

MycAssay™ Aspergillus

Roche LightCycler® 2.0

Respiratory Samples

REF 080-045

Intended Use:

MycAssay™ Aspergillus is indicated for use by qualified laboratory professionals for the qualitative detection of *Aspergillus* spp. 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 *Aspergillus* infection or allergy.

MycAssay™ Aspergillus has been validated for use with the Roche LightCycler® 2.0.

Summary and Explanation

Aspergillus spp. are ubiquitous opportunistic moulds which cause both allergic and invasive syndromes. The genus is comprised of approximately 300 species, of which 41 have been associated with human disease. The majority of infections are caused by *A. fumigatus*, *A. flavus*, *A. terreus* and *A. niger*; less commonly, *A. nidulans* and other rarer species such as *A. sydowii*, *A. versicolor*, *A. lentulus* and *A. pseudofischeri* have been implicated¹. Most infections and allergies caused by *Aspergillus* spp. affect the respiratory tract. Allergic syndromes include allergic bronchopulmonary aspergillosis (ABPA) and allergic *Aspergillus* sinusitis and are usually caused by *A. fumigatus*. Chronic pulmonary aspergillosis includes aspergilloma, chronic cavitary pulmonary aspergillosis and chronic fibrosing aspergillosis. Invasive aspergillosis (IA) occurs in at-risk patient groups including those treated with corticosteroids and those with

¹ Species Database in www.aspergillus.org.uk

neutropenia or phagocyte dysfunction (i.e. chronic granulomatous disease and HIV infection). About 80% of cases of invasive aspergillosis involve the lungs².

Routine diagnosis of invasive pulmonary aspergillosis includes Computed Tomography (CT) scanning, bronchoscopy and bronchoalveolar lavage (microscopy and culture), *Aspergillus* antigen testing of blood, or histological examination of surgical biopsy specimens. In this scenario, cultures are frequently falsely negative³. Indeed bronchoalveolar lavage is only positive by culture in approximately 40% of cases even when the diagnosis is proven by other means^{4,5,6,7}, showing the lack of sensitivity of culture in detecting invasive aspergillosis and chronic pulmonary aspergillosis. Cultures are, however, important if positive because many diagnostic tests do not indicate either the genus or species of fungus causing the disease, or the susceptibility profile of the isolate causing infection.

Allergic bronchopulmonary aspergillosis complicates asthma and cystic fibrosis⁸ and may rarely present with no underlying disease. Most cases are associated with *A. fumigatus*, with other fungi occasionally implicated. Episodic airway obstruction with thick sputum plugs full of *Aspergillus* hyphae, serum total IgE >1,000 IU/mL and detectable *A. fumigatus* specific IgE and IgG antibodies or a positive *Aspergillus* skin prick test are the characteristics of the disease. Sputum cultures may be positive for *A. fumigatus* and bronchiectasis may be seen on a CT scan of the chest.

Current methods of diagnosing chronic pulmonary aspergillosis are a mixture of radiology (which is not specific)⁹ and serology (*Aspergillus* IgG antibodies or precipitins) which is positive in most forms of aspergillosis (and thus not specific for any particular

² Hope WW, Walsh TJ, Denning DW. (2005). The invasive and saprophytic syndromes due to *Aspergillus* spp. *Medical Mycology*: 43 (suppl. 1): S207-38.

³ Hope WW, Walsh TJ, Denning DW. (2005). Laboratory diagnosis of invasive aspergillosis. *Lancet Infectious Diseases*: 9: 609-22.

⁴ Levy H, Horak DA, Tegtmeyer BR, Yokota SB, Forman SJ. (1992). The value of bronchoalveolar lavage and bronchial washings in the diagnosis of invasive pulmonary aspergillosis. *Respir Med*: 86: 243-8.

⁵ Greub G and Bille J. (1998) *Aspergillus* species isolated from clinical specimens: suggested clinical and microbiological criteria to determine significance. *Clin Microbiol Infect* 4: 710-716.

⁶ Perfect JR, Cox GM, Lee JY, Kauffman CA, de Repentigny L, Chapman SW, Morrison VA, Pappas P, Hiemenz JW, Stevens DA, and the Mycoses Study Group. (2001). The impact of culture isolation of *Aspergillus* species: A hospital-based survey of *Aspergillus*. *Clinical Infectious Diseases*; 33:1824-33.

⁷ Maertens J, Van Eldere J, Verhaegen J, Verbeken E, Verschakelen J, Boogaerts M. (2002). Use of Circulating Galactomannan Screening for Early Diagnosis of Invasive Aspergillosis in Allogeneic Stem Cell Transplant Recipients *The Journal of Infectious Diseases*. 186:1297-306.

⁸ Tillie-Leblond I, Tonnel AB. (2005). Allergic bronchopulmonary aspergillosis. *Allergy*: 60: 1004-13.

⁹ Greene R. (2005). The radiological spectrum of pulmonary aspergillosis. *Med Mycol*: 43 (Suppl 1): S147-54.

manifestation of aspergillosis)¹. Cultures are positive in only 40-65% of cases proven by radiology and serology^{10,11}. As the differential diagnosis is wide including tuberculosis, atypical mycobacteriosis, sarcoidosis, histoplasmosis, coccidioidomycosis, pneumoconiosis, rheumatoid lung, ankylosing spondylitis and others, documenting the presence of *Aspergillus* spp. in respiratory samples is important to prevent delay in the recognition of pulmonary aspergillosis and mistreatment .

MycAssay™ Aspergillus is a molecular diagnostic kit for the detection of *Aspergillus* spp. genomic DNA using Molecular Beacon¹² Real-Time PCR technology. The whole test procedure, including extraction of DNA from the clinical sample, can be completed within 4 hours, compared to fungal culture which can take several days to produce positive results. This assay offers advantages over currently available diagnostic methods for acute invasive and chronic pulmonary aspergillosis. These advantages include faster detection of *Aspergillus* spp. and the potential for increased sensitivity for *Aspergillus* spp. in highly immunocompromised patients suspected of having invasive pulmonary aspergillosis.

Principles of the Procedure

Following mixing of the reagents in the MycAssay™ Aspergillus kit with a sample containing the *Aspergillus* target DNA sequence (a section of the *Aspergillus* ribosomal 18S gene), thermocycling will result in DNA amplification occurring. The assay also contains an Internal Amplification Control (IAC), a DNA fragment not present in *Aspergillus*, 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 using Molecular Beacon technology. Molecular Beacons are single-stranded oligonucleotide hybridisation 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

¹⁰ Denning DW, Riniotis K, Dobrashian R, Sambatakou H. (2003). Chronic cavitary and fibrosing pulmonary and pleural aspergillosis: Case series, proposed nomenclature and review. Clin Infect Dis: 37 (Suppl 3): S265-80.

¹¹ Camuset J, Lavolé A, Wislez M, Khalil A, Bellocq A, Bazelly B, Mayaud C, Cadranet J. (2007). Bronchopulmonary aspergillosis infections in the non-immunocompromised patient. Rev Pneumol Clin. 63:155-166.

¹² Tyagi S, Kramer FR. (1996). Molecular beacons: Probes that fluoresce upon hybridization. Nature Biotechnology: 14: 303-308.

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 physically separates the fluorophore and the quencher enabling them to fluoresce upon excitation. The amount of fluorescence at any given cycle, or following cycling, depends on the amount of specific amplicons present at that time. The Real-Time PCR System simultaneously monitors the fluorescence emitted by each beacon.

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 Roche LightCycler® 2.0 and LightCycler® v4.1 software.
- Do not use reagents or controls if the protective pouches are open or broken when received.
- Reagents and controls are not interchangeable between kits with different 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 re-frozen or re-used 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-tips 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 *Aspergillus* or IAC amplicons, do not open the reaction tubes after amplification.

- Additional controls may be tested according to guidelines or regulations of local, state, provincial, federal 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 removed from the box and 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 (Green Cap)	<0.01% Primers <0.01% Molecular Beacons <0.0001% Internal Amplification Control (IAC) The Internal Amplification Control is a recombinant DNA plasmid containing a non-infective sequence unrelated to target (<i>Aspergillus</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 containing the <i>Aspergillus</i> target sequence	25 µL

Tris-HCl Buffer

The kit also contains:

- MycAssay™ Aspergillus 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, when 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 at a later date.

Equipment/Materials required but 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™ Aspergillus assay is total genomic DNA extracted from clinical BAL and other lower respiratory tract samples. The following isolation kit and equipment 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 Adaptor Plate (REF: 080-015 available from Myconostica)

MycAssay Colour Compensation (CC) kit

Accurate analysis of data produced using MycAssay™ Aspergillus 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™ Aspergillus 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 laboratory¹³, separated from areas used for DNA extractions, that is regularly cleaned with DNA decontaminating reagents.
- However, avoid using DNA decontaminating reagents when performing 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 positive control DNA material and contamination could cause false positive test results.**
- Wear gloves at all times.
- All reagent tubes 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.

¹³ 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 LightCycler® 2.0 Real-Time PCR System (instrument, associated computer and centrifuge) and launch the relevant software. Enter 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™ Aspergillus 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 this run file later.
- 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 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:

Number of Pouches	Maximum number of patient samples
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 patient 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 patient 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 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 patient samples. The positive control should always be set up last.
- 1.11 Reagent and DNA volumes are shown in the table below:

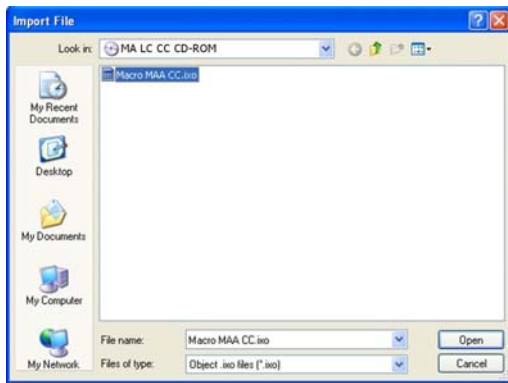
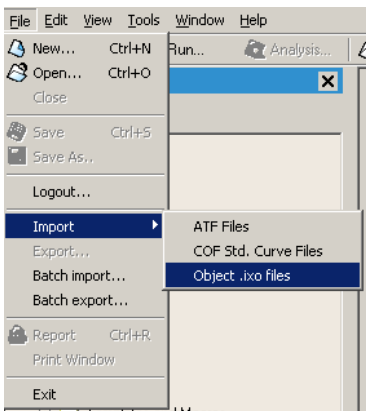
Reagent	Reaction		
	Negative control	Patient samples	Positive control
Tube 1 (Orange cap)	7.5 µL	7.5 µL	7.5 µL
Tube 2 (Green cap)	7.5 µL	7.5 µL	7.5 µL
Tube 3 (Clear cap)	10 µL	-	-
Patient Samples	-	10 µL	-
Tube 4 (Black cap)	-	-	10 µL
Total volume	25 µL	25 µL	25 µL

- 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.

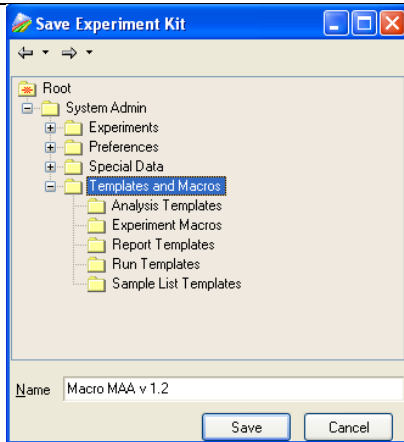
- 1.13 Use a new pipette tip for every liquid transfer. Re-cap each reagent tube after use and immediately discard it, and any remaining contents, into a sealable clinical waste container. Unused reagents cannot be saved for later use.
- 1.14 Take extra care when pipetting Tube 4 (positive control DNA) 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™ Aspergillus 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 Aspergillus Myconostica Protocol CD-ROM**.
2.2 Go to **Import** via the **File** directory and select **Object .ixo** files. Import the **Macro MAA v1.2.ixx** file from the CD to your database.



- 2.3 Go to **Save** via the **File** directory and save the macro in the desired location in your database.

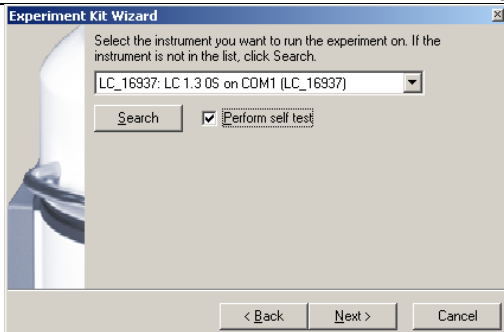


2.4 Select the **Run Macro** option from the Toolbar.



2.5 Select the **Macro MAA 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.



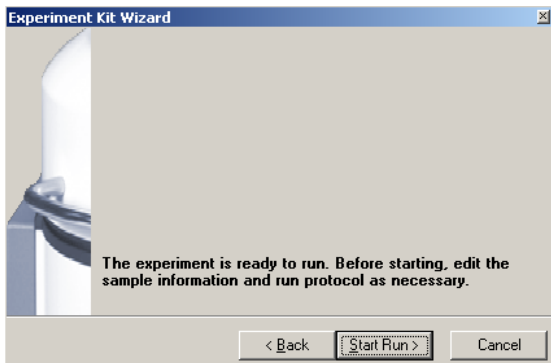


- 2.7 Name and save the run file in a desired location.
- 2.8 Go to the Samples section by clicking the tab in the left of the screen. Edit sample number in the **Samples Count** box and names in the **Capillary view** tab. Name the samples as it states in your experimental plan as positioned in the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

Sample Name	Repl. Of	Sample Note
1 Negative Control		
2 Repl. of Negative Control	1	
3 Repl. of Negative Control	1	
4 Asp 10e3 copies		
5 Repl. of Asp 10e3 copies	4	
6 Repl. of Asp 10e3 copies	4	
7 Asp 10e4 copies		
8 Repl. of Asp 10e4 copies	7	
9 Repl. of Asp 10e4 copies	7	
10 Asp 10e5 copies		
11 Repl. of Asp 10e5 copies	10	
12 Repl. of Asp 10e5 copies	10	
13 Asp 10e6 copies		
14 Repl. of Asp 10e6 copies	13	
15 Repl. of Asp 10e6 copies	13	
16 Positive Control		

- 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

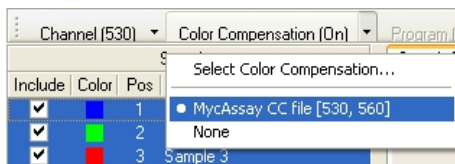
- 3.2 **Remember:** a colour compensation object must be applied prior to analysing results for MycAssay™ Aspergillus on the LightCycler® 2.0. If you have not yet created one, please do so now before continuing with Data Analysis and Interpretation.

When the run has finished check for the contents of the popped up report and print it if desired.

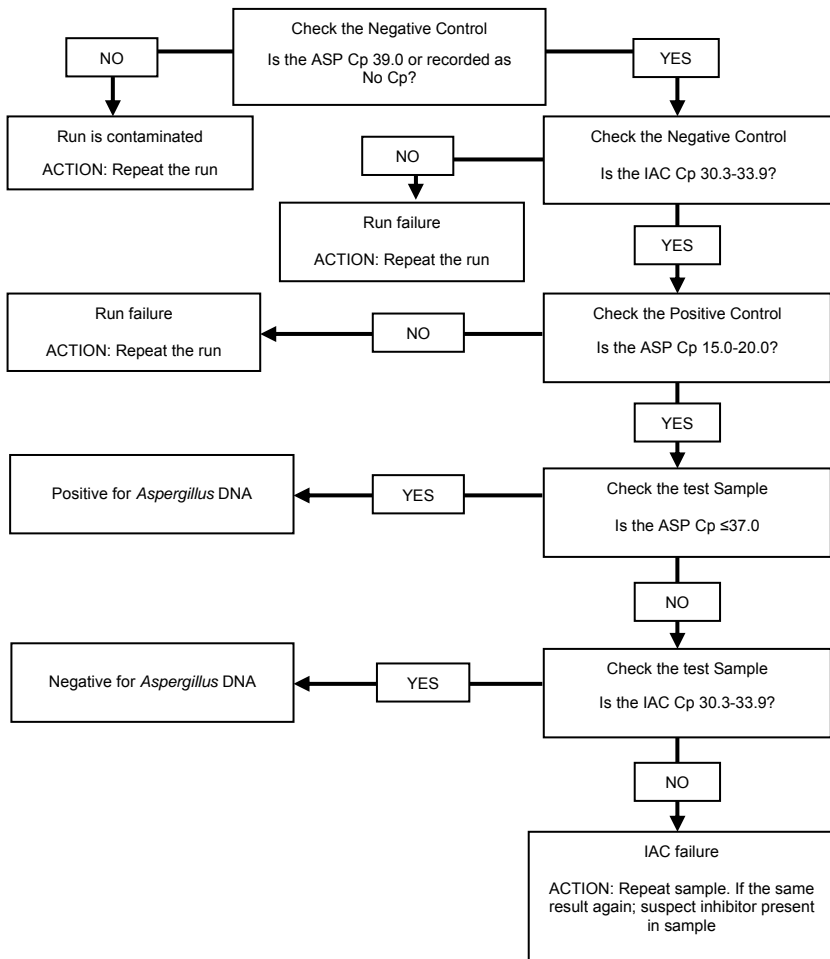
- 3.3 The Aspergillus results can be viewed in the Pathogen (530) analysis section and the IAC results in the IAC (560) analysis section.



- 3.4 In both sections, **ASP (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).



Sample	Pathogen (530) Cp	IAC (560) Cp	Interpretation	Further Action
Negative Control	39.0 or No Cp	Within 30.3-33.9	Negative Control acceptable	Patient results are valid
Negative Control	39.0 or No Cp	<30.3 or >33.9	Failure in Negative Control	Repeat entire run
Negative Control	<39.0	Within 30.3-33.9	Contamination	Repeat entire run
Positive Control	Within 15.0-20.0	N/A	Positive Control acceptable	Patient results are valid
Positive Control	<15.0 or >20.0	N/A	Failure in Positive Control	Repeat entire run
Patient Sample	≥39.0 or No Cp	Within 30.3-33.9	Negative for <i>Aspergillus</i>	Report result: Outcome 1
Patient Sample	≤37.0	N/A	Positive for <i>Aspergillus</i>	Report result: Outcome 2
Patient Sample	39.0 or No Cp	<30.3 or >33.9	IAC failure in sample	Repeat sample: Outcome 3

*CCO = clinical cut-off. All results at or below this level are considered clinically positive. Other samples may report Cp values >37.0, but these are reflective of normal or background levels of *Aspergillus* load in the respiratory sample.

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:

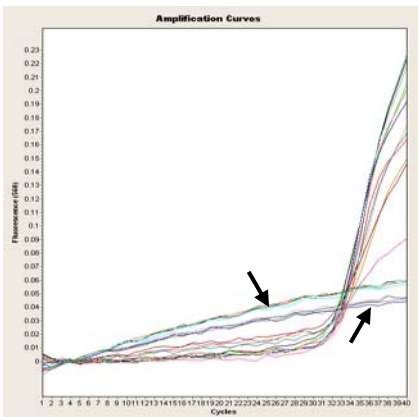
- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.
- Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.
- An error occurred during step 1.11/1.13 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 ASP (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.

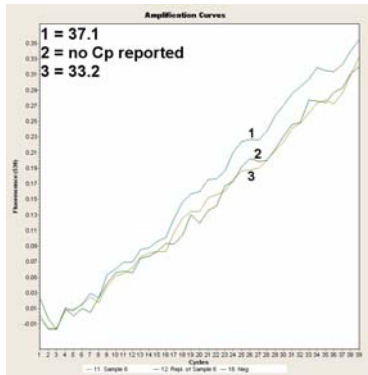
- Ensure that the work area and instruments are thoroughly dry after the use of decontaminating agents prior to PCR set up.
- Run the patient sample again. If the problem repeats, the PCR inhibitor is present in the sample. Report the sample as Undetermined.

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

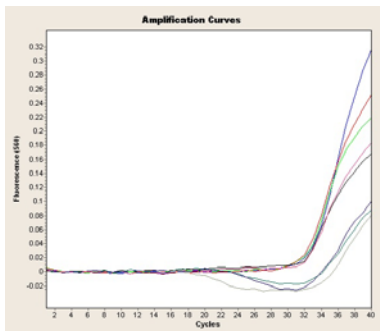
- There was no or an incorrect Colour Compensation file was applied to the experiment results.
- In both analysis sections check if the Colour Compensation is ON and that the same MycAssay CC file is applied in both channels.

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 Aspergillus, 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)

Performance Characteristics and Limitations

The kit was initially validated using the Cepheid SmartCycler®. Certain of the assay performance claims were re-validated on the 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 LightCycler® 2.0 protocol described above, and PCR templates generated at Myconostica, the LoB for the MycAssay™ Aspergillus was determined to be a Cp of 39.0, while the LoD was determined to be <25 copies of target DNA, using the AF293 strain of *A. fumigatus*.

Analytical Selectivity

Genomic DNA extracted from *Penicillium* spp. generates positive results. This is due to the fact that the sequences of the molecular targets are highly conserved between *Aspergillus* and *Penicillium*. Therefore, it must be noted that a positive result with this assay may be the result of infection by *Penicillium*, rather than *Aspergillus*.

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; *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 apiosperinu*, *S. prolificans*, *Sporothrix schenckii*, *Trichosporon capitatu*. The following bacterial species did not report a positive result; *Bordetella pertussis*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Haemophilus influenza*, *Lactobacillus plantarum*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Mycoplasma pneumonia*, *Neisseria meningitides*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumonia*, *S. pyogenes*, *S. salivarius*.

Human DNA and the following 3 species *Pneumocystis jirovecii*, *Histoplasma capsulatum* and *Alternaria alternata* were not tested on the LightCycler® 2.0 system, but had been tested on the SmartCycler®: . All gave negative results with the assay.

Reproducibility and Repeatability

Repeatability and reproducibility were determined by 4 different operators testing a panel of 7 different templates, in triplicate, for a total of 168 assays. Experiments were performed using 1 manufactured batch of MycAssay™ Aspergillus kit, on 2 different instruments situated at 2 different locations in the UK.

The results were analysed against the Limit of Blank (LoB) and the clinical cut-off (CCO). At a concentration of 6 times the Limit of Detection (LoD), the results from 100% of all samples tested were in agreement (positive) for LoB, and 87.5% of the samples were in agreement at the CCO. At concentrations higher than 6 x LoD, results from 100% of all samples were in agreement at both the CCO and LoB.

Transfer of the Clinical Cut-Off

The clinical cut-off of 36.0 had been established on the SmartCycler®. This cut-off was transferred analytically to the LightCycler® 2.0 platform using a template with an *Aspergillus* concentration that had been shown to yield ≥95% positive results at the CCO on the SmartCycler®. A Cp value of 37.0 was determined, which was then confirmed using templates of 3 different concentrations.

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, sodium chloride, sodium cromoglicate, terbutaline, Tobramycin.

Performance Evaluation

Respiratory samples (BAL) that had been collected from 2 hospitals, extracted using the MycXtra™ kit, and stored were used to evaluate the performance of the MycAssay™ Aspergillus kit with clinical samples. Comparisons were made to both clinical diagnosis and culture.

The cut-off value of a Ct of 36.0 was established following review of a dataset of samples sourced from different sites and different patient populations. Different cut-offs were evaluated for the probability of differentiating between disease state and non-disease state.

PCR v Clinical Diagnosis

	Clinical positive	Clinical negative		
PCR positive	31	1	0.97	PPV
PCR negative	2	10	0.83	NPV
	0.94	0.91		
	Sensitivity	Specificity		

PCR v *Aspergillus* Culture

	Culture positive	Culture negative		
PCR positive	29	3	0.91	PPV
PCR negative	2	10	0.83	NPV
	0.94	0.77		
	Sensitivity	Specificity		

Of the samples tested, 0.8% contained PCR inhibitors as reported by the IAC, following extraction using the MycXtra™ kit.

Clinical Reporting

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

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

Outcome No 1

“*Aspergillus* spp. not detected”

Outcome No 2

“*Aspergillus* spp. detected; Positive result. This assay also detects *Penicillium* spp.”

Outcome No 3

“Test failed; inhibitors or other unknown substance present”

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.

- False positive results are possible if the infecting agent is *Penicillium* spp. which cannot be differentiated from *Aspergillus* spp. using this kit.

- 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 *Aspergillus* contaminated air, poor experimental technique with respect to the positive control or external (especially pipettor) contamination with *Aspergillus* DNA.
- As a true positive result may be obtained from patients who are transiently or persistently colonised by *Aspergillus* spp., clinical judgment is required in interpretation of the test results, in the context of disease.

LICENSING

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