

Individual primer and probe designs for detection of *P. jirovecii*, human β globin gene and a synthetic internal extraction control template (introduced at the sample extraction stage) have been combined into a single assay and their DNA can be detected through the different fluorescent channels as described in the kit contents.

IMMY's *Pneum*ID makes use of the most widely used qPCR chemistry, which is based on the detection of light emitted by hydrolysis probes (Figure 1).

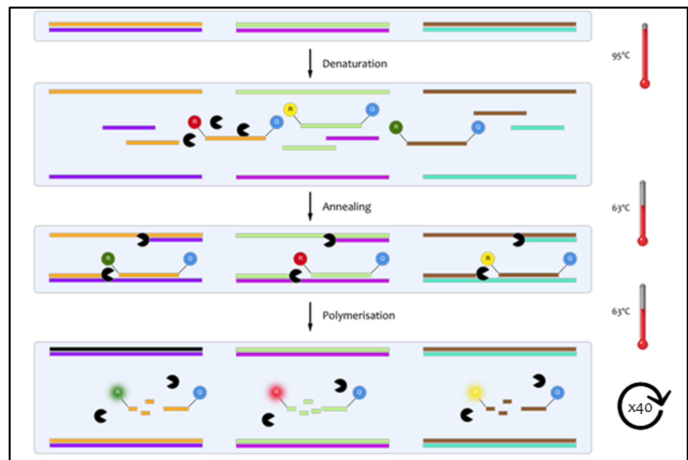

Figure 1. Principle of hydrolysis probe-based test. Double stranded genomic DNA, represented by the coloured strands, is denatured as the sample is briefly heated to 95°C. During the annealing phase of the PCR the temperature is reduced and forward and reverse primers (short coloured lines) hybridise to any complementary target DNA present. The same reaction mixture also contains fluorogenic probes, which consist of target-specific DNA oligonucleotides labelled with a 5`-fluorescent reporter dye (R) and a quencher (Q). In the case of IMMY's *Pneum*ID kit, these probes are specific for *P. jirovecii* (FAM), HBG (HEX) and the internal extraction control (ROX). During the polymerisation phase of the PCR, any bound probes are displaced and cleaved by the 5' nuclease activity of the *Taq* polymerase (black circles), resulting in the physical separation of the reporters and quenchers. This results in the emission of light at fluorophore-specific wavelengths (see Table 1), which can be detected in the appropriate channels of a qPCR instrument.

POSITIVE CONTROL

The kit contains a positive control tube with templates for each of the two assay targets (*P. jirovecii* and HBG). The positive control is handled like a normal nucleic acid extract and indicates that the primers and probes for detecting both targets are working properly in the run. Inclusion of the positive control depends on the preference of the end user and is not mandatory in every run. However, IMMY recommends its use in every run as it provides more confidence in the results if all samples are negative. The positive control does not need to be subjected to a nucleic acid extraction procedure. Care should be taken to avoid cross-contamination of other samples when adding the positive control to the run. Any risk can be minimised by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

NO TEMPLATE CONTROL (NTC)

To confirm the absence of contamination, at least one no template control (NTC) reaction must be included in every PCR run. For this reaction, RNase/DNase-free water should be used instead of template.

INTERNAL EXTRACTION CONTROL (IEC)

The internal extraction control (IEC) is added to distinguish true negative samples from false negative samples, which can result from nucleic acid degradation, failure of nucleic acid extraction step, PCR inhibition or qPCR instrument malfunction. The primers and probe necessary to detect the IEC are included in the multiplex primer and probe mix. The IEC template is added either to the DNA lysis/extraction buffer or to the sample once it has been resuspended in lysis buffer. The IEC will give a quantification cycle (Cq) value of >25, with the variation depending on the efficiency of sample extraction and level of sample dilution.

ENDOGENOUS INTERNAL CONTROL FOR SAMPLE QUALITY

The human β-globin is co-amplified in the multiplex reaction as an endogenous internal control. Detection of the human β -globin gene is included as an internal control to allow assessment of the specimen quality and the nucleic acid extraction as well as the amplification processes. This allows the user to make some assessment of the quality of the BAL respiratory sample obtained (ie. if the extract contains no detectable human DNA it is likely to be a poor-quality sampling, leading one to question whether a 'negative Pneumocystis result' from that sample is indeed reliable).

TESTING PROTOCOL

Remove the *Pneum*ID kit from the freezer and allow the reagents to thaw. Thawed reagents must be kept on ice. Briefly vortex and centrifuge all *Pneum*ID reagent tubes prior to use.

DNA EXTRACTION

The IEC DNA can be added either to the DNA lysis/extraction buffer or to the sample once it has been resuspended in lysis buffer.
DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

1. Add 5 µL of the IMMY Internal Extraction Ctrl DNA (IEC) to each sample in DNA lysis/extraction buffer for an elution volume of 50 µL. For eluting into different volumes adjust volume of the IEC DNA accordingly.
2. Complete the DNA extraction according to the manufacturer's recommended protocols.

PCR DETECTION PROTOCOL

1. Prepare a PCR premix according to Table 2
   Volumes are given per reaction well and should be multiplied by the number of reaction wells required, include sufficient reactions for positive and negative controls. The prepared PCR premix should be thoroughly mixed and briefly spun down.

Component	Volume/Reaction
qPCR Master Mix	10 µL
Primer/Probe Mix	2 µL
RNase/DNase-Free Water	2 µL
Final Volume	14 µL

Table 2. *Pneum*ID assay premix (volumes per reaction).
Due to small variations in pipetting accuracy, we recommend that you allow an additional 10% for the final volume, i.e. if you are assaying ten samples, make sufficient reaction mix for 11 tests.

2. Pipette 14 µL of prepared PCR premix into each reaction well according to your qPCR experimental plate set up.
3. Prepare sample DNA templates for each of your samples (see DNA extraction step).
4. Pipette 6 µL of DNA template into each well according to your experimental plate set up.
5. For negative control wells use 6 µL of RNase/DNase -free water. For positive control use 6 µL of *Pneum*ID Positive Control. The final volume in each well is 20 µL.
6. Ensure that your PCR reaction plate is sealed and briefly centrifuged before transferring to a validated thermocycler for amplification.

AMPLIFICATION PROTOCOL

1. If using an instrument that uses ROX as a passive reference then the passive reference must be turned off or set to "none" for no passive reference, as the IEC uses the ROX channel.
2. Please refer to your instrument manual for instructions on setting up an amplification run. Amplification should be carried out according to the conditions detailed in Table 3.

	Step	Time	Temp
Cycling x40	Enzyme Activation	2 mins	95 °C
	Denaturation	5 secs	95 °C
	Data Collection*	20 secs	60 °C

Table 3. PCR protocol
** Fluorogenic data should be collected during this step through the FAM, HEX, and ROX channels*

INTERPRETATION OF RESULTS

Please refer to your thermocycler instruction manual for information on how to operate the qPCR instrument and perform data analysis. *Pneum*ID operates in the FAM, HEX and ROX channels. Remember to apply colour compensation if appropriate to your qPCR instrument.

It is important to visually inspect the amplification plots for each sample to ensure that the results recorded are due to true amplification and cannot be attributed to background noise recorded above the defined thresholds.

Note: Cq estimations stated below are calculated with threshold positioned at the centre of the log-linear range of the PCR amplification curve.

POSITIVE CONTROL

The supplied positive control well should record characteristic amplification plots through both the FAM (*P. jirovecii*) and HEX (HBG) channels, with Cqs of 28 ± 2. There is no internal control template within the positive control so the ROX channel should record no signal (flat amplification plots). The positive control signals indicate that the kit is working correctly for *Pneumocystis* and human β globin gene detection.

NO TEMPLATE CONTROL (NTC)

The NTC wells should give a flat line (flat amplification plots) through all channels. Signals in the NTC may indicate cross contamination during plate set up.

SAMPLE DATA (summarised in Table 4)

1. Successful sample extraction is indicated by a positive signal in the ROX channel (Cq>25), with lower Cqs indicative of more efficient extraction.
2. Absence of a signal in both FAM and HEX channels suggests poor sampling. In this case, resampling is recommended.
3. A signal in the FAM channel, with no signal in the HEX channel indicates a *Pneumocystis* positive sample. However, the absence of detectable HBG target may suggest poor quality sampling.
4. A signal in both FAM and HEX channels indicates a *Pneumocystis* positive sample.
5. It is possible to obtain a signal in the HEX and/or the FAM channel without also recording a signal in the ROX channel. Typically, this occurs when a high fungal load results in early amplification (i.e. low Cqs) and the accumulation of target DNA inhibits the amplification of the IEC DNA.
6. If there is no signal in any of the channels, the assay has failed and no conclusions are possible.

Detection Channels			
FAM	HEX	ROX	Result
+	+/-	+	*Pneumocystis* **POSITIVE** sample. **IEC PASS. Valid result.**
-	+	+	*Pneumocystis* **NEGATIVE** sample. **Valid sample. IEC PASS. Valid result.**
-	-	+	Indication of poor sampling. **IEC PASS. Resampling recommended.**
+	+/-	-	*Pneumocystis* **POSITIVE** sample. IEC outcompeted by high *Pneumocystis* load. **Valid result.**
-	-	-	Invalid

Table 4. Summary of data interpretation

INTERNATIONAL SYMBOL USAGE

Symbol	Meaning	Symbol	Meaning
	Manufactured by	LOT	Lot Number
REF	Reference Number		Expiration Date
EC REP	EU Authorized Representative	IVD	In Vitro Diagnostic
	Consult Instructions for Use		Irritant

MANUFACTURER INFORMATION

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