

MycAssay™ Aspergillus Roche LightCycler® 2.0

REF 080-050

Intended Use:

MycAssay™ Aspergillus is a Real-Time PCR kit for the detection of *Aspergillus* DNA using the Roche LightCycler® 2.0.

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 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 for Research Use Only. It is Not for Use in Diagnostic Procedures.
- The kit is intended for use only by laboratory professionals. Procedures are required for non-aerosol manipulations of specimens. Standard precautions and institutional guidelines should be followed in handling all samples. A Material Safety Data Sheet is available from Myconostica Ltd.
- This test is only for use with the Roche LightCycler® 2.0 and the 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.
- 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 extracts 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 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

- 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)

Sample

The sample for the MycAssay™ Aspergillus assay is total genomic DNA.

MycAssay 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 laboratory¹, 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 filter tips are recommended for use to ensure that no DNA is lost during the set-up procedure.
- **Exercise caution when handling Tube 4. This contains 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.

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 required database. If this

¹ For example see Mifflin, T. E. (2003). Setting up a PCR Laboratory. *In* PCR Primer, 2nd Ed. (eds. Dieffenbach and Dveksler). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. USA.

- is the first run of a day, perform an instrument Self-Test first before setting a run.
- 1.2 Ensure the work area has been cleaned using DNA decontaminating reagents and allowed to dry completely; avoid use during assay set-up as excess cleaning solution may inhibit the PCR reactions.
- 1.3 A pouch contains one each of Tube 1, Tube 2, Tube 3 and Tube 4. There are sufficient reagents in one pouch to run 8 reactions. At least one positive control and one negative control reaction must be performed per run where the reagents are from a single kit lot. One pouch therefore can analyse 6 test samples. If more than 6 samples need to be tested, more than one pouch can be used if the pouches used are from the same kit lot. However, the LightCycler® 2.0 can only hold up to 32 samples in a single run. Therefore, a maximum of 30 test samples can be performed in a single run (4 pouches).
- 1.4 Calculate the number of reactions required, referring to the table below:

Number of Pouches	Maximum number of test samples
1	6
2	14
3	22
4	30

- 1.5 Remove the appropriate number of pouches from the freezer. Do not use any pouch that is no longer sealed. If the test samples were frozen after extraction, also remove these from the freezer.
- 1.6 Tear open the required number of pouches and remove the tubes. If more than one pouch is being used, but only one set of positive and negative controls are being run, it is only necessary to remove Tubes 3 and 4 from one pouch. **Exercise caution when handling Tube 4. This contains positive control DNA material and contamination could cause false positive test results.**
- 1.7 Allow the tubes' contents to thaw by placing on the laboratory bench for 5-10 minutes, ensuring that the contents of each tube are completely thawed before proceeding. Vortex mix the tubes' contents and the test samples; follow by a short spin in a microcentrifuge to ensure collection of all the contents at the base of the tubes before use.

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

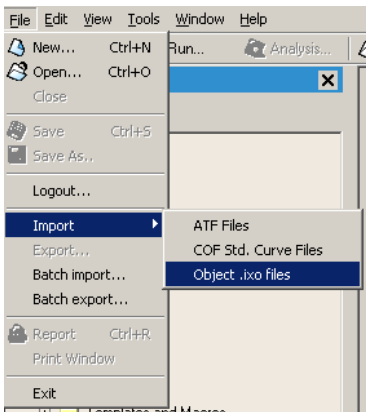
Reagent	Reaction		
	Negative control	Test samples	Positive control
Tube 1 (Orange cap)	7.5 µL	7.5 µL	7.5 µL
Tube 2 (Green cap)	7.5 µL	7.5 µL	7.5 µL
Tube 3 (Clear cap)	10 µL	-	-
Test Samples	-	10 µL	-
Tube 4 (Black cap)	-	-	10 µL
Total volume	25 µL	25 µL	25 µL

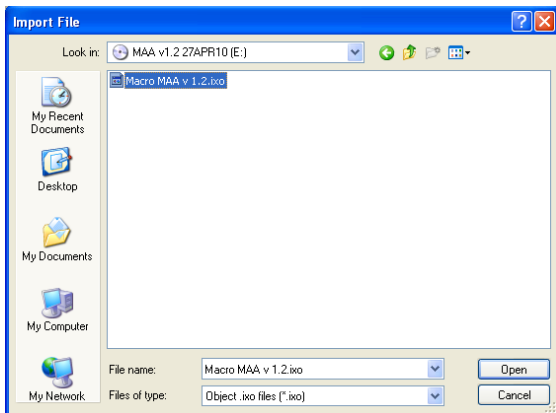
- 1.11 Add reagents in the order shown in the table above; Tube 1, then Tube 2, followed by the template (Negative control, Test sample, or Positive control). Take care when taking aliquots from Tube 1; the liquid is slightly viscous and can stick on the inner ridge of the tube. If this happens, re-spin to collect the final contents in the base of the tube before attempting to remove the final aliquots.
- 1.12 Use a new pipette tip for every liquid transfer. Re-cap each reagent tube after use and immediately discard it, and any remaining contents, into a sealable clinical waste container. Unused reagents cannot be saved for later use.
- 1.13 Take extra care when pipetting Tube 4 (positive control DNA) to ensure it does not contaminate any other reaction. Capping all the other capillaries before opening Tube 4 can reduce the risk of cross-contamination.
- 1.14 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.

- 1.15 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 1.16.
- 1.16 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.
- 1.17 If not already spun down in 1.15, spin the samples using the LC 2.0 carousel centrifuge.
- 1.18 Proceed to Section 2 promptly. MycAssay™ Aspergillus reactions are stable on the bench for up to 60 minutes.
- 1.19 Following the PCR set-up ensure the work area is thoroughly cleaned using DNA decontaminating reagents.

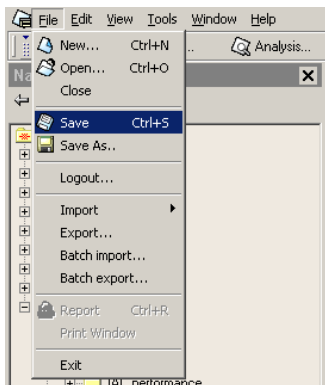
2. Performing the run

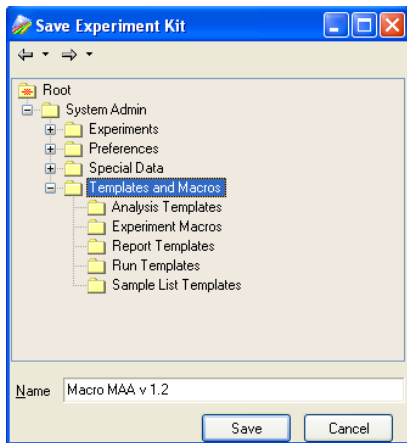
- 2.1 Insert the **MycAssay Aspergillus Myconostica Protocol CD-ROM**.
Go to **Import** via the **File** directory and select **Object .ixc files**. Import the **Macro MAA v1.2.ixc** file from the CD to your database.





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

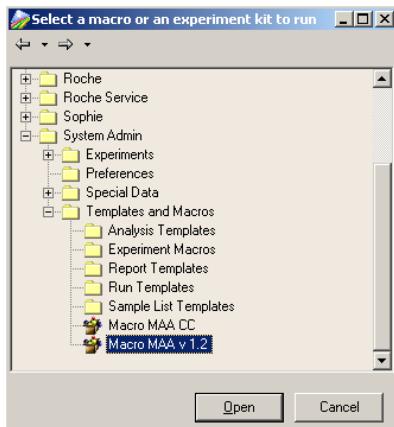




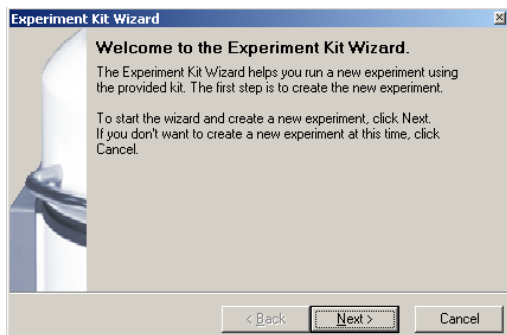
2.4 Select the **Run Macro** option from the Toolbar.

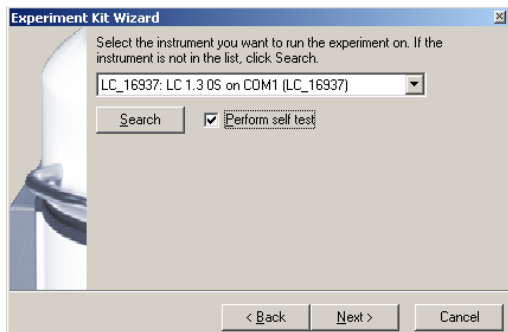


2.5 Select the **Macro MAA v1.2** template file and press **Open**.

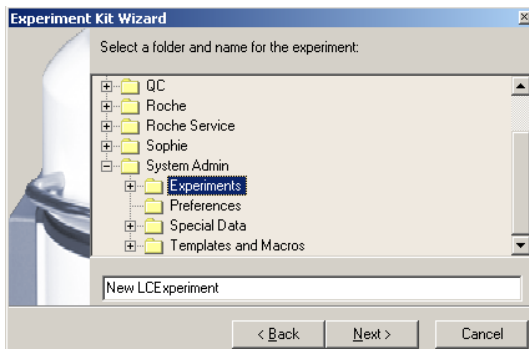


- 2.6 Follow the wizard instructions. Tick the **Perform Self-Test** box if this is first run if a day.

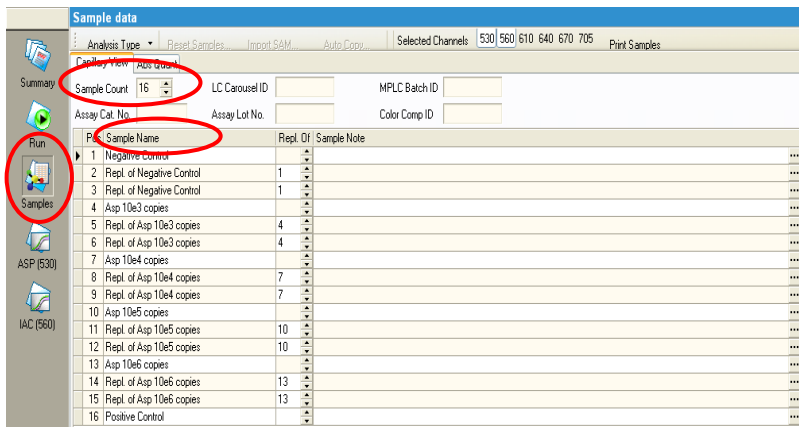




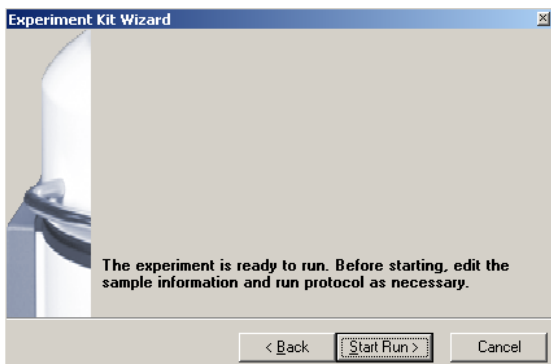
- 2.7 Name and save the run file in a desired location.



- 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.

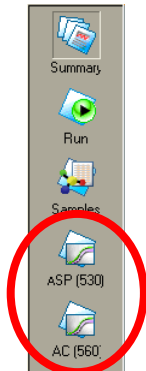


- 2.9 Place the spun down carousel in the LC 2.0 instrument. Ensure that the notch below sample position 1 on the carousel locks into position with the pin on the thermal chamber. Make sure that the carousel is inserted firmly in the chamber and close the lid.
- 2.10 When finished, press the **Start**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



3. Data Analysis and Interpretation

- 3.1 When the run has finished check for the contents of the popped up report and print it if desired.
- 3.2 The Aspergillus results can be viewed in the ASP (530) analysis section and the IAC results in the IAC (560) analysis section.

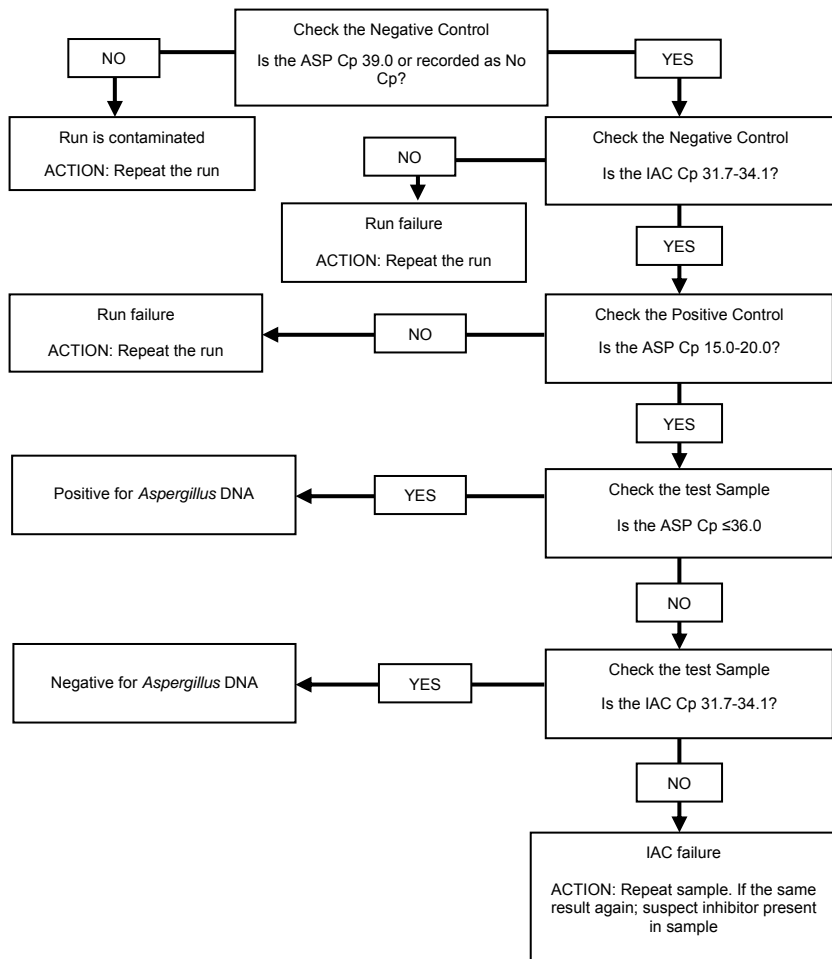


- 3.3 In both sections select the correct MycAssay Colour Compensation file to be applied to the experiment.

The screenshot shows the 'Absolute Quantification [MAA CC QC08APR10-15]' window. On the left is a navigation pane with icons for Summary, Run, Samples, ASP (530), and IAC (560). The main area displays a table with columns for 'Include', 'Color', and 'Pos'. A dropdown menu is open over the table, showing 'Select Color Compensation...' with 'MAA CC QC08APR10-15 [530, 560]' selected. The table lists 16 rows, each with a sample name and a numerical value.

Include	Color	Pos		
<input type="checkbox"/>	Blue	1		
<input type="checkbox"/>	Green	2		
<input type="checkbox"/>	Red	3	Repl. of Sample 8	33.61
<input type="checkbox"/>	Black	4	Sample 9	
<input type="checkbox"/>	Pink	5	Repl. of Sample 9	
<input type="checkbox"/>	Green	6	Sample 10	30.45
<input type="checkbox"/>	Purple	7	Repl. of Sample 10	30.94
<input type="checkbox"/>	Grey	8	Sample 11	31.47
<input type="checkbox"/>	Orange	9	Repl. of Sample 11	31.69
<input type="checkbox"/>	Pink	10	Sample 12	30.21
<input type="checkbox"/>	Yellow	11	Repl. of Sample 12	30.13
<input type="checkbox"/>	Teal	12	Sample 13	32.51
<input type="checkbox"/>	Red	13	Repl. of Sample 13	32.36
<input type="checkbox"/>	Grey	14	Sample 14	33.67
<input type="checkbox"/>	Pink	15	Repl. of Sample 14	33.91
<input type="checkbox"/>	Green	16	Positive Control	17.89

- 3.4 Analyse each sample, starting with the controls, as shown in the flowchart (details can also be found in the table shown following the flowchart):



Sample	ASP (530) Cp	IAC (560) Cp	Interpretation
Negative Control	39.0 or No Cp	Within 31.7-34.1	Negative Control acceptable
Negative Control	39.0 or No Cp	<31.7 or >34.1	Failure in Negative Control
Negative Control	<39.0	Within 31.7-34.1	Contamination
Positive Control	Within 15.0-20.0	N/A	Positive Control acceptable
Positive Control	<15.0 or >20.0	N/A	Failure in Positive Control
Test	≥39.0 or No Cp	Within 31.7-34.1	Negative for <i>Aspergillus</i>
Test	<39.0	N/A	Positive for <i>Aspergillus</i>
Test	39.0 or No Cp	<31.7 or >34.1	IAC failure in sample

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.

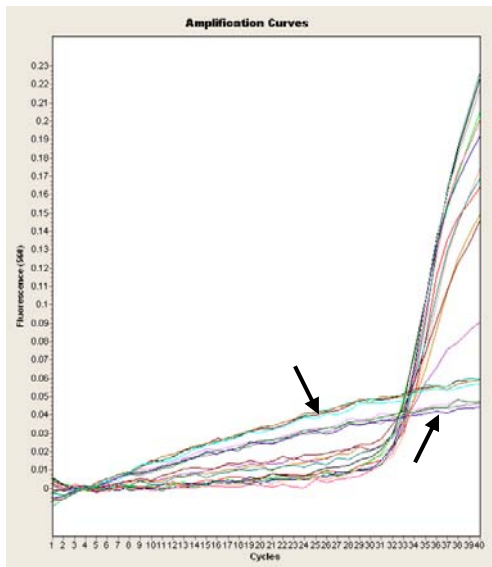
4.3 The Positive Control is negative:

- 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 1.16 and the Positive Control template (Tube 4) was placed in the wrong capillary.
- 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 Test sample(s) are negative and the IAC is out of range:

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

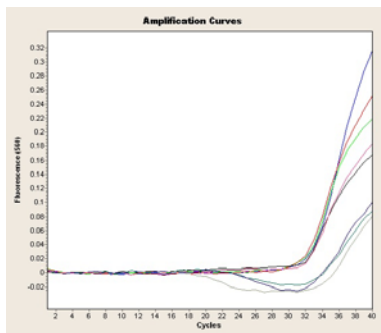
4.6 The Test Sample is negative in the ASP (530) section and the IAC (560) plot drifts away significantly from the regular baseline (as shown on the example picture below; arrows indicate abnormal plots):



- 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 test sample again. If the problem repeats, the PCR inhibitor is present in the sample. Report the sample as Undetermined.

4.7 The results in the IAC (560) section almost exactly match the results in the ASP (430) 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.

4.8 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)

LICENSING

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