

MycAssay™ Aspergillus

Stratagene Mx3000 series

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 Stratagene Mx3000 series instruments, either Mx3000P® or Mx3005P®, (using MxPro software version 4.10).

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, Lavalé 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 Stratagene Mx3000 series instruments, either Mx3000P® or Mx3005P®, (using MxPro software version 4.10).
- During validation studies the following was noted, specific to this instrument;
 - If the instrument has been used immediately before, please allow the lamp to cool for at least 1 hour before setting up a MycAssay™ Aspergillus run, otherwise there can be a loss in sensitivity resulting in false negatives.
 - There is less precision at low concentrations at and around the clinical cut-off relative to other instruments we have tested.
- 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.
- This kit was validated using 0.2ml 8-strip PCR tubes with attached caps (Starlab Cat # A1402-3700). Use of other sources/types of plastic could invalidate the thresholds and, therefore, the claims made in the IFU. It is recommended that should an alternative source be used, that local validation should be conducted with the positive and negative control reactions.

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 Tris-HCl Buffer	25 µL

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

- Stratagene Mx3000 series Real-Time PCR System (including user manual, attached computer and MxPro software version 4.10)
- 0.2ml 8-strip PCR tubes with attached caps (Starlab Cat # A1402-3700)
- Support rack for 0.2ml tubes (strips of 8)
- Mini-centrifuge adapted for 8-strip 0.2ml tubes
- Micro centrifuge
- Vortex mixer
- Micropipettes (volumes required 7.5 µL – 20 µL)
- Sterile low-retention filtertips
- Disposable gloves, powderless
- Proprietary DNA decontaminating solution
- Permanent marker pen
- DNA isolation kit (see below)

Specimen

The specimen for the MycAssay™ 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)

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.
- On the Stratagene Mx3000 series: number the strips of plastic which attach the cap to the tube (not the cap itself) and make a note of what each number corresponds to.
- Please allow the lamp to cool for at least 1 hour after a run before setting up MycAssay™ Aspergillus to minimise false negative results. . Following this cooling period, the lamp will require a 20 minute warm up period as noted in 1.1 below

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 Real-Time PCR System (instrument and associated computer) and launch the relevant software. Enter usernames and passwords as required. The lamp will require 20 minutes to warm up. Do not start the run until the lamp is ready.
- 1.2 Ensure the work area has been cleaned using DNA decontaminating reagents and allowed to dry completely; avoid use during assay set-up as excess cleaning solution may inhibit the PCR reactions.
- 1.3 A pouch contains one each of Tube 1, Tube 2, Tube 3 and Tube 4. There are sufficient reagents in one pouch to run 8 reactions. At least one positive control and one negative control reaction must be performed per run where the reagents are from a single kit lot. One pouch therefore can analyse 6 patient samples. If more than 6 samples need to be tested, more than one pouch can be used if the pouches used are from the same kit lot. A maximum of 38 patient samples may be tested using the 5 pouches in a kit.
- 1.4 Calculate the number of reactions required, referring to the table below:

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

- 1.5 Remove the appropriate number of pouches from the freezer. Do not use any pouch that is no longer sealed. If the patient samples were frozen after extraction, also remove these from the freezer.
- 1.6 Tear open the required number of pouches and remove the tubes. If more than one pouch is being used, but only one set of positive and negative controls are being run, it is only necessary to remove Tubes 3 and 4 from one pouch. **Exercise caution when handling Tube 4. This contains positive control DNA material and contamination could cause false positive test results.**

- 1.7 Allow the tubes' contents to thaw by placing on the laboratory bench for 5-10 minutes, ensuring that the contents of each tube are completely thawed before proceeding. Vortex mix the tubes' contents and the patient samples; follow by a short spin in a microcentrifuge to ensure collection of all the contents at the base of the tubes before use. Place the required number of 0.2 mL PCR tubes in a support rack. Take care not to leave any marks on the optical cap.
- 1.8 Always set up the negative control first, followed by the patient samples. The positive control should always be set up last.
- 1.9 Reagent and DNA volumes are shown in the table below:

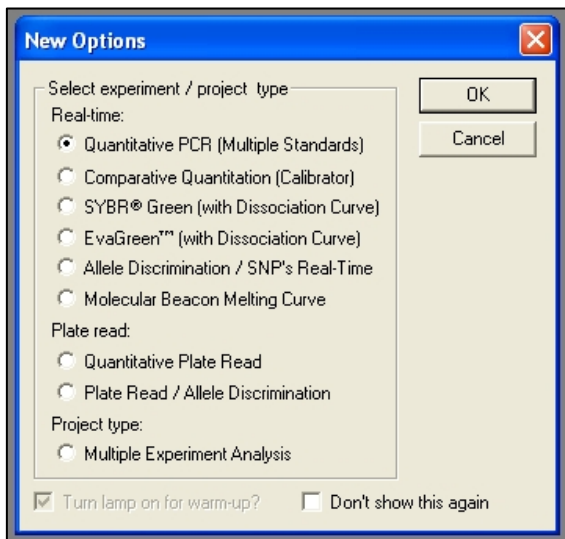
Reagent	Reaction		
	Negative control	Patient sample	Positive control
Tube 1 (Orange cap)	7.5 µL	7.5 µL	7.5 µL
Tube 2 (Green cap)	7.5 µL	7.5 µL	7.5 µL
Tube 3 (Clear cap)	10 µL	-	-
Patient Sample	-	10 µL	-
Tube 4 (Black cap)	-	-	10 µL
Total volume	25 µL	25 µL	25 µL

- 1.10 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.
- 1.11 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.
- 1.12 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.
- 1.13 Make sure all reaction tube lids are firmly closed but do not mark the actual lids. Instead, use the strip of plastic that attaches each cap to each tube. Spin down the tubes for 10 seconds using the mini-centrifuge adapted for 0.2 mL PCR tubes.
- 1.14 Proceed to Section 2 promptly. MycAssay™ Aspergillus reactions are stable on the bench for up to 60 minutes.
- 1.15 Following the PCR set-up ensure the work area is thoroughly cleaned using DNA decontaminating reagents.

2. Performing the run

- 2.1 Open up the MxPro software, version 4.10.
- 2.2 Insert the MycAssay Aspergillus Myconostica Protocol CD-ROM
- 2.3 In the New Options menu, select the first option: Real Time: Quantitative PCR (Multiple Standards), and click OK, as shown:



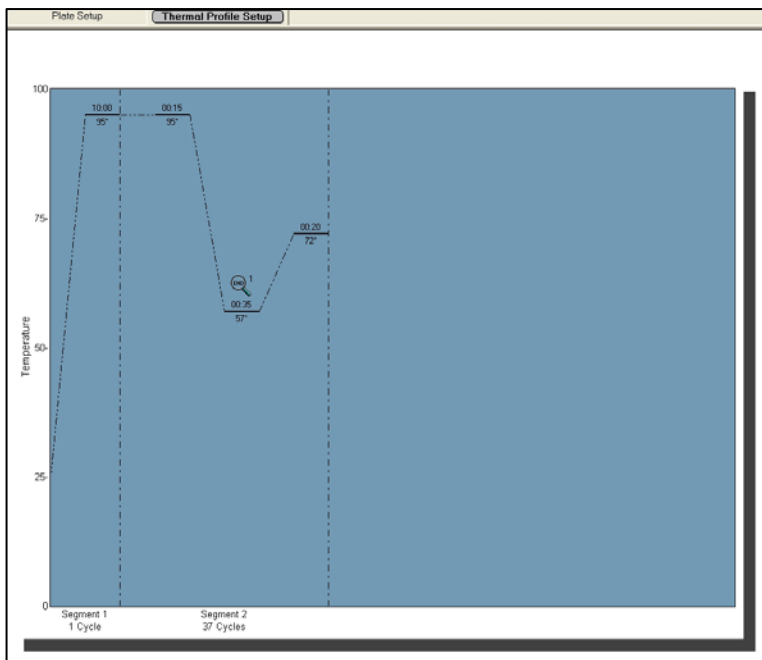
- 2.4 In the **Plate Setup** tab, click on the **Import** button on the right. Select the **MycAssay Asp Myconostica Protocol CD-ROM** from the drop-down list in **Look-In:** and then import the file named **MycAssay Aspergillus v2.mxp**. Click **Finish**. Once this is completed the **Plate Setup** should look like this example;

The screenshot displays the 'Plate Setup' window. The main area is a grid representing a 96-well plate. The columns are numbered 1 through 12, and the rows are labeled A through H. Each well in the grid contains the text 'Myc ASP' in red. Above the grid, there are tabs for 'Plate Setup' and 'Thermal Profile Setup'. To the right of the grid is a control panel with the following elements:

- Buttons for 'Import...' and 'Quick Details'.
- A 'Well type' dropdown menu set to '<blank>'. Below it is a 'Show Well Names' checkbox.
- A 'Collect Reference data' section with checkboxes for 'CVS', 'BOX', 'E 888', 'LSD', and 'MSE'.
- A 'Reference file' dropdown menu.
- An 'Assign Assay Names' section with a 'Standard quantity' input field.
- A 'Standard units' section with a 'Identify replicates' dropdown menu.
- A 'Replicate symbol' dropdown menu set to 'none'.
- An 'Auto-Increment' checkbox.
- A 'Clear Selected Wells' button.
- A 'Plate setup comments' text area.
- Buttons for 'Full Screen Plate' and 'Next'.

At the bottom left, there are 'Reference aliquot' and 'Well types aliquot' sections, each with a dropdown menu.

- 2.5 It is recommended that in those wells which are empty that the **Well Type** be set to **<blank>** to prevent refraction of light from the plastic interfering with the signals from those wells which do contain reactions.
- 2.6 Repeat the import process in the **Thermal Profile Setup** tab to import the PCR program for this assay; set the **Thermal Profile Design** to **Custom** and then **Import** from the same file as described in 2.4. Once this is completed the **Thermal Profile Setup** should look like this example;

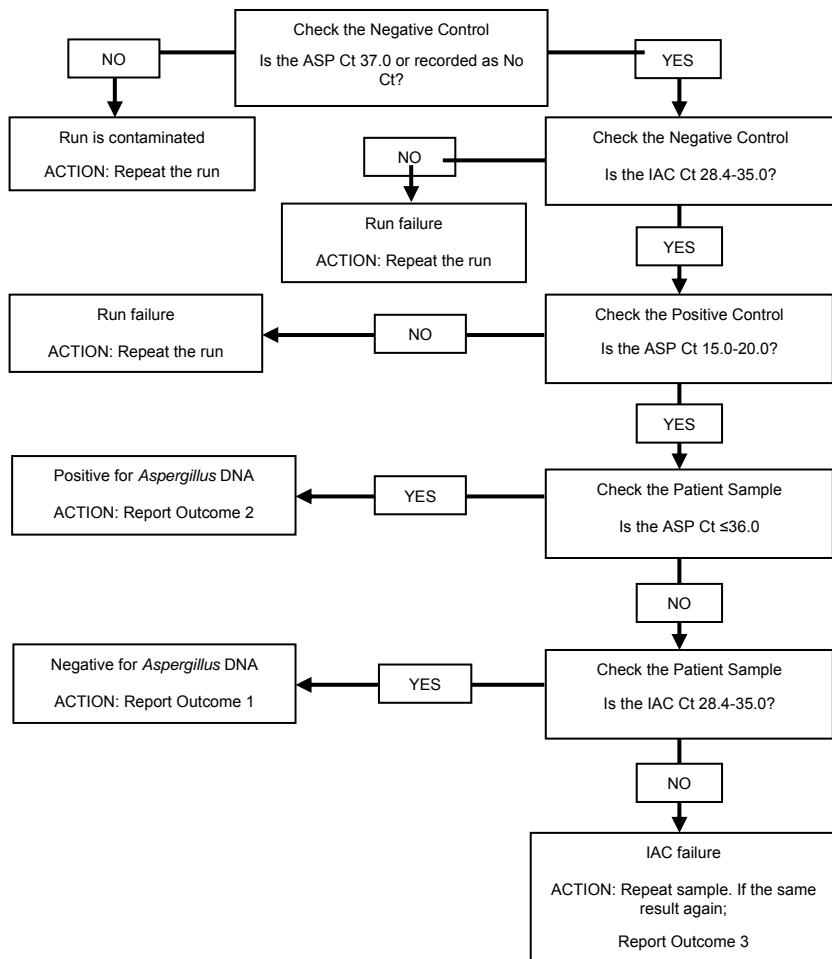


- 2.7 In the **Plate Setup** tab, name the wells appropriately; Right click on a highlighted well (or group of wells if replicates) and select **Well Information** from the list of options. Type in the name of the sample used in that well in the **Name:** section.
- 2.8 When all the wells are named appropriately, save the run, giving it an appropriate file name that includes the date and the operator's initials, and then start the run by selecting the **Run** button on the top right of the screen in the **Instrument** tab, ensure the check box is ticked to turn the lamp off after the run has completed, then click on the **Start** button in the bottom right corner.

- 2.9 Remember to allow the instrument to cool for at least 1 hour before starting at step 1.1 again.

3.0 Data Analysis and Interpretation

- 3.1 Once the run has finished, the results can be viewed by selecting the **Analysis** button on the top right of the screen, followed by the **Results** tab.
- 3.2 With the **Amplification Plots analysis area** selected, set the thresholds for each channel as follows, and lock by clicking on the padlock icon;
ASP = 1000
IAC = 100
- 3.3 The **Adaptive Baseline** should also be selected; this is usually the default setting for this software.
- 3.4 Save the changes. Each channel can be viewed separately by clicking the boxes in the Assays Shown section in the bottom left of the screen on or off.
- 3.5 Data can be exported for further manipulation in to Excel by **File>Export Text Report>Export Text Report to Excel**. Only those wells/dyes which have been highlighted will be exported, so ensure that all relevant/required wells/dyes are selected.
- 3.6 Open the saved .csv file with Excel or similar spreadsheet software.
- 3.7 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	ASP MycAssay Ct	IAC MycAssay Ct	Interpretation	Further Action
Negative Control	37.0 or Undetected	Within 28.4-35.0	Negative Control acceptable	Patient results are valid
Negative Control	37.0 or Undetected	<28.4 or >35.0	Failure in Negative Control	Repeat entire run
Negative Control	<37.0	Within 28.4-35.0	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	>36.0	Within 28.4-35.0	Negative for <i>Aspergillus</i> at CCO*	Report result: Outcome 1
Patient	≤36.0	N/A	Positive for <i>Aspergillus</i> at CCO*	Report result: Outcome 2
Patient	37.0 or Undetected	<28.4 or >35.0	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 Ct values >36.0, 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 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 OR that wells are annotated correctly within the software.
- Non-recommended tubes or plates were used.
- Thresholds are only valid when using the recommended Starlab tubes with caps (Cat # A1402-3700).

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.
- Non-recommended tubes or plates were used.

- Thresholds are only valid when using the recommended Starlab tubes with caps (Cat # A1402-3700).

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 1.10 or 1.12 and the Positive Control template (Tube 4) was placed in the wrong reaction tube.
- Repeat the run, taking great care during the set-up stage. Such errors can be detected by seeing a higher level of liquid in one reaction, and a lower level in another, compared to normal.
- Either Tube 1 or 2 reagent was not added to the reaction.
- Repeat the run taking care in the set-up stage. Such errors can be detected by seeing lower levels of liquid in this reaction compared to others.
- The Positive Control was incorrectly positioned in the instrument.
- Take care that the reaction tubes are placed in their designated sites OR that wells are annotated correctly within the software.
- Non-recommended tubes or plates were used.
- Thresholds are only valid when using the recommended Starlab tubes with caps (Cat # A1402-3700).

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

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

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

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

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

Performance Characteristics and Limitations

Mx3000 series Analytical Performance Data

The kit was initially validated using the Cepheid SmartCycler®. Certain of the assay performance claims were re-validated on the Mx3005P® platform, using 0.2ml 8-strip PCR tubes with attached caps (Star Lab Cat # A1402-3700), and are reported below.

Analytical Sensitivity

Using the Mx3000 series protocol described above, and PCR templates generated at Myconostica, the Limit of Blank (LoB) for the MycAssay™ Aspergillus was determined to be a Ct of 37.0, while the Limit of Detection (LoD) was determined to be <25 copies of target DNA, using the AF293 strain of *A. fumigatus*.

Reproducibility and Repeatability

Repeatability and reproducibility were determined by 6 different operators (OP1-6) testing a panel of 7 different templates, in triplicate, for a total of 252 assays. Experiments were performed using 1 manufactured batch of MycAssay™ Aspergillus kit, on 2 different instruments situated at 2 different locations.

The results were analysed against the Limit of Blank (LoB) and the clinical cut-off (CCO). At a concentration of ~6 times the LoD (~ 150 copies), the results from 75% of all samples tested were in agreement (i.e. positive) for LoB, and 67% of the samples were positive at the CCO. At a concentration of 20 times the LoD (~ 500 copies), 92% of samples tested were positive at the CCO. At concentrations higher than this, results from 100% of all samples were in agreement at both the CCO and LoB. For negative templates, all samples tested were negative at the CCO.

Template	OP1	OP2	OP3	OP4	OP5	OP6	Combined
0	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
150	35.9 ± 1.0	35.9 ± 0.7	34.7 ± 0.9	34.9 ± 0.7	36.3 ± 1.1	36.2 ± 0.8	35.7 ± 1.0
500	34.6 ± 0.5	34.7 ± 0.9	33.5 ± 0.7	33.8 ± 0.8	34.9 ± 1.5	34.9 ± 0.4	34.4 ± 1.0
1000	32.5 ± 0.4	32.4 ± 0.4	31.6 ± 0.4	31.9 ± 0.5	32.9 ± 0.6	33.0 ± 0.4	32.4 ± 0.6
1875	31.6 ± 0.3	32.0 ± 0.3	30.8 ± 0.4	31.0 ± 0.6	31.6 ± 0.9	32.7 ± 0.3	31.6 ± 0.8
3750	30.7 ± 0.3	31.1 ± 0.5	29.9 ± 0.2	30.3 ± 0.7	30.6 ± 0.5	31.4 ± 0.3	30.7 ± 0.7
7500	29.7 ± 0.4	29.9 ± 0.4	29.1 ± 0.4	29.5 ± 0.4	29.9 ± 0.8	30.3 ± 0.3	29.7 ± 0.6

Results of reproducibility testing for 6 operators presented by Template copy number. Panel members (column numbers are an approximation of *Aspergillus* DNA copies/Template panel member) varied from 0 copies to 7500 copies/reaction. For each operator the data represent the mean result and standard deviation of six runs / panel member. The standard deviations are larger across all operators and within each operator's runs for the panel members with the lower template copy numbers.

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 Mx3000 series platforms using a template with an *Aspergillus* concentration that had been shown to yield ≥95% positive results at the CCO on the SmartCycler®. A Ct value of 36.0 was determined, which was then confirmed using templates of 3 different concentrations.

The following Performance Claims were established using the Cepheid SmartCycler®

Analytical Specificity and Selectivity

Analytical specificity was tested using DNA extracted from 15 different Aspergilli species, including several strains each of *A. fumigatus*, *A. niger*, *A. terreus*, and *A. nidulans*. Signals detected above the LoB were recorded as a positive result.

All of the 15 *Aspergillus* spp. tested were positive with the assay. In addition to those previously mentioned, this includes *A. flavus*, *A. versicolor*, *A. glaucus*, *A. sclerotiorum*, *A. niveus*, *A. lentulus*, *A. unguis*, *A. candidus*, *A. wentii*, *A. tubingensis* and *A. foetidus*.

Genomic DNA extracted from *Penicillium* spp. also generated 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; *Alternaria alternata* *Blastomyces capitatus*, *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Cladosporium* spp., *Cryptococcus neoformans*, *Doratomyces microsporus*, *Fusarium solani*, *Histoplasma capsulatum* *Pneumocystis jirovecii*, *Rhizomucor pusillus*, *Rhodotonia rubra*, *Saccharomyces cerevisiae*, *Scedosporium apiosperinu*, *S. prolificans*, *Sporothrix schenkii*, *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 genomic DNA does not report a positive result with this assay.

Interfering Substances (contraindications for use)

The following compounds were tested at clinically relevant concentrations, and found not to inhibit the assay; 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.
- Clinical Performance Evaluation has not been confirmed using the MX3005P® instrument.
- 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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