MycAssay™ Pneumocystis
Cepheid SmartCycler®
REF 080-075

Intended Use:

MycAssay™ Pneumocystis is a Real-Time PCR kit for the detection of *Pneumocystis jirovecii* genomic DNA using the Cepheid SmartCycler® (Dx software versions 1.7b and 3.0).

Principles of the Assay

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.

Precautions

- The kit is for Research Use Only. It is Not for Use in Diagnostic Procedures.
- 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 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 IAC amplicons, do not open the reaction tubes post-amplification.
- 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 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.

		Volume
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 IAC is a recombinant DNA plasmid harbouring a non-infective sequence unrelated to either target (*Pneumocystis*) 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 *Pneumocystis* 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 filter-tips
- Disposable gloves, powderless
- Proprietary DNA decontaminating solution
- Permanent marker pen
- DNA isolation kit (see below)

Sample

The sample for the MycAssay™ Pneumocystis assay is *Pneumocystis* genomic DNA.

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 laboratory[1], 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 filter-tips 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.

[1] 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.

- Take care to identify the SmartCycler® reaction tubes appropriately when multiple samples are being processed.

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 test 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 test 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 test 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 test 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 test 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 tube with your hands.**

1.9 Always set up the negative control first, followed by the test 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	**Test 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	-	-
Test 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, Test 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 an ID for the test 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

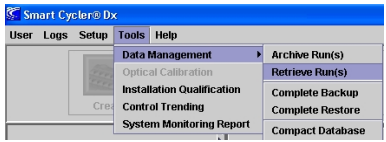
Please also be aware that certain user privileges are required in the software to **Retrieve Run(s)** or **Import** an assay. These can only be assigned by the **Administrator** of the instrument.

2.1 SmartCycler® Dx Diagnostic software version 1.7b

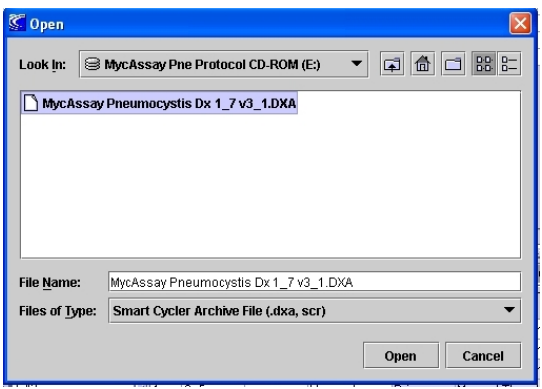
2.1.1 Open up the SmartCycler® Dx Diagnostic software version 1.7b and enter your username and password.

2.1.2 Insert the **MycAssay Pneumocystis Myconostica Protocol CD-ROM** and click on the **Define Assays** tab.

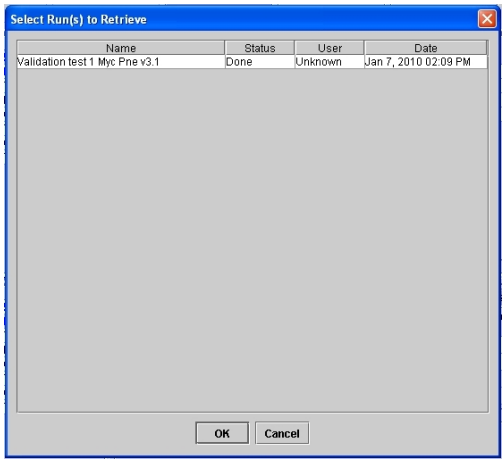
2.1.3 Got to **Retrieve Run(s)** via the **Tools** directory on the top menu bar and click **Proceed**:

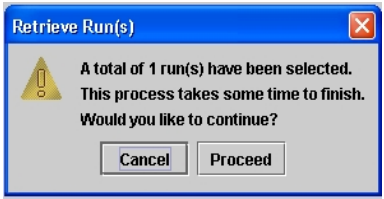


2.1.4 Select the file **MycAssay Pneumocystis Dx1_7 v3_1.DXA** from the CD-ROM as shown below. This file should be the only one recognised by the software (an example is shown below):



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.

2.2.3 Next **Import** the **MycAssay Pneu RUO Dx 3.0 v 3.1.sca** file from the CD-ROM.

2.2.4 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.

2.2.5 Select **MycAssay Pneu RUO Dx 3.0 v3.1** as the assay.

2.2.6 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-XXXXXXXX.

2.2.7 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.

2.2.8 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 Sample Results tab shows the overall Assay Result and these can be interpreted using the table below::

Result	Colour	Interpretation
Negative	Green	Negative for *Pneumocystis*
Positive	Red	Positive for *Pneumocystis*
Unresolved	Yellow (v3) or Light Grey (v1.7b)	IAC failure in sample; Repeat sample
Invalid	Light Grey	Failure in Positive or Negative Control; Repeat entire run
Valid	White	Controls have performed within defined parameters. Test sample results are valid.

3.4 To view the Ct results for either *Pneumocystis* or IAC separately, click on the individual tabs for each target; **<PNE>** and **<IAC>**. Amplification plots can also be viewed by selecting the **FAM** (or **Ch1 Optics**) and **CY3** (or **Ch2 Optics**) channels respectively.
3.5 If a sample reports an Unresolved result, this is due to a failed IAC reaction; run the sample again (plus the 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 Data can be exported for analysis with a spreadsheet package using the **Export** button. If a hardcopy of the results is also required, click on **Report** and **Print**.

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 1.11 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 Test sample(s) are negative and the IAC is out of range:

- It is likely that the test sample(s) contain PCR inhibitors.

→ Use a different DNA extraction method that removes inhibitors.

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, Rhodotonila rubra, Saccharomyces cerevisiae, Scedosporium apiospermum, S. prolificans, Sporothrix schenkii, 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*.

LICENSING

TopTaq™ Hot Start provided by QIAGEN. QIAGEN® is a registered trade mark of Qiagen GmbH, Hilden, Germany.

This product is sold under license from the Public Health Research Institute, Newark, New Jersey, USA and may be used under PHRI patent rights only for human *in vitro* diagnostics.

SmartCycler® is a registered Trademark of Cepheid, 904 Caribbean Drive, Sunnyvale, CA, 94089, USA.

Myconostica Limited, South Court, Sharston Road, Sharston, Manchester,
M22 4SN, United Kingdom.
Telephone: +44 (0) 161 998 7239 Facsimile: +44 (0) 161 902 2496
Email: mycotech@myconostica.co.uk