myconostica

MycAssay™ Aspergillus
Cepheid SmartCycler®
Serum
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 serum as an aid to diagnosis of pulmonary aspergillosis.

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

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 diseases 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 implicated[1]. Most diseases caused by *Aspergillus* spp. affect the respiratory tract. Invasive aspergillosis (IA) occurs in at-risk patient groups including those having treatment for leukemia and lymphoma, hematopoetic stem cell (HSCT) and solid organ transplant patients as well as patients treated with corticosteroids and those with neutropenia or phagocyte dysfunction (i.e. chronic granulomatous disease and HIV infection).

[1] Species Database in www.aspergillus.org.uk

Invasive fungal disease (IFD) rates are nearly seven times higher in allogeneic HSCT patients than in autologous transplant patients, and invasive aspergillosis (IA) is responsible for approximately half of infections[2]. Aspergillosis is largely confined to the early post transplant neutropenic phase in autologous HSCT patients. Allogeneic HSCT patients are at risk for much longer periods not only up to but also beyond 100 days, owing to their more frequent GvHD and slow T cell recovery. In patients receiving chemotherapy for acute leukaemia or salvage regimens for relapsed leukaemia or lymphoma, IA is a leading cause of death.

Consensus definitions of Invasive Fungal Diseases have been revised and published by the European Organisation for Research (EORTC) and the Mycoses Study Group (MSG) including defined criteria for diagnosis of proven, probable and possible IA in patients with hematologic malignancy or following HSCT[3]. Currently, the criteria for 'probable IA' are defined as one host factor plus one clinical criterion plus one microbiological test. Diagnosis of 'possible IA' does not require a microbiology criterion. The microbiological tests accepted in the probable IA criteria include a serum based ELISA test that detects the presence of galactomannan. Galactomannans are polysaccharides that are released as the Aspergillus fungus grows. In serum, the test has a sensitivity in adult patients of 79.3% (95% C.I. 61.6-90.2%) and a specificity in adult patients of 88.8% (95% C.I. 82.6-93.0), in patients not receiving prophylaxis with azoles active against moulds[4]. Two consecutive positive GM tests are recommended to improve diagnostic accuracy.

A meta analysis by Mengoli et al reported on >10,000 blood, serum and plasma samples from 1618 patients at risk for IA. They calculated the sensitivity and specificity of a single PCR positive blood sample to be 0.88 (95% C.I. 0.75-0.94) and 0.75 (95% C.I. 0.63 – 0.84) respectively and the diagnostic odds ratio for proven and probable cases to be 16.41 (95% C.I. 6.43 – 41.88)[5].

MycAssay™ Aspergillus is a molecular diagnostic kit for the detection of *Aspergillus* spp. genomic DNA using Molecular Beacon[6] Real-Time PCR technology. The whole test procedure, including extraction of DNA from the clinical sample, can be completed

[2] Kontoyiannis DP et al Clin Infect Dis 2010: 50(8); 1091-1100
[3] Ascioglu S et al Clin Infect Dis 2002: 34; 7-14
[4] BioRad Platelia *Aspergillus* EIA product information
[5] Mengoli C. et al The Lancet ID 2009: 9; 86-96
[6] Tyagi S, Kramer FR. (1996). Molecular beacons: Probes that fluoresce upon hybridization. Nature Biotechnology: 14: 303-308.

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 Aspergilli, 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 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.
- In analytical validation studies, levels of transaminase of 22.2 U per 0.5 mL serum were shown to have a possible degradation effect on *Aspergillus* DNA.
- This assay has been evaluated with serum collected in Greiner Red Top serum collection tubes. Other serum/blood collection tubes may contain inhibiting or competing substances that have not been tested.
- This assay has been validated for serum specimens. Validation data are not available for plasma or whole blood.
- This test is only for use with the Cepheid SmartCycler® system with Dx diagnostic software versions 1.7b and 3.0
- Do not use reagents or controls if the protective pouches are open or broken 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.
- Ensure all reagents used are free from fungal contamination.
- 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.
- Serum may be stored up to 48h in a refrigerator (2-8ºC) or freezer (-15 to -25ºC).
- 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.

		Volume
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 (*Aspergillus*) 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 *Aspergillus* 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

A. Equipment required
- SmartCycler® Real-Time PCR System (including user manual, attached
- computer and SmartCycler® Dx software versions 3.0 or 1.7b)
- SmartCycler® reaction tubes
- Mini centrifuge adapted for SmartCycler® reaction tubes
- Plastic support rack for SmartCycler® reaction tubes

B. Common equipment / materials required
- 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 serum samples. The following DNA extraction kit and equipment, used during validation, is recommended for this purpose:

- High Pure manual DNA extraction kit (Roche Diagnostics Cat. No. 11 796 828 001)
- Proteinase K solution (Sigma Aldrich Chemicals Cat. No. P4850-5ML)
- 2-Propanol (Sigma Aldrich Chemicals Cat.No. 19516-25ML)
- 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 (including DNA extraction) takes approximately 2½ 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[7], separated from areas used for DNA extractions, that is regularly cleaned with DNA decontaminating reagents.
- 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.

[7] 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.

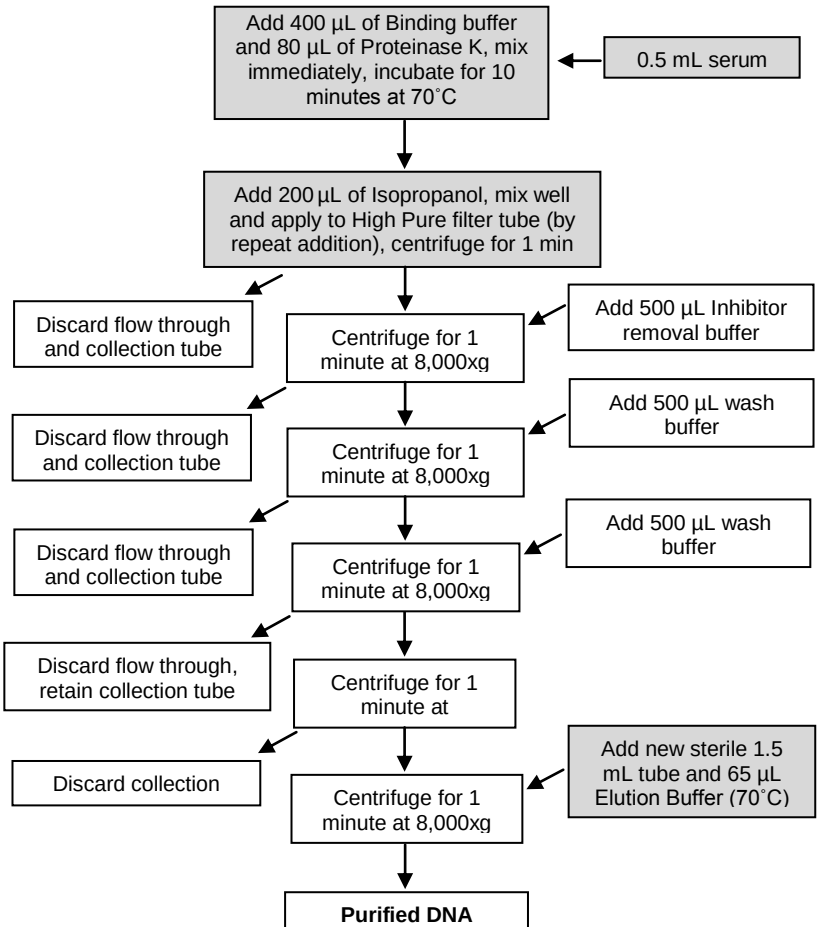
- All reagent tubes must be capped following use and prior to disposal.
- Take care to identify the SmartCycler® reaction tubes appropriately when multiple patient samples are being processed.
- Take care when selecting the protocol run file: Select **SERUM MycAssay Asp v1**. Do NOT select **MycAssay Aspergillus Dx1,7b v1.3** or **MycAssay Aspergillus v1_3**.

Procedure for Use

The procedure has 2 stages, DNA extraction from serum followed by Real-Time PCR quantification. DNA extraction is achieved using the Roche High Pure PCR Template Preparation Kit. The High Pure PCR Template Preparation Kit is designed to purify nucleic acids from a variety of sample types. The extraction protocol, detailed in this IFU, has been optimised to isolate *Aspergillus*.spp DNA from serum and is suitable for use with the MycAssay™ Aspergillus Serum kit.

IMPORTANT NOTE: The manufacturer's instructions have been modified to improve the yield of DNA recovered from a serum sample and to improve the sensitivity of the test. Certain reagents detailed in steps 1 and 2 of Section 2.3 in the High Pure PCR Template Preparation Kit IFU, ,will be depleted before others, and will need to be replaced. During the validation process, Proteinase K from Sigma Aldrich was used.

Extraction Protocol – shaded areas identify those steps that are modified from manufacturer's instructions.



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.

1.2 Ensure the work area has been cleaned using DNA decontaminating reagents and allowed to dry completely; avoid use during assay set-up as excess cleaning solution may inhibit the PCR reactions.

1.3 A pouch contains one each of Tube 1, Tube 2, Tube 3 and Tube 4. There are sufficient reagents in one pouch to run 8 reactions. At least one positive control and one negative control reaction must be performed per run where the reagents are from a single kit lot. One pouch therefore can analyse 6 patient samples. If more than 6 samples need to be tested, more than one pouch can be used if the pouches used are from the same kit lot. A maximum of 38 patient samples may be tested using the 5 pouches in a kit.

1.4 Calculate the number of reactions required, referring to the table below:

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

1.5 Remove the appropriate number of pouches from the freezer. Do not use any pouch that is no longer sealed. If the patient samples were frozen after extraction, also remove these from the freezer.

1.6 Tear open the required number of pouches and remove the tubes. If more than one pouch is being used, but only one set of positive and negative controls are being run, it is only necessary to remove Tubes 3 and 4 from one pouch. **Exercise caution when handling Tube 4. This contains positive control DNA material and contamination could cause false positive test results.**

1.7 Allow the tubes' contents to thaw by placing on the laboratory bench for 5-10 minutes, ensuring that the contents of each tube are completely thawed

before proceeding. Vortex mix the tubes' contents and the patient samples; follow by a short spin in a microcentrifuge to ensure collection of all the contents at the base of the tubes before use.

1.8 Place the required number of SmartCycler® reaction tubes in their support rack(s). Take care to only touch the neck of the reaction tubes with your hands.

1.9 Always set up the negative control first, followed by the patient samples. The positive control should always be set up last.

1.10 Reagent and DNA volumes are shown in the table below:

Reagent	Reaction		
	Negative control	**Patient sample**	**Positive control**
Tube 1 (Orange cap)	7.5 µL	7.5 µL	7.5 µL
Tube 2 (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

1.11 Add reagents in the order shown in the table above; Tube 1, then Tube 2, followed by the template (Negative control, Patient sample, or Positive control). Take care when taking aliquots from Tube 1; the liquid is slightly viscous and can stick on the inner ridge of the tube. If this happens, re-spin to collect the final contents in the base of the tube before attempting to remove the final aliquots.

1.12 Use a new pipette tip for every liquid transfer. Re-cap each reagent tube after use and immediately discard it, and any remaining contents, into a sealable clinical waste container. Unused reagents cannot be saved for later use.

1.13 Take extra care when pipetting Tube 4 (positive control DNA) to ensure it does not contaminate any other reaction tube. Closing the lids on the other reaction tubes before opening Tube 4 can reduce the risk of cross-contamination.

1.14 Make sure all reaction tube lids are firmly closed and then label each lid using a permanent marker pen e.g. POS for positive control, NEG for negative control and patient ID for patient samples. Spin down the reaction tubes for 10 seconds using the specially-adapted mini centrifuge. Visually check that there are no bubbles present in the reaction mixtures.

1.15 Proceed to Section 2 promptly. MycAssay™ Aspergillus reactions are stable on the bench for up to 60 minutes.

1.16 Following the PCR set-up ensure the work area is thoroughly cleaned using DNA decontaminating reagents.

2. Performing the run

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

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

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

2.1 SmartCycler® Dx Diagnostic software version 1.7b

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

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

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

2.1.4 Select the file **SERUM MycAssay Asp v1.DXA** from the CD-ROM. WARNING: This file will be one of two recognised by the software; ensure you pick the correct file.

2.1.5 On the next screen highlight the filename **SERUM MycAssay Asp. v1** and click **OK**, followed by **Proceed** and **OK**.

2.1.6 Close the software. When it is reopened the **SERUM MycAssay Asp v1** assay will be available for use when creating a new run.

2.1.7 Click on the **Create Run** tab. Enter an appropriate **Run Name** (it is recommended that this includes the date and operators initials as a minimum), or leave blank if you wish the name to be created automatically by the software.

2.1.8 Select **SERUM MycAssay Asp v1** as the assay.

2.1.9 Enter the **Lot Number** and **Expiration Date** of the kit as printed on the kit box and on each pouch. The lot number will be in the form of M-XXXXXXXX.

2.1.10 Enter the **Number of specimens** in the box and click **Apply**. The **Sample ID** for each specimen will automatically be named **SPEC** by the software. Therefore, rename each site appropriately for identification purposes; i.e. double click on **SPEC** to highlight it and then type in the sample ID.
The software will automatically include a Negative and Positive control in the Real-Time PCR run.

2.1.11 Carefully place the reaction tubes into the designated sites in the SmartCycler® block and click **Start Run**. N.B. Take care when placing the reaction tubes into the designated sites as they may not be in the same order as your set-up. Make a note of the run name and click **OK**. The run will now start and red lights will appear above each site in use on the block.

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

2.2 SmartCycler® Dx Diagnostic software version 3.0

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

2.2.2 Insert the **MycAssay Aspergillus Myconostica Protocol CD-ROM** and click on the **Define Assays** tab, and **Import** the **SERUM MycAssay Asp v1.sca** file from the CD-ROM. WARNING: This file will be one of two recognised by the software; ensure you pick the correct file.

2.2.3 Click on the **Create Run** tab. Enter an appropriate **Run Name** (it is recommended that this includes the date and operators initials as a minimum), or leave blank if you wish the name to be created automatically by the software.

2.2.4 Select **SERUM MycAssay Asp v1** as the assay.

2.2.5 Enter the **Lot Number** and **Expiration Date** of the kit as printed on the kit box and each pouch. The lot number will be in the form of M-XXXXXXXX.

2.2.6 Enter the **Patient (Sample) ID** and the **Number of specimens** (replicates) in the appropriate boxes and click **Apply**. Do this for all patient samples being tested. The software will automatically include a Negative and Positive control in the Real-Time PCR run.

2.2.7 Carefully place the reaction tubes into the designated sites in the SmartCycler® block and click **Start Run**. N.B. Take care when placing the reaction tubes into the designated sites as they may not be in the same order as your set-up. Make a note of the run name and click **OK**. The run will now start and red lights will appear above each site in use on the block.

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

3. Data Analysis and Interpretation

3.1 The results can be viewed in Dx software, by selecting the **View Results** tab.

3.2 Click on the **View Another Run** button at the bottom of the page, select the run you wish to view then click **OK**.

3.3 The **Patient Results** should already be selected in the **Views** list. The patient (sample) ID and the subsequent assay result will be clearly listed. The results can be interpreted using the table below:

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

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

3.4 To view individual sample results for either *Aspergillus* or IAC separately, select **Sample Results** from the **Views** list and click on the individual tabs for each target. The results will be displayed in the same format as the **Patient Results** but for each individual target.

3.5 If a Patient sample reports an Invalid result, this is due to a failed IAC result (indicated by Unresolved in the **Sample Results** tab); repeat the reaction (plus Positive and Negative controls). If the reaction continues to fail, an inhibiting substance may be present in the template and a Negative result cannot be relied upon.

3.6 To export run data to allow transfer to another computer, go to the **Tools** directory at the top of the screen and select **Data Management**, followed by **Archive Run(s)** from the drop down menu. A message screen will appear, click **Proceed.** Select the run to be archived by ticking the box to the left and click **OK**. A warning message will appear stating how many runs are to be archived; if this number is correct, click **Proceed**. Select a destination to save the run file e.g. USB data stick. Click **Save** and make a note of the file name. A message screen will appear stating how many runs are to be archived, if this number is correct, click **Proceed.**

3.7 To import run data, go to the **Tools** directory at the top of the screen and select **Data Management**, followed by **Retrieve Run(s)** from the drop down menu. A message screen will appear, click **Proceed.** Go to **Look In:** and select the storage device used to archive the run data (see section 3.4 above). Select the run file to be retrieved and click **Open**. Another screen will appear

prompting you to select the run you wish to retrieve. Select the run to be retrieved and click **OK**. A message screen will appear stating how many runs are to be retrieved, if this number is correct, click **Proceed.**

3.8 If a hardcopy of the results is also required, click on **Report** and **Print**.

4. Troubleshooting

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

- Contamination occurred during the set up. Results from the entire run cannot be relied upon as accurate.

→ Repeat the entire run taking great care when adding the templates, in particular, the Positive Control (Tube 4), to ensure that cross-contamination does not occur.

→ Make sure that the work area and instruments are properly decontaminated before and after use.

- The Negative Control was incorrectly positioned in the instrument.

→ Take care that the reaction tubes are placed in their designated sites.

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

- The PCR has been inhibited.

→ Ensure that the work area and instruments are thoroughly dry after the use of decontaminating agents prior to PCR set up.

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.

→ Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with unexpired kit if necessary.

- Either Tube 1 or 2 reagent was not added to the PCR reaction, or double the amount of Tube 2 was added.

→ Repeat the run taking care in the set-up stage. Such errors can be detected by seeing higher or lower levels of liquid in one reaction tube compared to others.

4.3 **The Positive Control is negative:**

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.

→ Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.

- An error occurred during step 1.12 and the Positive Control template (Tube 4) was placed in the wrong reaction tube.

→ Repeat the run, taking great care during the set-up stage. Such errors can be detected by seeing a higher level of liquid in one reaction, and a lower level in another, compared to normal.

- Either Tube 1 or 2 reagent was not added to the reaction.

→ Repeat the run taking care in the set-up stage. Such errors can be detected by seeing lower levels of liquid in this reaction compared to others.

- The Positive Control was incorrectly positioned in the instrument.

→ Take care that the reaction tubes are placed in their designated sites.

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

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

→ Follow the Roche High Pure manual DNA modified extraction procedure, and not manufacturer's instructions, for optimal DNA extraction.

→ Some blood collection tubes for serum may contain PCR inhibitors that have not been tested.

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 (productsupport@lab21.com)

Performance Characteristics and Limitations

Analytical Sensitivity

Using the 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 39.0, while the Limit of Detection (LoD), starting from a serum sample, was determined to be < 5 genome equivalents. This was determined using genomic DNA from the AF293 strain of *Aspergillus fumigates* spiked into serum of non-infected individuals The genome of the AF293 strain has been fully sequenced and it is known there are 37 copies of the target within the genome, determined by optical mapping[8].

Analytical Specificity

This was initially determined during the validation studies for use with respiratory samples, and was not repeated.

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*.

[8] Nierman WC, Pain A, Anderson MJ, et al. (2005). Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigates.* Nature: 438: 1151-6.

Analytical Selectivity

Analytical selectivity was tested using DNA extracted from a variety of different fungal and non-fungal species. The following species were tested during the initial validation for respiratory samples and 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, Rhodotonila rubra, Saccharomyces cerevisiae, Scedosporium apiosperinu, S. prolificans, Sporothrix schenkii, Trichosporon capitatu. The following bacterial species did not report a positive result; *Bordetella pertussi, 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*..

The following were specifically tested for potential presence in serum and did not report out a positive result.
Acinetobacter baumannii, Aeromonas hydrophilia, Burkholderia cepacia, Citrobacter koseri, Enterobacter cloacae, Enterococcus faecium, Klebsiella pneumoniae, Morganella morganii, Proteus mirabilis, Salmonela enterica Serratia marcescens, Stenotrophmonas maltophila,

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, salmerterol, sodium chloride, sodium cromoglicate, terbutaline, tobramycin.

The following were tested for potential presence in serum. Clinically relevant concentrations were tested and were found not to inhibit the PCR reaction.
Amoxicillin with clavulanic acid, atovaquone, azathioprine, azotreonam, ceftazidime, ciproflaxicin, chlorphenamine maleate, clindamycin phosphate, co-trimoxazole, creatinine, dapsone, dexamethasone sodium phosphate, fluconazole, meropenam,

metoclopramide hydrochloride, paracetamol, primaquine phosphate, prednisone sodium phosphate, prednisone, prochlorperazine vancomycin and voriconazole

The following were found to inhibit PCR reactions: cefuroxime, heparin, methylpredisolone sodium succinate, transaminase and urea. When these inhibiting substances were added at clinically relevant levels to serum containing *Aspergillus* DNA and extracted with the modified High Pure DNA extraction kit, no inhibition was observed. However, transaminase appeared to degrade the *Aspergillus* DNA prior to extraction, as 25% of the replicates were negative for *Aspergillus*.

Clinical Reporting

NOTE: When inspecting the Results Report ensure that the correct protocol has been used. For serum samples, **SERUM MycAssay Asp v1** must be used. Use of the wrong protocol will give invalid results.

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 levels of *Aspergillus* DNA in the blood are low, extraction efficiency may impact the result, and the test may give a false negative outcome.
  - Preliminary data indicate that freezing and storage of serum samples may affect the quantity of viable DNA available for assaying.
  - No data are available on the stability of *Aspergillus* DNA in unprocessed whole blood or serum. It is recommended therefore that samples are processed as quickly as possible after collection
  - No data are available on the performance of serum collected in blood collection tubes other than the recommended Greiner Red Top serum collection tubes.
  - No data are available on the performance characteristics of the assay starting with *Aspergillus* DNA extracted from plasma or whole blood.

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

- While the High Pure DNA extraction procedure may remove PCR inhibitors, not all drugs or patient populations have been evaluated.

- During analytical validation studies, it was noted that transaminase at 22.2 U/0.5mL serum may have caused *Aspergillus* DNA degradation prior to extraction.

- During validation, batches of Proteinase K were obtained and used that were subsequently found to be contaminated (at source) with *Aspergillus*. Source all materials carefully and use recommended sources wherever possible.

- 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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