

MycAssay™ Pneumocystis

Cepheid SmartCycler®

Respiratory Samples

REF 080-035

Intended Use

MycAssay™ Pneumocystis is indicated for use by qualified laboratory professionals for the qualitative detection of *Pneumocystis jirovecii* genomic DNA extracted from respiratory specimens from the lower respiratory tract (e.g., bronchial samples) as an aid to diagnosis in adult patients suspected of having *P. jirovecii* pneumonia.

MycAssay™ Pneumocystis has been validated for use with the Cepheid SmartCycler® (using Dx software versions 1.7b and 3.0)

Summary and Explanation

Pneumocystis jirovecii (formerly *carinii*) pneumonia (PCP) is a common opportunistic pneumonia in immunocompromised patients, especially those with advanced HIV infection and AIDS¹. It is typically community acquired, and sub-acute in presentation, leading to progressive respiratory failure and death² if untreated. Prophylaxis with trimethoprim-sulphamethoxazole (Bactrim or Septrin) is routinely given to many at risk patients, a practice which has substantially reduced the incidence of PCP, but breakthrough occurs and those who do not know they are HIV positive may present with AIDS with PCP³. PCP also occurs in other immunocompromised patients, including recipients of solid organ transplants, hypogammaglobulinaemia and chronic leukaemia.

Currently the diagnosis of PCP relies on microscopic methods as *P. jirovecii* cannot be cultured in routine microbiology laboratories. Bronchoalveolar lavage (BAL) is the preferred means of sample collection. Common methods for diagnosis include immunofluorescence (IF) or direct fluorescence and histological staining of samples⁴.

MycAssay™ Pneumocystis is a molecular diagnostic kit for the detection of *P. jirovecii* based on Molecular Beacon⁵ PCR technology. The whole test procedure, including extraction of DNA from the clinical sample, can be completed within 4 hours, or only 2 hours if extracted DNA is already available. This assay brings the direct benefit of enhanced laboratory efficiency combined with a rapid test leading to likely clinical benefits. The diagnostic accuracy of the test depends to a great extent on sample quality.

Principles of the Procedure

Following mixing of the reagents in the MycAssay™ Pneumocystis kit with a sample containing *Pneumocystis* target DNA sequence, (a portion of the *Pneumocystis* mitochondrial ribosomal large sub-unit), thermocycling will result in DNA amplification occurring. The assay also contains an Internal Amplification Control (IAC) sequence, a DNA fragment not present in *Pneumocystis*, other fungal, bacterial or human genomes, to detect PCR inhibitory substances and confirm the functionality of the assay reagents.

The amplified DNA targets are detected with Molecular Beacons; single-stranded oligonucleotide hybridization probes that form a stem-and-loop structure. The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore, which fluoresces when excited by light of the appropriate wavelength, is covalently linked to the end of one arm and a quencher, which suppresses the fluorescence of the fluorophore when in close physical proximity, is covalently linked to the end of the other arm. Molecular Beacons do not fluoresce when they are free in solution. However, when they hybridise to a nucleic acid strand containing a target sequence they undergo a conformational change that enables them to fluoresce. The amount of fluorescence at any given cycle, or following cycling, depends on the amount of specific amplicons present at that time. The SmartCycler® Real Time PCR System simultaneously monitors the fluorescence emitted by each beacon.

¹ Morris A, Lundgren JD, Masur H, Walzer PD, Hanson DL, Frederick T, Huang L, Beard CB, Kaplan JE. (2004). Current epidemiology of *Pneumocystis* pneumonia. *Emerg Infect Dis*: 10: 1713-20.

² Miller RF, Allen E, Copas A, Singer M, Edwards SG. Improved survival for HIV infected patients with severe *Pneumocystis jirovecii*. pneumonia is independent of highly active antiretroviral therapy. *Thorax* 2006; 61:716-21.

³ Kovacs JA, Gill VJ, Meshnick S, Masur H. (2001). New insights into transmission, diagnosis, and drug treatment of *Pneumocystis carinii* pneumonia. *JAMA*: 286: 2450-60.

⁴ Huang L, Morris A, Limper AH, Beck JM; ATS *Pneumocystis* Workshop Participants. An Official ATS Workshop Summary: Recent advances and future directions in pneumocystis pneumonia (PCP). *Proc Am Thorac Soc* 2006;3:655-64.

⁵ Tyagi S, Kramer FR. (1996). Molecular beacons: Probes that fluoresce upon hybridization. *Nature Biotechnology*: 14: 303-308.

Precautions

- The kit is intended for use only by laboratory professionals. Procedures are required for non-aerosol manipulations of specimens. Standard precautions and institutional guidelines should be followed in handling all samples. A Material Safety Data Sheet is available from Myconostica Ltd.
- This test is for *in vitro* diagnostic use only.
- This test is only for use with the Cepheid SmartCycler® system with Dx diagnostic software versions 1.7b and 3.0.
- Do not use reagents or controls if the protective pouches are open or broken upon arrival.
- Reagents and controls are not interchangeable between kits with differing lot numbers.
- Never pool reagents or controls from different tubes even if they are from the same lot.
- Never use the reagents or controls after their expiry date.
- Reagents and controls should not be refrozen or reused after opening.
- Wear protective clothing and disposable gloves while handling kit reagents.
- Avoid microbial and deoxyribonuclease (DNase) contamination of reagents when removing aliquots from tubes.
- The use of sterile DNase-free, low-retention disposable filter-tipped or positive displacement pipette tips is recommended.
- Use a new tip for each specimen or reagent.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- To avoid contamination with *Pneumocystis* or internal amplification control (IAC) amplicons, do not open the reaction tubes post-amplification.
- Additional controls may be tested according to guidelines or requirements of local, state, provincial and/or federal regulations or accrediting organisations.
- Do not eat, drink or smoke in areas where specimens or kit reagents are being handled.
- Low concentrations of DNA can be unstable if not stored correctly. It is recommended that DNA extractions from clinical samples are stored at -80°C to preserve their integrity. Multiple rounds of thawing and refreezing should also be avoided whenever possible.

Kit Contents

Description

The kit consists of five 3-compartment sealed foil pouches, each of which can be used separately. Each pouch contains sufficient reagents for 8 reactions.

		<u>Volume</u>
Tube 1 (Orange Cap)	dNTPs MgCl ₂ Buffered solution of DNA Polymerase complex	66 µL
Tube 2 (Blue Cap)	<0.01% Primers <0.01% Molecular Beacons <0.0001% Internal Amplification Control (IAC) The Internal Amplification Control is a recombinant DNA plasmid harbouring a non-infective sequence unrelated to either target (<i>Pneumocystis</i>) sequence Tris-HCl Buffer	66 µL
Tube 3 (Clear Cap)	Negative Control Water	25 µL
Tube 4 (Black Cap)	Positive Control <0.0001% Positive Control DNA The Positive Control molecule is a recombinant plasmid harbouring the <i>Pneumocystis</i> target sequences Tris-HCl Buffer	25 µL

The kit also contains:

- MycAssay™ Pneumocystis Myconostica Protocol CD-ROM
- Instructions for Use
- Certificate of Analysis

Storage

The kit should be stored frozen (-15 to -25 °C) until the expiry date indicated on the kit box label, at which time it should be disposed of according to local regulations.

Once a pouch has been opened, the contents must be used immediately, not re-frozen or re-used.

Equipment/Materials required and not provided

- Cepheid SmartCycler® Real Time PCR System (including user manual, attached desktop computer and Dx Diagnostic software, version 1.7b or 3.0d)
- Mini centrifuge adapted specifically for SmartCycler® reaction tubes
- Micro centrifuge
- Vortex mixer
- SmartCycler® reaction tubes
- Support rack for SmartCycler® reaction tubes
- Micropipettes (volumes required 7.5 µL – 20 µL)
- Sterile low-retention filtertips
- Disposable gloves, powderless
- Proprietary DNA decontaminating solution
- Permanent marker pen
- DNA isolation kit (see below)

Specimen

The specimen for the MycAssay™ Pneumocystis assay is total DNA extracted from clinical BAL samples. The following DNA isolation kit and equipment, supplied by Myconostica Ltd., is recommended for this purpose and was used during validation:

- MycXtra® Fungal DNA Extraction kit (REF: 080-005 available from Myconostica)
- Vortex-Genie 2 (Scientific Industries Inc., New York, USA)
- Vortex Adapter Plate (REF: 080-015 available from Myconostica)

Procedural Notes

- Read the entire protocol before commencing
- The entire MycAssay™ Pneumocystis process (excluding DNA extraction) takes approximately 2 hours, dependent on the number of samples tested.
- Setting up of the test should be performed in a PCR workstation or pre-PCR laboratory. If a PCR workstation is not available, then the test should be set-up in a dedicated area of the laboratory⁶, which is regularly cleaned with DNA decontaminating reagents.
- However, avoid using DNA decontaminating reagents during the Real-Time PCR set-up as they can inhibit the assay.
- Use micropipettes for the transfer of fluids. Dedicated micropipettes should be used for the set-up of these reactions and they should be regularly decontaminated.
- Low-retention filtertips are recommended for use to ensure that no DNA is lost during the set-up procedure.
- **Exercise caution when handling Tube 4. This contains template DNA material and contamination could result in false positive test results.**
- Wear gloves at all times.
- All tubes must be capped following use and prior to disposal.
- Take care to identify the SmartCycler® reaction tubes appropriately when multiple patient samples are being processed.

⁶ For example see Mifflin, T. E. (2003). Setting up a PCR Laboratory. *In* PCR Primer, 2nd Ed. (eds. Dieffenbach and Dveksler). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. USA.

Procedure for Use:

1. Real-Time PCR Set-Up

- 1.1 To begin, switch on the SmartCycler® Real-Time PCR System (instrument and associated computer) and launch the relevant software. Enter usernames and passwords as required.
- 1.2 Ensure the work area has been cleaned using DNA decontaminating reagents and allowed to dry completely; avoid use during assay set-up as excess cleaning solution may inhibit the PCR reactions.
- 1.3 A pouch contains one each of Tube 1, Tube 2, Tube 3 and Tube 4. There are sufficient reagents in one pouch to run 8 reactions. At least one positive control and one negative control reaction must be performed per run where the reagents are from a single kit lot. One pouch therefore can analyse 6 patient samples. If more than 6 samples need to be tested, more than one pouch can be used if the pouches used are from the same kit lot. A maximum of 38 patient samples may be tested using the 5 pouches in a kit.
- 1.4 Calculate the number of reactions required, referring to the table below:

Number of Pouches	Maximum number of patient samples
1	6
2	14
3	22
4	30
5	38

- 1.5 Remove the appropriate number of pouches from the freezer. Do not use any pouch that is no longer sealed. If the patient samples were frozen after extraction, also remove these from the freezer.
- 1.6 Tear open the required number of pouches and remove the tubes. If more than one pouch is being used, but only one set of positive and negative controls are being run, it is only necessary to remove Tubes 3 and 4 from one pouch. **Exercise caution when handling Tube 4. This contains positive control DNA material and contamination could cause false positive test results.**
- 1.7 Allow the tubes' contents to thaw by placing on the laboratory bench for 5-10 minutes, ensuring that the contents of each tube are completely thawed before proceeding. Vortex mix the tubes' contents and the patient samples; follow by a short spin in a microcentrifuge to ensure collection of all the contents at the base of the tubes before use.
- 1.8 Place the required number of SmartCycler® reaction tubes in their support rack(s). **Never touch the diamond-shaped reaction chamber of the reaction tubes with your hands.**
- 1.9 Always set up the negative control first, followed by the patient samples. The positive control should always be set up last.
- 1.10 Reagent and DNA volumes are shown in the table below:

Reagent	Reaction		
	Negative control	Patient sample	Positive control
Tube 1 (Orange cap)	7.5 µL	7.5 µL	7.5 µL
Tube 2 (Blue cap)	7.5 µL	7.5 µL	7.5 µL
Tube 3 (Clear cap)	10 µL	-	-
Patient Sample	-	10 µL	-
Tube 4 (Black cap)	-	-	10 µL
Total volume	25 µL	25 µL	25 µL

- 1.11 Add reagents in the order shown in the table above; Tube 1, then Tube 2, followed by the template (Negative control, Patient sample, or Positive control). Take care when taking aliquots from Tube 1; the liquid is slightly viscous and can stick on the inner ridge of the tube. If this happens, re-spin to collect the final contents in the base of the tube before attempting to remove the final aliquots.
- 1.12 Use a new pipette tip for every liquid transfer. Re-cap each reagent tube after use and immediately discard it, and any remaining contents, into a sealable clinical waste container. Unused reagents cannot be saved for later use.

- 1.13 Take extra care when pipetting Tube 4 (positive control DNA) to ensure it does not contaminate any other reaction tube. Closing the lids on the other reaction tubes before opening Tube 4 can reduce the risk of cross-contamination.
- 1.14 Make sure all the SmartCycler® reaction tube lids are firmly closed and then label each lid using a permanent marker pen e.g. POS for positive control, NEG for negative control and patient ID for patient samples. Spin down the reaction tubes for 10 seconds using the specially-adapted mini centrifuge. Visually check that there are no bubbles present in the reaction mixtures.
- 1.15 Proceed to Section 2 promptly. MycAssay™ Pneumocystis reactions are stable on the bench for up to 60 minutes.
- 1.16 Following the PCR set-up ensure the work area is thoroughly cleaned using DNA decontaminating reagents.

2. Performing the run

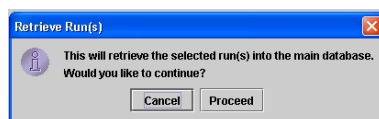
Before proceeding with the following section, please check which version of the Dx software you have installed on your computer. Open the software, choose **Help** from the toolbar and click **About**.

For version 1.7b, follow the instructions below in Section 2.1
For version 3.0, follow the instructions below in Section 2.2

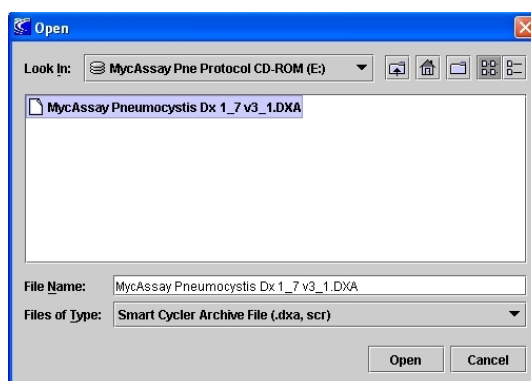
Please also be aware that certain user privileges are required in the software to **Retrieve Run(s)** or **Import** an assay. These can only be assigned by the **Administrator** of the instrument.

2.1 SmartCycler® Dx Diagnostic software version 1.7b

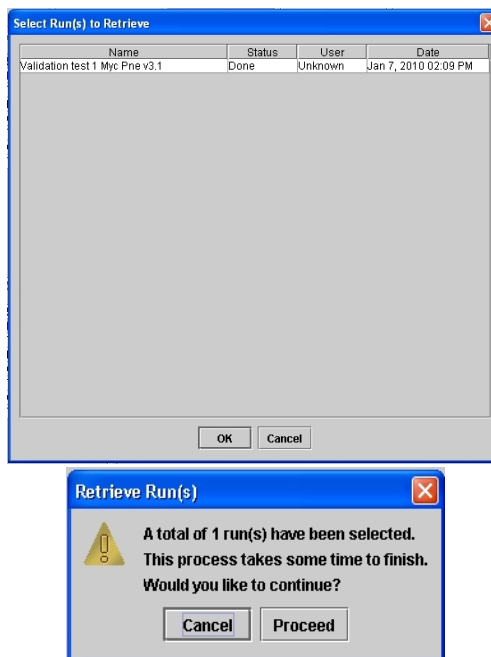
- 2.1.1 Open up the SmartCycler® Dx Diagnostic software version 1.7b and enter your username and password.
- 2.1.2 Insert the **MycAssay Pneumocystis Myconostica Protocol CD-ROM** and click on the **Define Assays** tab.
- 2.1.3 Got to **Retrieve Run(s)** via the **Tools** directory on the top menu bar and click **Proceed**:



- 2.1.4 Select the file **MycAssay Pneumocystis Dx1_7 v3_1.DXA** from the CD-ROM as shown below. This file should be the only one recognised by the software (an example is shown below):



- 2.1.5 On the next screen highlight the filename **Validation test 1 Myc Pne v3.1** and click **OK**, followed by **Proceed** and **OK**:



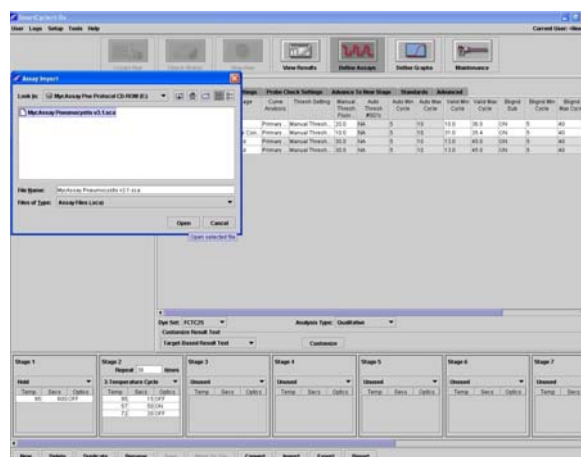
- 2.1.6 Close the software. When it is reopened the **MycAssay Pneumocystis Dx1.7b v3.1** assay will be available for use when creating a new run.
- 2.1.7 Click on the **Create Run** tab. Enter an appropriate **Run Name** (it is recommended that this includes the date and operators initials as a minimum), or leave blank if you wish the name to be created automatically by the software.
- 2.1.8 Select **MycAssay Pneumocystis Dx1.7 v3.1** as the assay.
- 2.1.9 Enter the **Lot Number** and **Expiration Date** of the kit as printed on the kit box and on each pouch. The lot number will be in the form of M-XXXXXXXX.
- 2.1.10 Enter the **Number of specimens** in the box and click **Apply**. The **Sample ID** for each specimen will automatically be named **SPEC** by the software. Therefore, rename each site appropriately for identification purposes; i.e. double click on **SPEC** to highlight it and then type in the sample ID.
- 2.1.11 The software will automatically include a Negative and Positive control in the Real-Time PCR run.
- 2.1.12 Carefully place the reaction tubes into the designated sites in the SmartCycler® block and click **Start Run**. N.B. **Take care** when placing the reaction tubes into the designated sites as they may not be in the same order as your set-up. Make a note of the run name and click **OK**. The run will now start and red lights will appear above each site in use on the block.

To determine how long the run will take to complete, click on the **Check Status** tab. The run name and subsequent run time will be listed.

2.2 SmartCycler® Dx Diagnostic software version 3.0

- 2.2.1 Open up the SmartCycler® Dx Diagnostic software version 3.0 and enter your username and password.

- 2.2.2 Insert the **MycAssay Pneumocystis Myconostica Protocol CD-ROM** and click on the **Define Assays** tab, and **Import** the **MycAssay Pneumocystis v3_1.sca** file from the CD-ROM, as shown below:



- 2.2.3 Click on the **Create Run** tab. Enter an appropriate **Run Name** (it is recommended that this includes the date and operators initials as a minimum), or leave blank if you wish the name to be created automatically by the software.
- 2.2.4 Select **MycAssay Pneumocystis v3.1** as the assay.
- 2.2.5 Enter the **Lot Number** and **Expiration Date** of the kit as printed on the kit box and each pouch. The lot number will be in the form of M-XXXXXXX.
- 2.2.6 Enter the **Patient (Sample) ID** and the **Number of specimens** (replicates) in the appropriate boxes and click **Apply**. Do this for all patient samples being tested. The software will automatically include a Negative and Positive control in the Real-Time PCR run.
- 2.2.7 Carefully place the reaction tubes into the designated sites in the SmartCycler® block and click **Start Run**. N.B. **Take care** when placing the reaction tubes into the designated sites as they may not be in the same order as your set-up. Make a note of the run name and click **OK**. The run will now start and red lights will appear above each site in use on the block.

To determine how long the run will take to complete, click on the **Check Status** tab. The run name and subsequent run time will be listed.

3. Data Analysis and Interpretation

- 3.1 The results can be viewed in Dx software, by selecting the **View Results** tab.
- 3.2 Click on the **View Another Run** button at the bottom of the page, select the run you wish to view then click **OK**.
- 3.3 The **Patient Results** should already be selected in the **Views** list. The patient (sample) ID and the subsequent assay result will be clearly listed. The results can be interpreted using the table below:

Out-come	Patient Result	Colour	Interpretation	Further Action
1	Negative	Green	Negative for <i>Pneumocystis</i>	Report result
2	Positive	Red	Positive for <i>Pneumocystis</i>	Report result
3	Invalid	Light Grey*	IAC failure in sample	Repeat sample
4	Invalid	Light Grey*	Failure in Positive or Negative Control	Repeat entire run

*If the result is reported as ND, in light grey, this corresponds to error code 3079, the result of high fluorescence in one or more channels. If a Ct value of ≤ 39.0 is recorded in the Pneumocystis channel, report as positive.

- 3.4 To view individual sample results (Ct values) for either *Pneumocystis* or IAC separately, select **Sample Results** from the **Views** list and click on the individual tabs for each target (i.e. <Pne> or <IAC>). The results will be displayed in the same format as the **Patient Results** but for each individual target.
- 3.5 If a Patient sample reports an Invalid result, this is due to a failed IAC result (indicated by Unresolved in the **Sample Results** tab); repeat the reaction (plus Positive and Negative controls). If the reaction continues to fail, an inhibiting substance may be present in the template and a Negative result cannot be relied upon.
- 3.6 The lower the Ct value the higher the probability of disease. Ct values close to the cut-off of 39.0 are more likely to represent colonisation than infection, but some patients may have disease with very little *P. jirovecii* present, representing a poor specimen, prior treatment or the nature of fungal load in that particular patient.

- 3.7 To export run data to allow transfer to another computer, go to the **Tools** directory at the top of the screen and select **Data Management**, followed by **Archive Run(s)** from the drop down menu. A message screen will appear, click **Proceed**. Select the run to be archived by ticking the box to the left and click **OK**. A warning message will appear stating how many runs are to be archived; if this number is correct, click **Proceed**. Select a destination to save the run file e.g. USB data stick. Click **Save** and make a note of the file name. A message screen will appear stating how many runs are to be archived, if this number is correct, click **Proceed**.
- 3.8 To import run data, go to the **Tools** directory at the top of the screen and select **Data Management**, followed by **Retrieve Run(s)** from the drop down menu. A message screen will appear, click **Proceed**. Go to **Look In:** and select the storage device used to archive the run data (see section 3.4 above). Select the run file to be retrieved and click **Open**. Another screen will appear prompting you to select the run you wish to retrieve. Select the run to be retrieved and click **OK**. A message screen will appear stating how many runs are to be retrieved, if this number is correct, click **Proceed**.
- 3.9 When a run has completed its run data file will be automatically downloaded and can be located in the **SmartCycler Dx Folder** on the desktop. This can be copied into any folder and stored electronically if required.
- 3.10 If a hardcopy of the results is also required, click on **Report** and **Print**. This will include both the overall **Patient result** (Positive, Negative or Invalid) and the Ct value for each **Sample Result**.

4. Troubleshooting

4.1 The Negative Control has generated a positive signal in the FAM channel:

- Contamination occurred during the set up. Results from the entire run cannot be relied upon as accurate.
- Repeat the entire run taking great care when adding the templates, in particular, the Positive Control (Tube 4), to ensure that cross-contamination does not occur.
- Make sure that the work area and instruments are properly decontaminated before and after use.
- The Negative Control was incorrectly positioned in the instrument.
- Take care that the reaction tubes are placed in their designated sites and are annotated correctly within the software.

4.2 The Negative Control IAC Ct value is not within the acceptable range:

- The PCR has been inhibited.
- Ensure that the work area and instruments are thoroughly dry after the use of decontaminating agents prior to PCR set up.
- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.
- Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with unexpired kit if necessary.
- Either Tube 1 or 2 reagent was not added to the PCR reaction, or double the amount of Tube 2 was added.
- Repeat the run taking care in the set-up stage. Such errors can be detected by seeing higher or lower levels of liquid in one reaction tube compared to others.

4.3 The Positive Control is negative:

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.
- Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.
- An error occurred during step 1.12 and the Positive Control template (Tube 4) was placed in the wrong reaction tube.
- Repeat the run, taking great care during the set-up stage. Such errors can be detected by seeing a higher level of liquid in one reaction, and a lower level in another, compared to normal.
- Either Tube 1 or 2 reagent was not added to the reaction.
- Repeat the run taking care in the set-up stage. Such errors can be detected by seeing lower levels of liquid in this reaction compared to others.
- The Positive Control was incorrectly positioned in the instrument.
- Take care that the reaction tubes are placed in their designated sites.

4.4 Patient sample(s) give Outcome 3 - "Invalid":

- It is likely that the extracted clinical sample(s) contain PCR inhibitors.
- We recommend that DNA from clinical samples is extracted using the MycXtra™ Fungal DNA Extraction kit.

4.5 There are no results for any channel with any samples or controls:

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.
- Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.
- The equipment used is not functioning optimally.
- Please check that your Real-Time PCR instrument has an up-to-date service history and has been fully calibrated as described in its Installation and Maintenance Guide.
- An incorrect protocol file was used during the software set up.
- Please refer to Section 2 and choose the correct Protocol file, as specified for each software type/version, from the Myconostica Protocol CD-ROM. Only the file appropriate to the software can be loaded. Repeat the run using the correct protocol file.

If you have further questions, or you experience any problems, please contact Technical Support (mycotech@myconostica.co.uk)

Performance Characteristics and Limitations

Analytical Sensitivity

Using the protocol described above, and a recombinant *Pneumocystis* DNA molecule generated at Myconostica, the Limit of Detection (LoD) for *Pneumocystis* was determined to be < 35 copies. This value was determined using a recombinant DNA plasmid harbouring the target sequence. The *Pneumocystis* target sequence is mitochondrial, therefore, there will be numerous copies per cell, but it is not known how many.

Analytical Selectivity

Analytical selectivity was tested using DNA extracted from a variety of different fungal and non-fungal species. The following species did not report out a positive result;

Alternaria alternata, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Blastomyces capitatus*, *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Cladosporium spp.*, *Cryptococcus neoformans*, *Doratomyces microsporus*, *Fusarium solani*, *Rhizomucor pusillus*, *Rhodotonia rubra*, *Saccharomyces cerevisiae*, *Scedosporium apiospermum*, *S. prolificans*, *Sporothrix schenckii*, *Trichosporon capitatum* The following bacterial species did not report a positive result; *Bordetella pertussis*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Haemophilus influenzae*, *Lactobacillus plantarum*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *S. pyogenes*, *S. salivarius*.

Human genomic DNA does not report a positive result with this assay.

Interfering Substances (contraindications for use)

The following compounds were tested at clinically relevant concentrations, and found not to inhibit the assay; acetylcysteine, amphotericin, beclometasone dipropionate, budesonide, colistimethate sodium, fluticasone propionate, formoterol fumarate dehydrate, ipratropium bromide, lidocaine, mannitol, salbutamol sulphate, salmeterol, septrin (trimethoprim-sulphamethoxazole), sodium chloride, sodium cromoglicate, terbutaline and tobramycin.

Performance Evaluation

The clinical cut-off at a Ct of 39.0 was established following analysis of a set of clinical samples sourced from different patient populations.

Clinical samples collected by bronchoalveolar lavage (BAL) that had been obtained at 2 hospitals, extracted using the MycXtra® kit, and stored, were used to evaluate the performance of the MycAssay™ *Pneumocystis* kit. Comparisons were made of the PCR results to immunofluorescent microscopy.

PCR v Microscopy Diagnosis

	Microscopy positive	Microscopy negative	
PCR positive	45	8	0.85 PPV
PCR negative	2	33	0.94 NPV
	0.96 Sensitivity	0.80 Specificity	

Table 1: Diagnostic specificity and sensitivity of the MycAssay™ *Pneumocystis* kit compared to immunofluorescent microscopy.

Table 1 represents data obtained from patients with diagnosed HIV, patients not infected with HIV and patients with undetermined HIV status. Patients with *Pneumocystis* pneumonia have highly variable amounts of organism detectable; the lower the Ct value the higher the likelihood of disease. Patients with HIV and *Pneumocystis* pneumonia tend to have higher numbers of organisms detectable than patients who are not infected with the virus, but the overlap is

considerable. The scatter plot in Figure 1 below demonstrates this overlap. For completion, as the data-set in Table 1 included patients whose HIV status was unknown, the scatter plot for this group is included in Figure 1 (column 3):

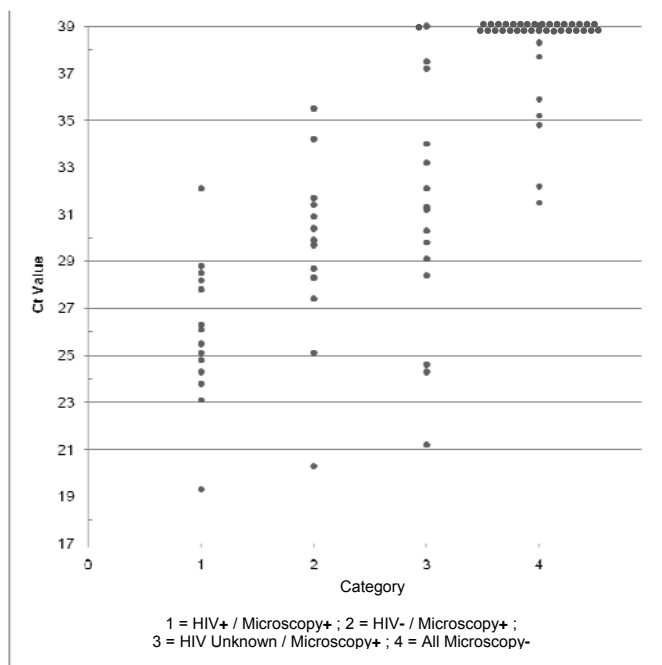


Figure 1: Scatter plot of Ct values obtained from DNA extracted from patient respiratory samples. Four groups are described.

Clinical Reporting

The MycAssay™ Pneumocystis kit is intended as an aid to diagnosis of Pneumocystis pneumonia. The results need to be taken in context of the clinical condition of the patient and other diagnostic tests results.

The following are recommended reports, each depending on the assay result interpretation.

Outcome No 1

“*Pneumocystis jirovecii* not detected.”

Outcome No 2

“*Pneumocystis jirovecii* detected. Positive result. State Ct value”

Outcome No 3

“Test failed; inhibitors or other unknown substance present.”

The lower the Ct value the higher the probability of disease. Ct values close to the cut-off of 39.0 are more likely to represent colonisation than infection, but some patients may have disease with very little *P. jirovecii* present, representing a poor specimen, prior treatment or the nature of fungal load in that particular patient.

Limitations of Procedure

- The principal limitation of this procedure relates to the quality of the primary sample:
 - If the sample is very small or not collected from the affected area of lung, the test will be less sensitive and may be falsely negative.
 - BAL samples should be centrifuged prior to DNA extraction from the pellet.
 - Data also demonstrated that a reduction in the volume of supernatant used in the extraction process, achieved by the centrifugation step, decreases the proportion of inhibitors entering the system.
- While the MycXtra™ Fungal DNA extraction procedure is designed to remove PCR inhibitors, not all drugs or patient populations have been evaluated.
- The procedure has not been fully assessed with sputa nor has it been assessed with induced saline samples or on samples from children.
- False positive results may arise from external contamination of the original sample or test. Such contamination could arise from *P. jirovecii* contaminated air, poor experimental technique with respect to the positive control or external (especially pipette) contamination with *P. jirovecii* DNA.
- As a true positive result may be obtained from patients who are transiently or persistently colonized by *P. jirovecii*; clinical judgment is required in interpretation of the test result.

LICENSING

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