

# MycAssay™ Pneumocystis

## Roche LightCycler® 2.0

### 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 Roche LightCycler® 2.0.

#### Summary and Explanation

*Pneumocystis jirovecii* (formerly *carinii*) pneumonia (PCP) is a common opportunistic pneumonia in immunocompromised patients, especially those with advanced HIV infection and AIDS<sup>1</sup>. It is typically community acquired, and sub-acute in presentation, leading to progressive respiratory failure and death<sup>2</sup> 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 PCP<sup>3</sup>. PCP also occurs in other immunocompromised patients, including recipients of solid organ transplants, hypogammaglobulinaemia and chronic leukaemia.

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<sup>1</sup> 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.

<sup>2</sup> 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.

<sup>3</sup> 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.

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 samples<sup>4</sup>.

MycAssay™ Pneumocystis is a molecular diagnostic kit for the detection of *P. jirovecii* based on Molecular Beacon<sup>5</sup> 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 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 Roche LightCycler® 2.0 System simultaneously monitors the fluorescence emitted by each beacon.

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<sup>4</sup> 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.

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

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## **Precautions**

- The kit is for *in vitro* diagnostic use.
- 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 Roche LightCycler® 2.0.
- 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.

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

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## **Equipment/Materials required and not provided**

- Roche LightCycler® 2.0 Real-Time PCR system (including User Manual, attached computer and LightCycler® Software v4.1)
- LC 2.0 carousel centrifuge (optionally capillary adaptors for mini centrifuge)
- Sample carousel for 20 µL capillaries
- LC 2.0 20 µL capillaries with caps
- Capillary rack holder
- Capillary releaser
- Capping tool
- Micro centrifuge
- Vortex mixer
- Micropipettes (volumes required 7.5 µL – 20 µL)
- Sterile low-retention filtertips
- Disposable gloves, powderless
- Proprietary DNA decontaminating solution
- DNA isolation kit (see below)
- MycAssay™ CC-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)

## MycAssay™ Colour Compensation (CC) kit

Accurate analysis of data produced using MycAssay™ Pneumocystis assay requires the application of a colour compensation file produced using the Myconostica MycAssay™ CC kit [ref 080-080]. Once created, the file can be applied to multiple runs on the same machine. Please contact your local distributor for details.

### 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<sup>6</sup>, 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 capillaries must be capped following use and prior to disposal.
- Accurately note the positions of all the capillaries containing relevant samples within the 32-position carousel on the experimental plan.
- Accurate analysis of the data requires the application of a colour compensation file created using the Myconostica MycAssay™ CC kit.

### Procedure for Use:

#### 1. Real-Time PCR Set-Up

- 1.1 To begin, switch on the LightCycler® 2.0 Real-Time PCR System (instrument, associated computer and centrifuge) and launch the relevant software. Enter

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<sup>6</sup> 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.

username and password as required and choose the Diagnostic database. If this is the first run of a day, perform an instrument Self-Test first before setting a run.

- 1.2 **Remember:** a colour compensation run must be completed prior to analysing results for MycAssay™ Pneumocystis on the LightCycler® 2.0. However, this does not have to be performed prior to using this product, and can be carried out and applied to run files retrospectively.
- 1.3 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.
- 1.4 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 patient samples need to be tested, more than one pouch can be used if the pouches used are from the same kit lot. However, the LightCycler® 2.0 can only hold up to 32 samples in a single run. Therefore, a maximum of 30 patient samples can be performed in a single run (4 pouches).
- 1.5 Calculate the number of reactions required, referring to the table below:

| <b>Number of Pouches</b> | <b>Maximum number of Patient samples</b> |
|--------------------------|--|
| 1                        | 6  |
| 2                        | 14                                       |
| 3                        | 22                                       |
| 4                        | 30                                       |

- 1.6 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.
- 1.7 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.**

- 1.8 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 to 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.
- 1.9 Place the required number of 20 µL capillaries in a capillary rack holder. Take care not to leave any marks on the glass.
- 1.10 Always set up the negative control first, followed by the test samples. The positive control should always be set up last.
- 1.11 Reagent and DNA volumes are shown in the table below:

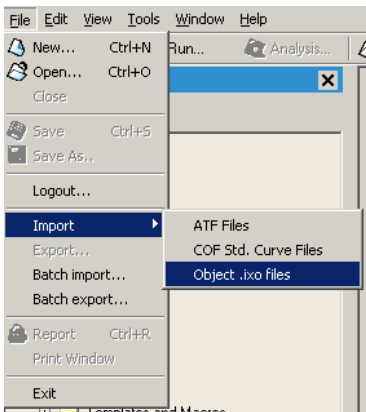
| Reagent                    | Reaction         |                |                  |
|----------------------------|------------------|----------------|------------------|
|                            | Negative control | Patient sample | Positive control |
| <b>Tube 1 (Orange cap)</b> | 7.5 µL     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

- 1.12 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.
- 1.13 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.

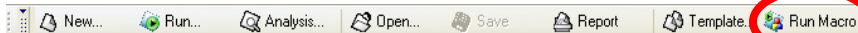
- 1.14 Take extra care when pipetting Tube 4 (positive control DNA) to ensure it does not contaminate any other reaction. Capping all the other capillaries before opening Tube 4 can reduce the risk of cross-contamination.
- 1.15 Carefully cap the capillaries with the caps provided in the capillary box using a capping tool. Ensure the capillaries are firmly capped. Capillaries can be capped once the template has been added to the reaction if desired to reduce the potential for cross/environmental-contamination.
- 1.16 If the carousel centrifuge is not available, spin the samples down in a mini centrifuge using the adapters provided with a rack holder. Otherwise, proceed to 1.17.
- 1.17 Very carefully, transfer all the capillaries to the carousel in exactly the same order that they are in the rack holder starting with the first capillary in the position 1 and continue in the ascending order leaving no gaps. Push each capillary all the way down till it firmly rests in its place.
- 1.18 If not already spun down in 1.16, spin the samples using the LC 2.0 carousel centrifuge.
- 1.19 Proceed to Section 2 promptly. MycAssay™ Pneumocystis reactions are stable on the bench for up to 60 minutes.
- 1.20 Following the PCR set-up ensure the work area is thoroughly cleaned using DNA decontaminating reagents.

## **2. Performing the run**

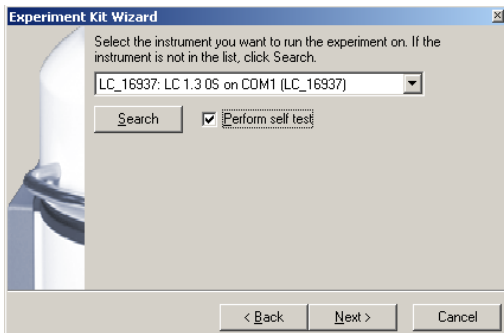
- 2.1 Insert the **MycAssay Pneumocystis Myconostica Protocol CD-ROM**.
- 2.2 Go to **Import** via the **File** directory and select **Object .ixo files**. Import the **Macro MAP v1.2.ixi** file from the CD to your database.



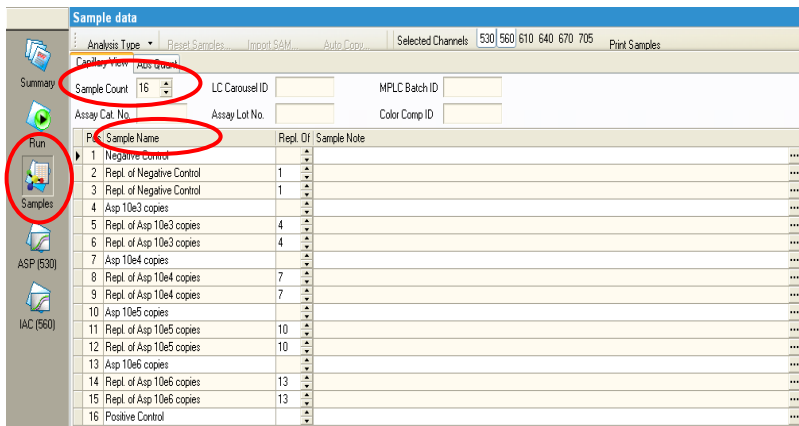
- 2.3 Go to **Save** via the **File** directory and save the macro template in the desired location in your database.
- 2.4 Select the **Run Macro** option from the Toolbar.



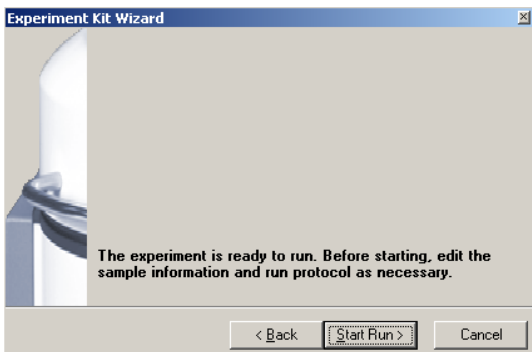
- 2.5 Select the **Macro MAP v1.2** template file and press **Open**.
- 2.6 Follow the wizard instructions. Tick the **Perform Self-Test** box if this is first run if a day.



- 2.7 Name and save the run file in a desired location.
- 2.8 Go to the Samples section by clicking the tab in the left of the screen. Edit sample number in the **Samples Count** box and names in the **Capillary view** tab. Name the samples as it states in your experimental plan as positioned in the carousel.



- 2.9 Place the spun down carousel in the LC 2.0 instrument. Ensure that the notch below sample position 1 on the carousel locks into position with the pin on the thermal chamber. Make sure that the carousel is inserted firmly in the chamber and close the lid.
- 2.10 When finished, press the **Start button**. Make sure that the instrument has found all the capillaries in the carousel and the program has started.

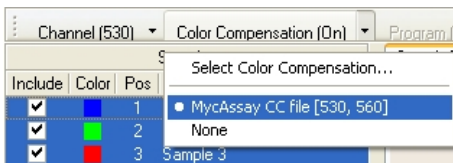


### 3. Data Analysis and Interpretation

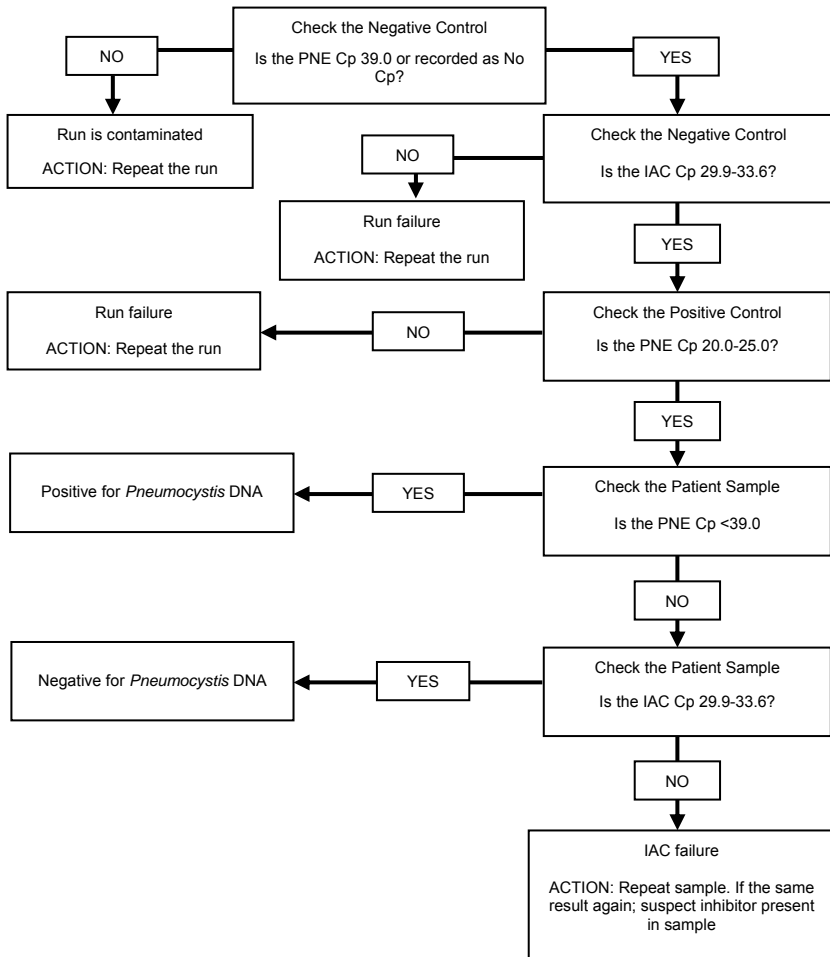
- 3.1 **Remember:** a colour compensation object must be applied prior to analysing results for MycAssay™ Pneumocystis on the LightCycler® 2.0. If you have not yet created one, please do so now before continuing with Data Analysis and Interpretation.
- 3.2 When the run has finished check for the contents of the popped up report and print it if desired.
- 3.3 The Pneumocystis results can be viewed in the PNE (530) analysis section and the IAC results in the IAC (560) analysis section.



- 3.4 In both sections, **PNE (530)** and **IAC (560)**, select the correct Colour Compensation file (**MycAssay CC file**) to be applied to the experiment.



- 3.5 Analyse each sample starting with the controls, as shown in the flowchart below (details can also be found in the table shown beneath the flowchart).



| <b>Sample</b>    | <b>PNE (530) Cp</b> | <b>IAC (560) Cp</b> | <b>Interpretation</b>            | <b>Further Action</b>     |
|------------------|---------------------|---------------------|----------------------------------|---------------------------|
| Negative Control | ≥39.0 or No Cp      | Within 29.9-33.6    | Negative Control acceptable      | Patient results are valid |
| Negative Control | ≥39.0 or No Cp      | <29.9 or >33.6      | Failure in Negative Control      | Repeat entire run         |
| Negative Control | <39.0               | Within 29.9-33.6    | Contamination                    | Repeat entire run         |
| Positive Control | Within 20.0-25.0    | N/A                 | Positive Control acceptable      | Patient results are valid |
| Positive Control | <20.0 or >25.0      | N/A                 | Failure in Positive Control      | Repeat entire run         |
| Patient Sample   | ≥39.0 or No Cp      | Within 29.9-33.6    | Negative for <i>Pneumocystis</i> | Report result: Outcome 1  |
| Patient Sample   | <39.0               | N/A                 | Positive for <i>Pneumocystis</i> | Report result: Outcome 2  |
| Patient Sample   | ≥39.0 or No Cp      | <29.9 or >33.6      | IAC failure in sample            | Repeat sample: Outcome 3  |

See Clinical Reporting (Outcome 1, 2 or 3)

## **4. Troubleshooting**

### **4.1 The Negative Control has generated a positive signal in the 530 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 capillaries are annotated correctly within the software.

### **4.2 The Negative Control IAC Cp 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 capillary compared to others.
- The correct CC-file was not applied to the data.
- Create a CC-file using the Myconostica MycAssay CC kit and apply to the results and reanalyse. See your local distributor for details of this kit.

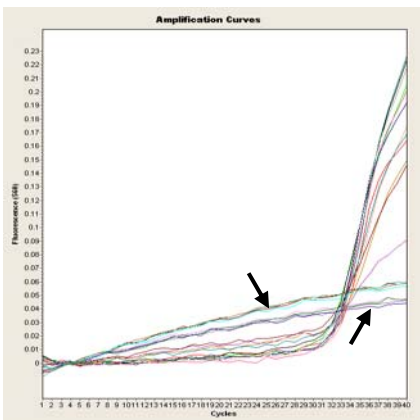
**4.3 The Positive Control is negative:**

- 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 set-up 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 capillary compared to others.
- The Positive Control was incorrectly positioned in the instrument.
- Take care that the capillaries are annotated correctly within the software.

**4.4 Patient sample(s) are negative and the IAC is out of range (Outcome 3):**

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

**4.5 The Patient Sample is negative in the PNE (530) section and the IAC (560) plot drifts away significantly from the regular baseline (as shown on the example picture below; arrows indicate abnormal plots):**



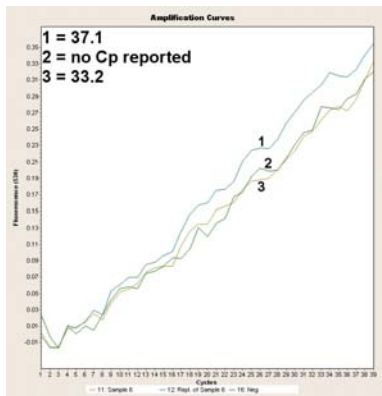
- The PCR reaction was inhibited.
- Ensure that the work area and instruments are thoroughly dry after the use of decontaminating agents prior to PCR set up.
- Run the patient sample again. If the problem repeats, the PCR inhibitor is present in the sample. Report the sample as Undetermined.

#### 4.6 The results in the IAC (560) section almost exactly match the results in the PNE (530) section.

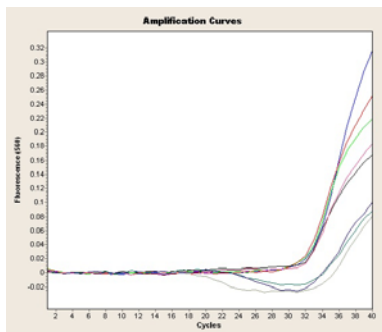
- There was no or an incorrect Colour Compensation file was applied to the experiment results.
- In both analysis sections check if the Colour Compensation is ON and that the same MycAssay CC file is applied in both channels.

#### 4.7 The baseline for some samples match the Negative control, indicating no amplification has occurred. However, the software has reported out a positive Cp value (as in figure below):

- An output of a positive Cp for a negative amplification plot was seen only twice in 154 negative reactions performed during validation studies.
- If this happens, please repeat the sample/s to confirm a *Negative* result.



- 4.8 When I apply my CC object some of the data in the 560 IAC channel dips, resulting in Cp values which are outside the acceptable range:



- This is entirely normal for reactions containing high concentrations of target DNA and will not interfere in the interpretation of patient results.

- Follow the normal analysis; you will see that for samples which are positive for Pneumocystis, the IAC result is not required for an outcome decision to be made for the patient.

**4.9 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](mailto:mycotech@myconostica.co.uk))

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## Performance Characteristics and Limitations

### LightCycler® 2.0 Analytical Performance Data

The kit was initially validated using the Cepheid SmartCycler®. Certain of the assay performance claims were re-validated on the Roche LightCycler® 2.0 platform, using 20 µL glass capillaries (Roche Cat # 04929292001 or 11909339001), and are reported below. Where the differences between platforms were not expected to affect the performance of the assay, and therefore the claim, the study was not repeated. These results, obtained using the SmartCycler®, are considered transferable to the LightCycler® 2.0 platform.

### 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 < 30 copies. This value was determined using a recombinant DNA plasmid harboring 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*.

**The following Performance Claims were established using the Cepheid SmartCycler®**

### **Interfering Substances (contraindications for use)**

The following compounds were tested at clinically relevant concentrations, and found not to inhibit the assay; acteylcysteine, 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.

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

### **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                | 0.80                |      |     |
|              | Sensitivity         | 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 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):

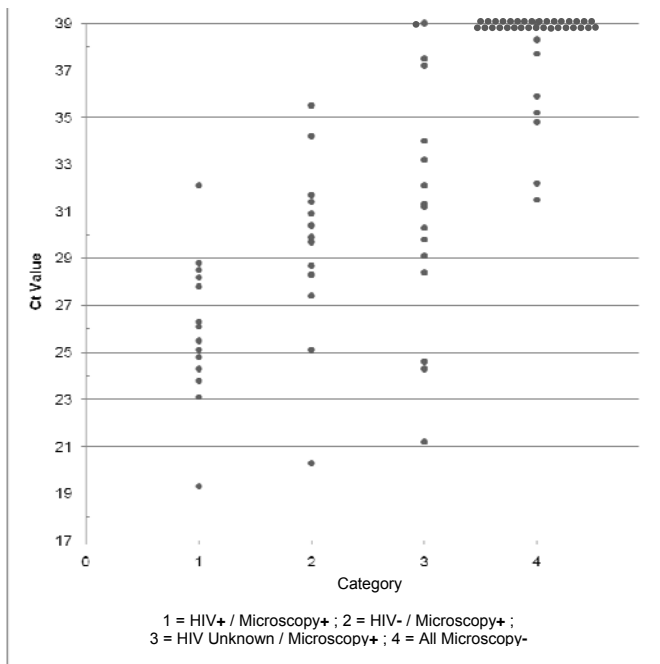


Figure 1: Scatter plot of Ct values obtained from DNA extracted from patient respiratory samples. Four groups are described.

## **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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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

