PathoSEEK® 2-Color Aspergillus Multiplex Assay with MaGiC Lysis Kit

User Guide v1

Real Time PCR (qPCR) assay for the detection of *Aspergillus niger*, *A. flavus*, *A. fumigatus* and *A. terreus* in dried cannabis flower and MIP matrices

Table of Contents

Introduction 3
Process Overview 3
    Limit of Detection 4
Materials and Methods 5
    Kit Components 5
    Shelf Life and Storage 6
    Required Equipment and Supplies 6
Safety Precautions and Recommendations for Best Results 8
    Environment 8
    Safety Precautions 8
    Intended User 9
Sample Preparation 9
    MaGiC Sample Lysis 10
        All Matrices 10
    Real-Time Quantitative PCR (qPCR) Setup Protocol 12
    Running the Agilent AriaMX 14
    Data Analysis: Agilent AriaMX 17
        PathoSEEK® Aspergillus 2-Color and Aspergillus Singleplex Assays Data Analysis Reference Table: 17
    Running the BioRad CFX96 23
    Data Analysis BioRad CFX96 27
        PathoSEEK® Aspergillus 2-Color Data Analysis Quick Reference Table: 27
    Running the BioMolecular Systems MIC 30
    Data Analysis with BioMolecular System MIC 31
Troubleshooting Guide 34
Glossary and Definitions 35
Revision History 36
DISCLAIMER 36

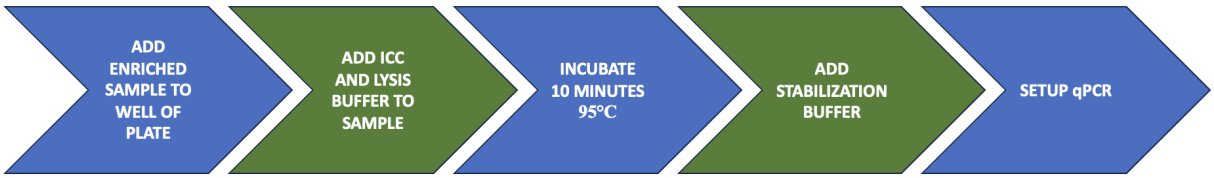
Introduction

Current regulations require cannabis flower and cannabis products to be free of select species of *Aspergillus*. The PathoSEEK® 2-Color Aspergillus Multiplex Assays with MaGiC Lysis are designed to detect *A. fumigatus*, *A. niger*, *A. flavus,* and *A. terreus* in a single qPCR (Quantitative Polymerase Chain Reaction) assay in cannabis flower, cannabis concentrates, infused edibles, and infused non-edibles.

Process Overview

The PathoSEEK® 2-Color Aspergillus Multiplex Detection Assays use a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of four species of *Aspergillus* as well as cannabis DNA in every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting pathogens, resulting in the minimization of false negative results due to reaction setup errors or failing experimental conditions. Below is an overview of the MaGiC Lysis process as well as a simplified depiction of the qPCR assays.

Figure 1: Overview of MaGiC Lysis



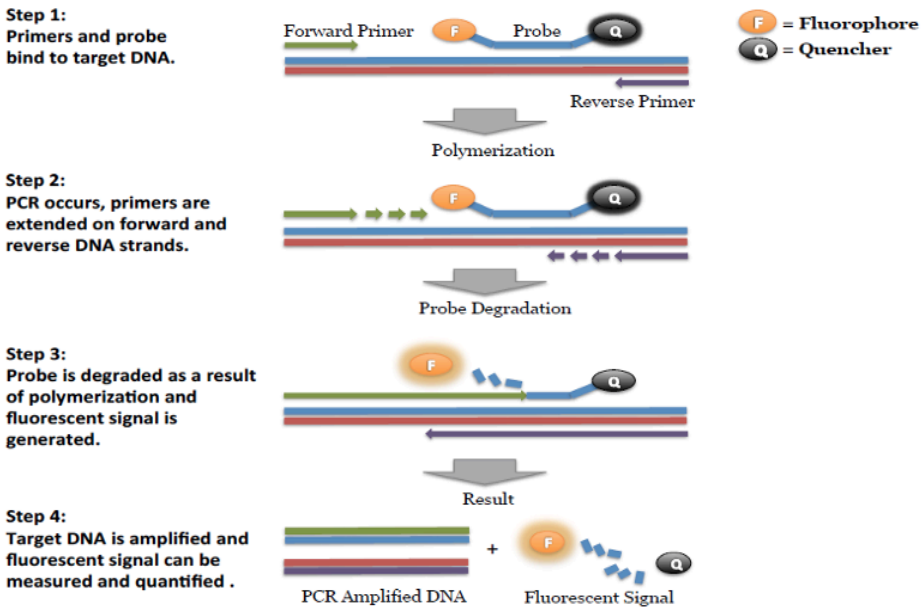


Figure 2: Overview of qPCR

Limit of Detection

The method is able to detect down to 1 CFU per test portion.

Materials and Methods

Kit Components

PathoSEEK® Aspergillus 2-Color Detection Assay v2 with MaGiC Lysis Kit, **P/N 420523** (contains sufficient reagents for 200 reactions)

Component Name	Qty Provided	Storage Conditions
MaGiC Lysis Reagent	1 Bottle (12 mL)	RT
MaGiC Stabilization Buffer	1 Bottle (24 mL)	RT
PathoSEEK® Amplification Mix	3 Vials (67 rxns/each)	RT / -20 ºC*
PathoSEEK® Aspergillus 2-Color Detection Assay v2	1 Tube (200 µL)	-20 ºC

Note: Some actual fill volumes include overage

*The PathoSEEK® Amplification Mix can be stored lyophilized at Room Temperature for up to 2 years. Once re-hydrated it must be stored at -20 ºC for up to 3 months.

Additional Required Reagents Not in Kit:

Item P/N	Item Name	Qty Provided	Storage Conditions
420032	Potato Dextrose Broth (PDB)	500 mL Bottle	2-25°C
420033	Chloramphenicol 34 mg/mL in Solution (CAMP)	100 mL Bottle	-20 ºC
420337	Internal Cannabis Control	1 Tube (50 µL)	-20 ºC
420330	PathoSEEK® *Aspergillus* Multiplex Positive Control v3	1 Tube (50 µL)	-20 ºC
420184	PCR Grade Water	500 mL Bottle	2-25°C

Optional Aspergillus Assays for Speciation:

Item P/N	Item Name	Qty Provided	Storage Conditions
420157	*Aspergillus fumigatus* Detection Assay v2	1 Tube (200 µL)	-20°C
420158	*Aspergillus niger* Detection Assay v2	1 Tube (200 µL)	-20 ºC
420159	*Aspergillus flavus* Detection Assay v2	1 Tube (200 µL)	-20 ºC
420160	*Aspergillus terreus* Detection Assay v2	1 Tube (200 µL)	-20 ºC
420310	*Aspergillus fumigatus* Control	1 Tube (50 µL)	-20 ºC
420309	*Aspergillus niger* Control	1 Tube (50 µL)	-20 ºC
420311	*Aspergillus flavus* Control	1 Tube (50 µL)	-20 ºC
420329	*Aspergillus terreus* Control	1 Tube (50 µL)	-20 ºC

Shelf Life and Storage

Once received, each kit component must be stored at its designated storage condition. Reagents stored properly can be used until the expiration date indicated on each component label.

Required Equipment and Supplies

Equipment:

- Agilent AriaMx Real-Time PCR System G8830A—Containing the following Optical Channels: FAM, ROX, HEX, Cy5 and ATTO425. **Medicinal Genomics P/N 420387**
    - Agilent HP Notebook PC option 650 or lab supplied Windows PC
    - Optical Strip Caps.—Agilent #401425.

      Note: If using adhesive seals use Applied Biosystems MicroAmp Optical Film Compression Pad, Fisher Scientific, #43-126-39 to prevent evaporation and cross contamination between wells.
- Bio-Rad CFX96 Touch™ Real-Time System.
    - Bio-Rad supplied or own Windows PC

- Bio Molecular Systems Mic 4-Channel PCR Instrument - **Medicinal Genomics P/N 420241**
    - BMS supplied or lab supplied Windows PC
    - Mic Tubes and Racked Caps - **Medicinal Genomics P/N 420244**
    - Mic Tubes and Caps (Bulk) - **Medicinal Genomics P/N 420243**
- Adjustable, variable volume pipettes (single or multichannel).—P10, P20, P50, P200, P300 and P1000
- Adjustable, variable volume filter pipettes tips.—For P10, P20, P50, P200, P300 and P1000
- Freezer—Capable of maintaining -20 ± 2 °C
- Tabletop Mini Plate Centrifuge—Fisher Scientific #14-100-143 or equivalent
- Tabletop Mini Centrifuge—VWR #10067-588 or equivalent
- Vortex-Genie Pulse—Scientific Industries, SKU: SI-0236 or equivalent
- Incubator—Capable of maintaining 37 ± 2 °C, VWR #97025-630 or equivalent.
- 96-Well PCR Plate Cryogenic Rack (optional) —VWR #89004-570
- 1.5 mL Tube Benchtop Cryogenic Rack (optional if crushed ice is not an option) — VWR #89004-558 or equivalent
- Eppendorf Tube Rack
- Scientific Scale—Capable of measuring to milligrams
- Refrigerator—Capable of maintaining 2–8 °C

Supplies:

- 96-well Optical qPCR plate — **Medicinal Genomics P/N 100164**
- Adhesive optical seals for qPCR plates — **Medicinal Genomics P/N 100177**
- Filter Bags—**Medicinal Genomics P/N 100008** (Whirl-Pak #B01385WA)
- Crushed ice or alternative
- Beaker or Solo Cup. (optional, used to hold Whirl-Pak bags or 15/50 mL tubes upright while weighing samples)
- 1.5 mL Eppendorf Tubes
- 15 mL or 50 mL conical tubes
- Pipette - Aid Portable Pipetting device for serological pipettes, VWR 89166-464 or equivalent
- 25mL Serological Pipette—VWR 89130-890 or 89130-900 or equivalent
- 10% bleach

Safety Precautions and Recommendations for Best Results

Environment

The quality of results depends on the strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR:

- Never circulate lab equipment from one workstation to another
- Always use a positive and negative control for each series of amplification reactions
- Periodically verify the accuracy and precision of pipette, as well as correct functioning of the instruments
- Change gloves often, especially if you suspect contamination
- Clean workspaces periodically with 10% bleach and other decontaminating agents
- Use powder-free gloves and avoid fingerprints and writing on tube caps. Both can interfere with data acquisition.

Safety Precautions

- *Aspergillus* spp. includes many species, about 40 of which have been implicated in human or animal infections. Aspergillosis is a common term used to describe infections caused by different species of *Aspergillus*. Most cases of aspergillosis are caused by *A. fumigatus*, with *A. flavus* and *A. niger* being the second most common pathogenic *Aspergillus* spp. worldwide. Diseases caused by *Aspergillus* spp. include clinical allergies (allergic bronchopulmonary aspergillosis, rhinitis, Farmer's lung), superficial and local infections (cutaneous infections, otomycosis, tracheobronchitis), infections associated with damaged tissue (aspergilloma, osteomyelitis), and invasive pulmonary and extrapulmonary infections. Invasive infections due to *Aspergillus* spp. occur mainly in immunocompromised individuals and are the most severe forms of infections caused by *Aspergillus* spp.
- Assay users should observe standard microbiological practices and safety precautions when performing this assay. Wear protective gloves, lab coats, eye/face protection as indicated by your quality system.

- It is the responsibility of each laboratory to handle waste and effluents processed according to their nature and degree of hazardousness and to treat and dispose of them in accordance with applicable local, state, and federal regulations.

Intended User

The PathoSEEK® 2-Color Aspergillus Multiplex Assay or Aspergillus singleplex assays with MaGiC Lysis and all components required to perform the method are intended for use by trained personnel familiar with laboratory techniques associated with pathogenic organism detection.

Sample Preparation

1. Wipe down the workspace with a 10% bleach solution, including the bench top and all equipment being used.
2. Prepare PDB and Chloramphenicol (Enrichment Media)
    a. To prepare 500 mL of PDB + Chloramphenicol (prepares about 55 samples):
        i. Add 4.8 mL 34 mg/mL Chloramphenicol to 500 mL PDB
        ii. Users may scale down this dilution as needed (ex: 480 µL Chloramphenicol + 50 ml PDB)
    b. This solution is stable for one month at 4°C
3. Prepare consumables. Label all the filter bags with "[sample name] [date]".
4. Prepare consumables. Label PCR plate with Sample batch and date.
5. Before weighing out the sample to be tested, make sure that the entire sample is broken up and thoroughly homogenized. A well-homogenized sample will ensure more accurate testing.
6. *Cannabis flower,* ***n*** *grams* —Weigh flower sample material into one side of the mesh liner inside the Whirl-Pak bag. Add 9 x *n* mL of prepared PDB with chloramphenicol to each test portion. Close the Filter bag by folding the top over three times. Mix for 1 minute by hand. Incubate for **24 – 48 h** at 37 ± 1°C.
7. *Cannabis MIP,* ***n*** *grams* —Weigh MIP sample material into either a 15 mL or a 50 mL conical tube. Add 9 x *n* mL of prepared PDB with chloramphenicol to each test portion. Close the tube and vortex for 1 minute. Incubate for **48 h** at 37 ± 1°C.

8. If processing multiple samples, be sure to change gloves between each sample to ensure that there is no cross-contamination between samples during the weighing process.

MaGiC Sample Lysis

All Matrices

1. Remove samples from the incubator.
    a. Flower matrix: hand homogenize thoroughly for 1 minute.
    b. Non flower matrix: Vortex 15 or 50 mL tube to homogenize sample in media.
    Note: Aspergillus cells tend to clump together, this homogenization and/or vortexing will aid in spreading them throughout the media in the WP bag or tubes.
2. Aspirate **50 µL** from the side of the filter bag free of plant debris or from the 15 or 50 mL tube and dispense into a well of a 96 well qPCR plate.
3. **MIP ONLY:** Dilute stock Internal Cannabis Control (ICC) to 1:50,000
    Note: Prepared 1:50k dilution of ICC has been tested up to 24 freeze thaws with minimal effect on resulting Cq value when stored at -20°C when not in use. If prepared in advance, allow diluted ICC to thaw completely and vortex to mix before use.
    a. Ensure stock ICC is fully thawed, vortexed and spun down before use.
    b. Dilute stock ICC 1:100.
        i. Add 2 µL of ICC to 198 µL of Nuclease free Water. Close tube, vortex and quick spin.
    c. Dilute the 1:100 dilution of ICC to 1:10,000.
        i. Add 2 µL of 1:100 ICC dilution to 198 µL of water. Close tube, vortex and quick spin.
    d. Dilute 1:10,000 dilution of ICC to 1:50,000.
        i. Add 20 µL of 1:10,000 ICC dilution to 80 µL of water. Close tube, vortex and quick spin.
4. Add 5 µL of 1:50,000 dilution of ICC to the 50 µL of each non flower (MIP) sample.
5. Add 100 µL of MaGiC Lysis buffer to each sample.

6. Pipette mix 5 times. Seal plate.
7. Place plate in thermal cycler and run the following program:
    a. 95°C for 10 minutes.
    b. 4°C for 10 minutes or "forever". If the thermal cycler does not go down to 4°C set to 25°C.

    Note: When using the Agilent AriaMX, a compression pad should be placed over the adhesive seal to prevent evaporation during thermal cycling.
8. Remove the lysed sample plate from the thermal cycler when the program has been at 4°C or 25°C for 5 minutes or more. Spin the plate in a plate centrifuge to ensure that the contents are not adhered to the plate seal. Carefully remove the seal and pipette mix samples five times. Transfer 50 µL of sample/lysis buffer to a new plate.
9. Add 100 µL of stabilization buffer to the transferred 50 µL lysed sample and pipette tip mix well.
    a. Samples with stabilization buffer added are stable at -20 ºC for 1 month.
    b. Extracted samples which have been frozen must be tip mixed 15 times prior to setting up qPCR or performing dilutions.
10. Proceed to qPCR.

Real-Time Quantitative PCR (qPCR) Setup Protocol

1. Remove reagents including Amplification mix, water, Aspergillus 2-Color Detection Assay v2, and Aspergillus Multiplex Positive Control v3 from the -20ºC freezer. Allow the tubes to thaw at room temperature. Once thawed, immediately place tubes on ice.
2. Before preparing the reaction, invert or vortex and spin down the reagents.
3. Make a master mix in a 1.5 mL tube

   Note: It is best to add the largest volume reagent first, in this case amplification mix.

   Table 2: PCR Reagent Volumes

Reagents	1 Reaction	24 Reactions (Plus 1 excess rxn)	48 Reactions (Plus 2 excess rxn)
Amplification Mix	10 µL	250 µL	500 µL
Assay Probe Mix	1 µL	25 µL	50 µL
Water	4 µL	100 µL	200 µL
Total Assay Probe MM	15 µL	375 µL	750 µL

4. Once combined, gently tip mix or invert the tube 5 times to homogenize the assay master mix.
   a. Pulse spin down tube in microcentrifuge.
   b. Place qPCR Master Mix tubes on ice until used.
   c. Make a 1:10 dilution of the stock positive control
      i. Add 1 µL of Positive Control to 9 µL nuclease free water (found in the kit) in a 1.5 mL tube, vortex to mix well and spin down the tube.
      ii. For the negative control, use water (found in the kit).

   Note: It is best to add the largest volume reagent first, in this case the 9 µL water then the 1 µL of positive control, pipette mix or vortex control dilution to ensure control DNA is in solution.
5. Use a 96-well optical qPCR plate and label the plate "qPCR Plate_ [date]".

6. Carefully remove the seal from the Lysis Plate. If frozen, let the contents thaw completely and spin the plate to avoid cross contamination between samples. Transfer 5 µL of each sample into the corresponding well on the qPCR plate.
    a. Add 5 µL of the diluted Positive Control to the corresponding positive control well. Then add 5 µL of water to the corresponding negative control well.

    Note: ALWAYS use a fresh tip for every liquid transfer into the qPCR plate
7. Add 15 µL of prepared master mix to each corresponding sample well, positive control well, and negative control well in the qPCR plate. Gently tip mix a few times after each addition of qPCR master mix. Be careful not to introduce bubbles during this mix.
8. Seal the plate with strip caps or an adhesive seal.
9. Spin down for at least 1 minute in plate microcentrifuge to bring well contents to the bottom of wells and help to get rid of reaction bubbles.

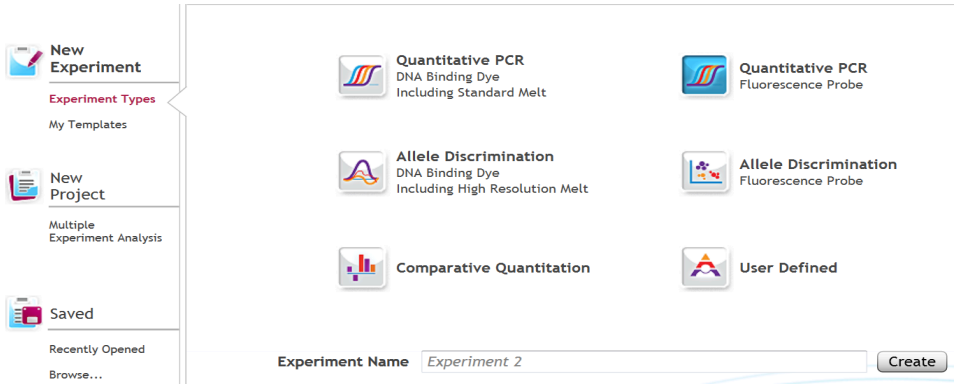
    Note: Check for bubbles at the bottom of the wells (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the wells, *spin down* for another minute.
10. For the Agilent AriaMX: If using an adhesive seal; place the reusable compression pad (gray side down) on top of the adhesive seal on the plate directly lining up the holes in the pad with the wells in the plate.
11. Place the sealed plate onto the qPCR instrument, positioning the A1 well in the top left corner.
12. Follow the software specific instructions to initiate the run.

Running the Agilent AriaMX

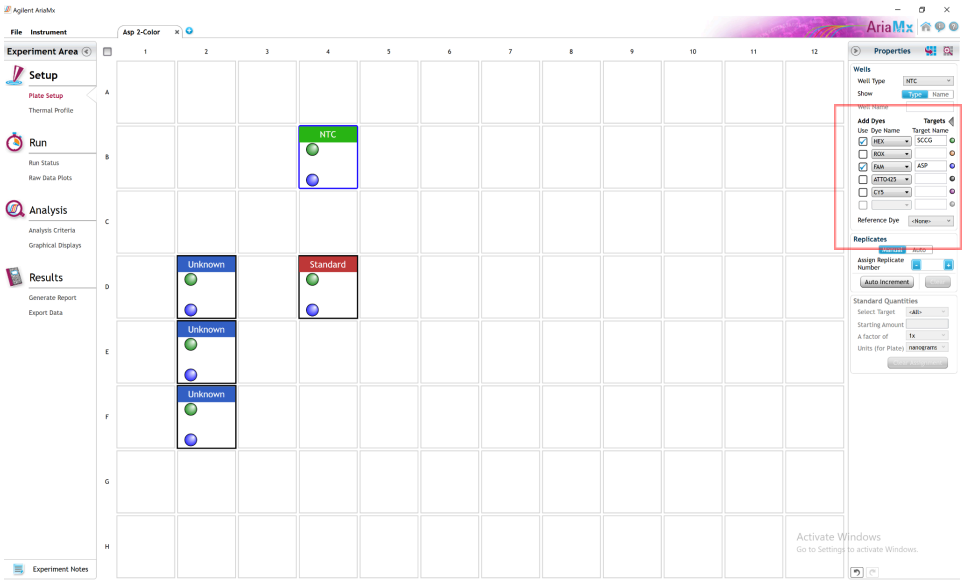
The following species will be detected on the following Fluorophores:

- *A. flavus, A. fumigatus, A. terreus, and A. niger*: FAM
- Cannabis DNA: HEX

1. Create a New Experiment on the Agilent qPCR instrument.
    a. Select “Quantitative PCR: Fluorescence Probe” from Experiment Types.



    b. Under Setup > Plate Setup, highlight **only** wells that contain reactions and select FAM and HEX under **Add Dyes**. Having fluorescence detection turned on in empty wells can affect the calculations which the software makes to obtain the corrected data, ΔR log.



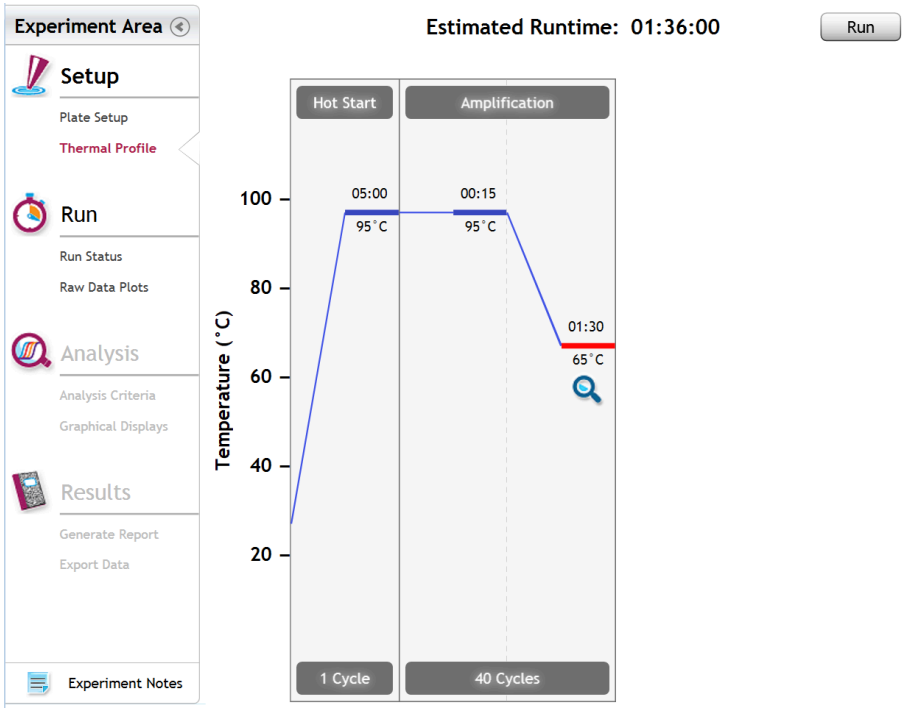
2. Change the well types to reflect your plate setup. All sample wells should be set to Unknown. The negative control can be set to NTC well type. The positive control can be set to Unknown or Standard well type. Add Target names to the dyes under **Targets:**

    a. FAM : *Aspergillus spp. (flavus, fumigatus, niger, terreus)*

    b. HEX : Internal Cannabis Control (ICC)

3. Under Setup > Thermal Profile, create the following PCR thermal profile

    a. Hot start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC for 15 seconds and 65ºC for 90 seconds.



4. Close the lid and click “Start Run”
5. Save the experiment with the [User] and [date]
6. When the qPCR run is complete, immediately dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

Data Analysis: Agilent AriaMX

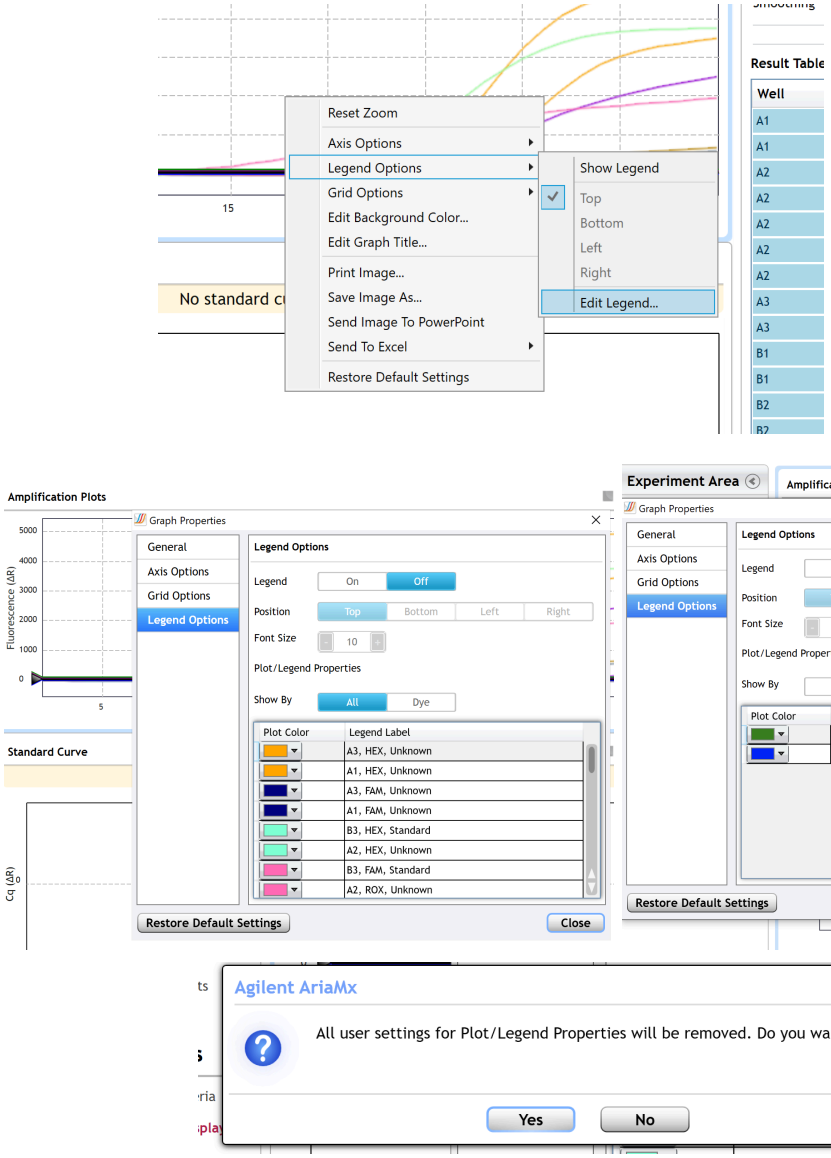
PathoSEEK® Aspergillus 2-Color and Aspergillus Singleplex Assays Data Analysis Reference Table:

Table 1: All Matrices

PathoSEEK™ Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Aspergillus 2 - Color Assay Aspergillus spp	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus flavus	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus fumigatus	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus niger	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus terreus	≤ 40	FAM	No Cq	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Control	≤35	FAM		

1. Open the Data Analysis window when the run is complete.
2. Highlight the wells of interest in the Analysis Criteria under Analysis, then select Graphical Displays.
    a. Amplification plots will be available for viewing
    b. The Cq values will appear to the right in the table
    c. Right-click inside the graph, select Edit Legend under Legend Options
    d. Change “All” to “Dye”
    e. All user settings for Plot/Legend Properties will be removed. Do you want to proceed? Select “Yes”.

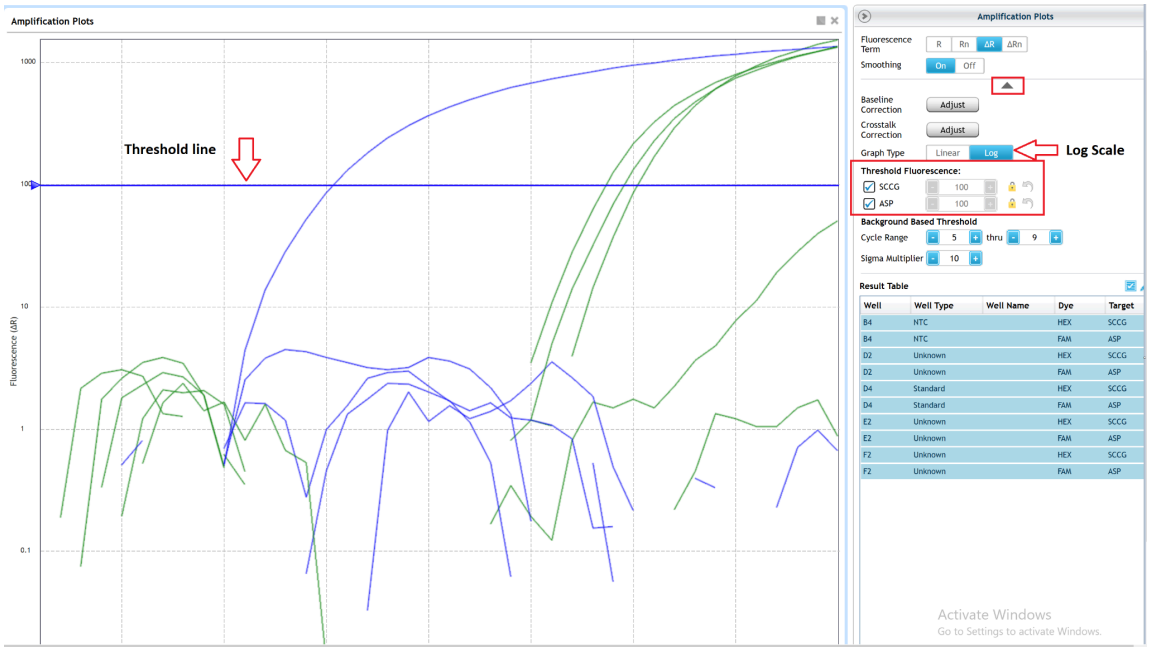
f. This will assign a single color to each fluorophore.



3. Data Analysis

a. Start by turning the graph to Log Scale with a right-click on the chart, select Axis options, enable y-axis log scale. Expand the amplification plots settings by clicking on the triangle

b. Manually adjust thresholds to 100 RFU for the HEX and FAM fluorophores (which will have the target name because they were added in the plate setup). Click the lock next to each target to prevent future changes.



c. Controls
   i. Positive Control, on the FAM Fluorophore, has Cq value ≤ 35.
      1. Visually confirm with the curve on the graph.
   ii. Negative Control, on the FAM Fluorophore, has no Cq Value.
      1. Visually confirm with the curve on the graph.

d. Unknown Aspergillus Targets
   i. A "presence" or failing result for the unknown Aspergillus target(s).
      1. Any Cq value for the FAM Fluorophore ≤ 40.

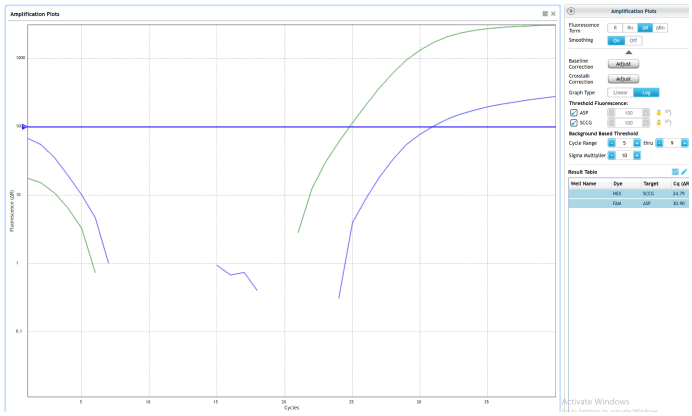


Figure: ΔR background correct amplification plots for Hex and Fam in the selected sample

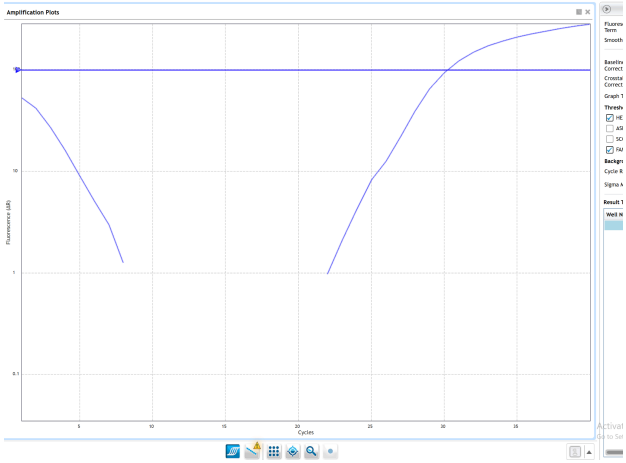


Figure: ΔR background correct amplification plots for only Fam in the selected sample

e. Visually confirm with the curve on the graph. It is very important to check the raw data view (R) to confirm with the amplification curve when a presence result occurs.

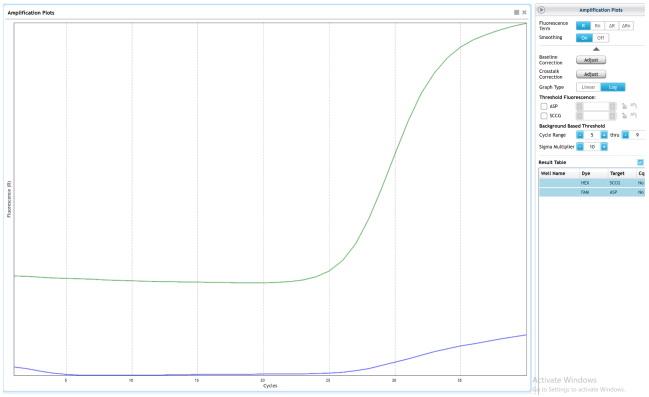


Figure: Raw data (R) plots of Fam and Hex for the selected sample

f. Review the raw data for each sample:

i. Select the wells of interest, use the plate view of the Raw Data Plots to scroll across the plate to confirm amplification in sample wells and positive control wells. This review also ensures that thermal cycling is uniform across the plate.

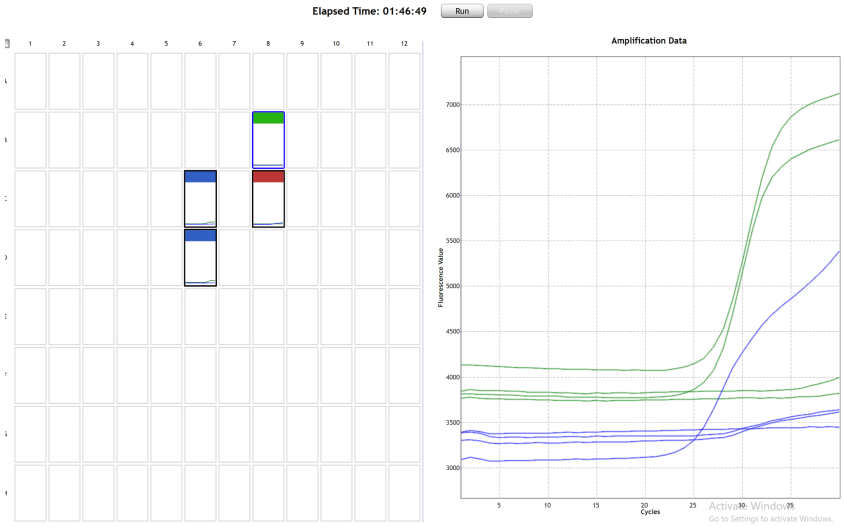


Figure: Raw data (R) plots for which the cycle quantification (Cq) will be generated.

ii. Look at the raw data in the "Graphical Displays" view. For every dye that gives a Cq make sure the baseline is flat and the fluorescence signal grows rapidly over 5-7 cycles as seen below. (To view the raw data, select "R" next to "Fluorescence Term" at the top right-hand side of the screen.)

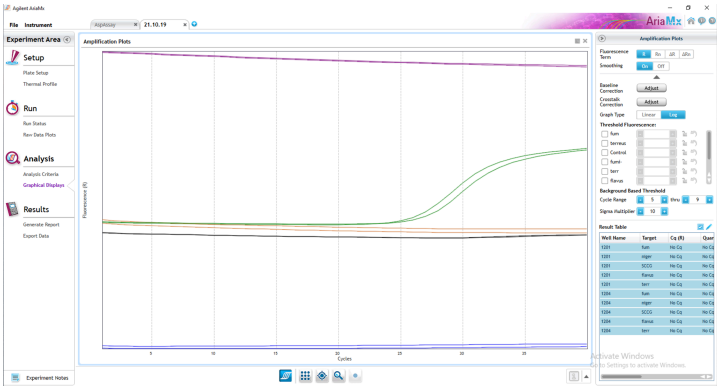


Figure: Example of good baseline with sigmoidal-shaped curve increase in the internal control fluorescence.

iii. Check the amplification plots for a semi-logarithmic curve with two distinct phases that crosses the threshold:

1. Select the ΔR Fluorescence Term and look at the shape of the amplification plot. The graph should have the classic semi-logarithmic shape with two visually distinct phases.
2. Phase 1 shows exponential growth in the fluorescence, in a span of around 5 cycles.
3. Phase 2 is a plateau where the amplification signal growth ends but remains level.

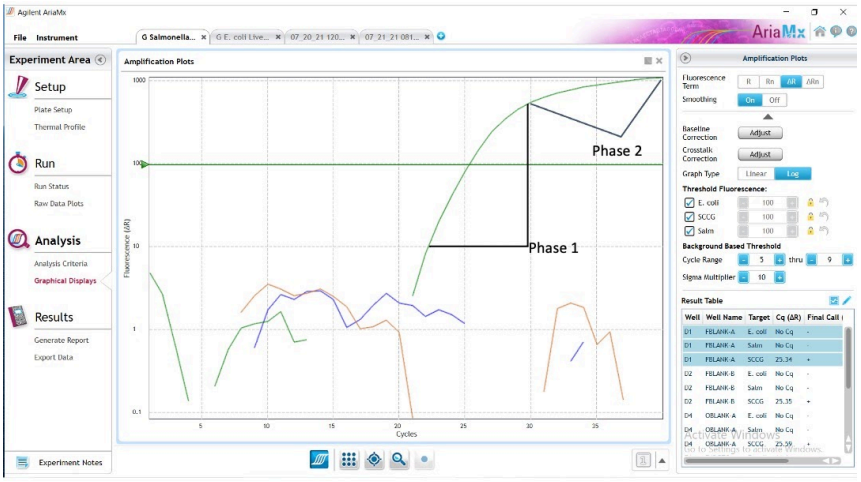


Figure: Example of the semi-logarithmic curve from the amplification of the internal control on HEX

g. An "absence" or passing result for the unknown Aspergillus targets.
   i. No Cq value for the FAM Fluorophore
      1. Visually confirm that there is no curve on the graph.
   ii. Cq of ≤ 35 for the internal control on the HEX fluorophore, < 40 for all other matrices .
      1. Visually confirm the curve on the graph.

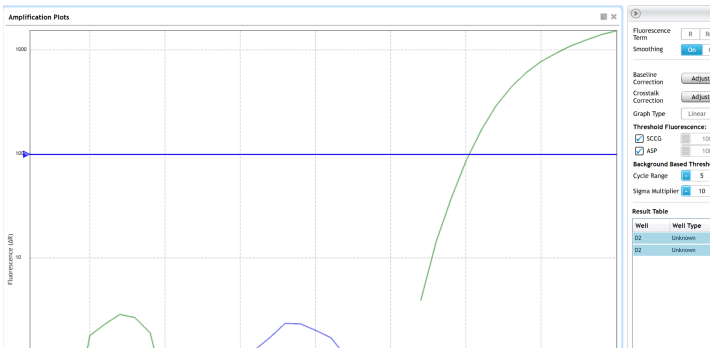


Figure: Example of a sample in which Aspergillus was not detected

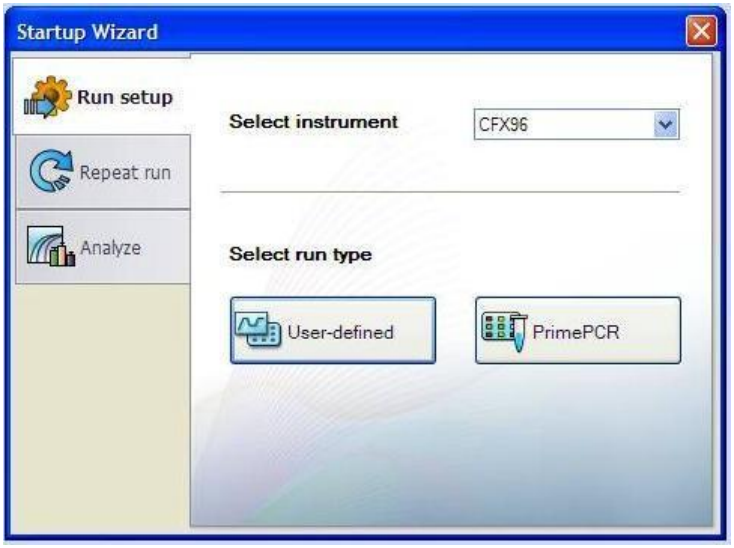
Running the BioRad CFX96

The following targets will be detected on the following fluorophores:

- *Aspergillus spp. (flavus, fumigatus, niger, terreus)*
- Cannabis DNA: HEX

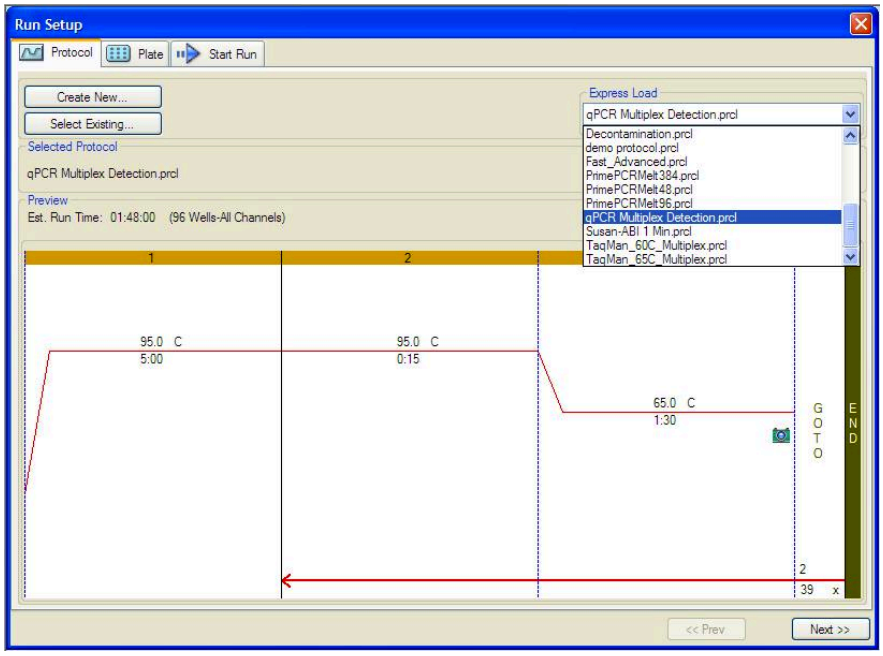
1. Start the qPCR Cycling program

    a. Select User-Defined in the Startup Wizard under Run setup

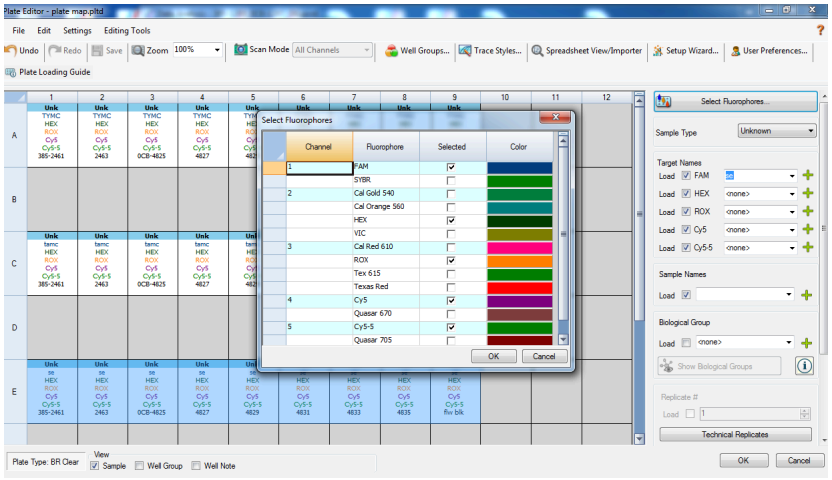


2. Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click "Next".
    a. If not already pre-programmed, create a cycling program with the following specifications and save as "qPCR Multiplex Detection"

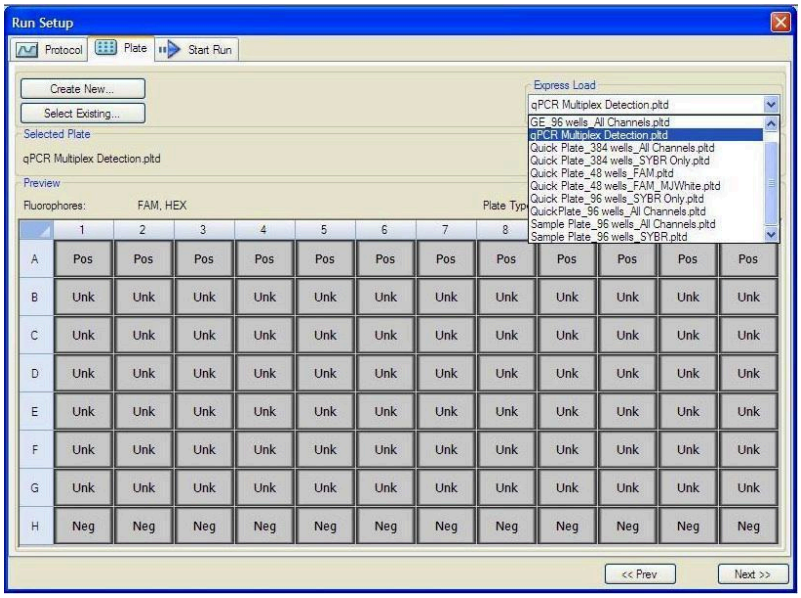
        i. Hot start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC for 15 seconds and 65ºC for 90 seconds.



3. Design your plate under the plate tab in the Run Setup
    a. Select the qPCR Multiplex Detection from the dropdown menu. If not already present, click "Create New"
    b. Plate editor window will appear. Choose FAM and HEX Fluorophores and click "OK".



    c. If plate layout was previously saved, click "Edit Selected" to move to the Plate Editor Screen.

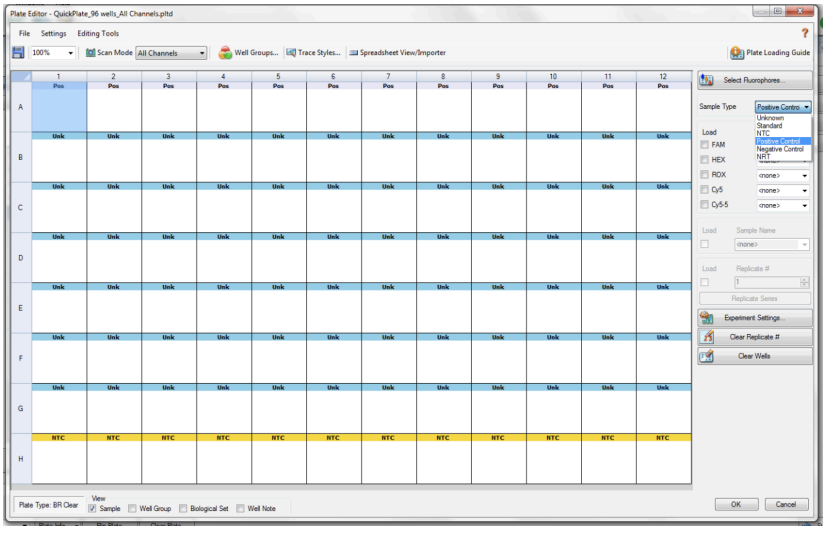


d. On the Plate Editor Screen, change the Sample Type to correlate with your specific plate setup.

NOTE: To select the Sample Type, highlight the wells you would like to define, then choose from the dropdown menu one of three types:

Unknown
Positive Control
Negative Control

e. Make sure All Channels is selected from the dropdown menu at the top.

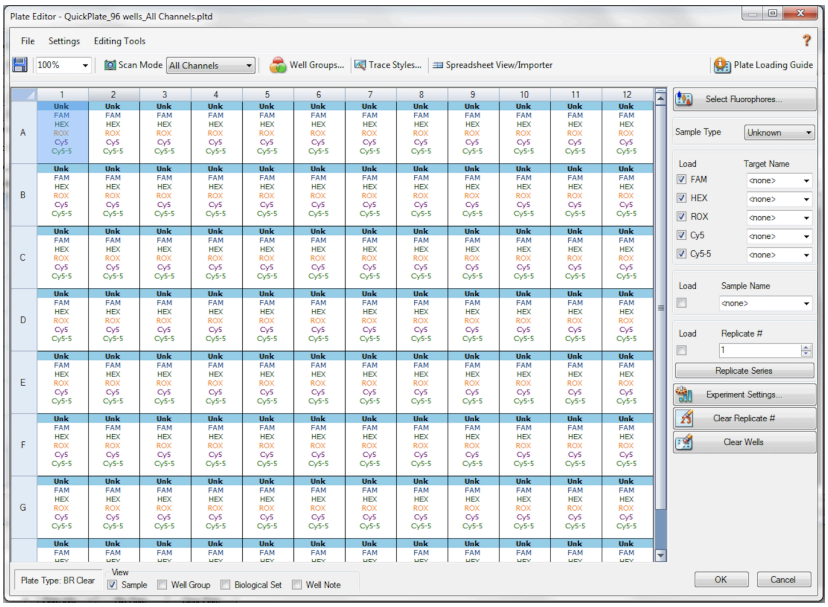


f. Attach the fluorophores to the wells being used.

i. Highlight all the wells being used
ii. For Aspergillus 2-Color Assay, **Highlight the well locations and click on FAM and HEX**

g. When the plate is designed correctly, click OK.

h. Click "yes" to save your plate. If creating plate layout for the first time, save as "qPCR Multiplex Detection". If you do not save the plate, it will return to the default plate.



Note: Saving will override the template (that is acceptable).

i. Close the lid and click Start Run.
j. Save the experiment with the [User] and [date]
k. When the run is complete, immediately dispose of the plate after qPCR. **Do not remove the plate seal after the run to avoid contamination in the lab.**

Data Analysis BioRad CFX96

PathoSEEK® Aspergillus 2-Color Data Analysis Quick Reference Table:

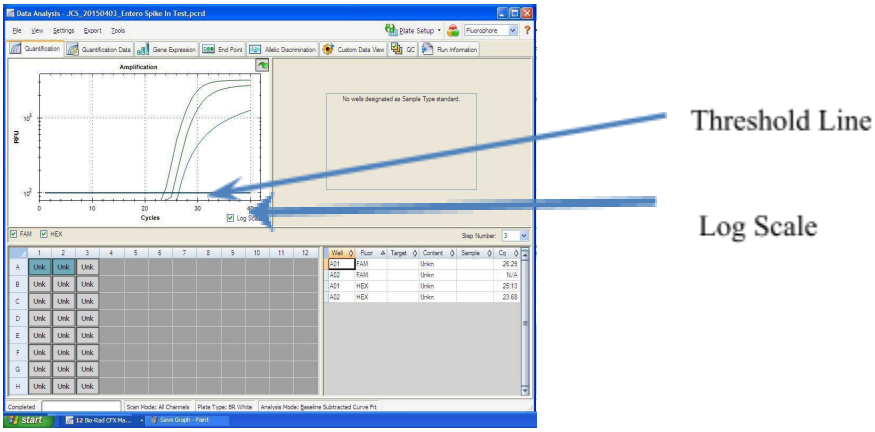
Table 1: All Matrices

PathoSEEK® Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Aspergillus 2 - Color Assay Aspergillus spp	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus flavus	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus fumigatus	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus niger	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus terreus	≤ 40	FAM	No Cq	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Control	≤35	FAM		

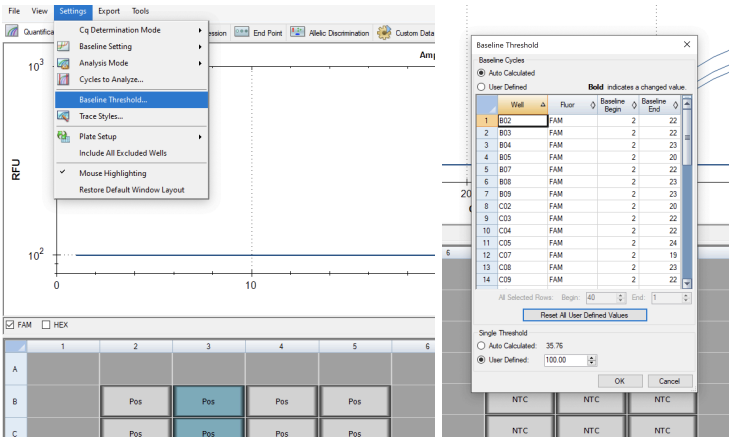
1. The Data Analysis window will open automatically when the run is complete.

2. Highlight the wells of interest.
    a. The graph will appear above.
    b. The Cq values will appear to the right.

3. To analyze the results
    a. Start by turning the graph to Log Scale and manually moving the threshold to 10^2 for all fluorophores.
        i. To turn the graph to Log Scale, click on the box at the bottom right of the graph.



ii. To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis. Alternatively, set an exact threshold value by selecting a single fluorophore at a time beneath the graph, then Settings > Baseline Threshold. In the next window select User Defined and enter 100.



b. Controls

i. Assay-specific Positive Control, on the FAM fluorophore, has a Cq value ≤ 35.

1. Visually confirm with the curve on the graph.

ii. Assay-specific Negative Control, on the FAM fluorophore, has no Cq value.

1. Visually confirm with the curve on the graph.

iii. Unknown Aspergillus Targets: The FAM fluorophore detects the following species of Aspergillus: Aspergillus niger, flavus, fumigatus and terreus

1. A “presence” or failing result for the unknown Aspergillus targets

a. Any Cq value for the FAM fluorophore ≤ 40.

i. Visually confirm with the curve on the graph. It is very important to confirm with the amplification curve when a presence result occurred. Sometimes the background amplification will give a false positive reading.

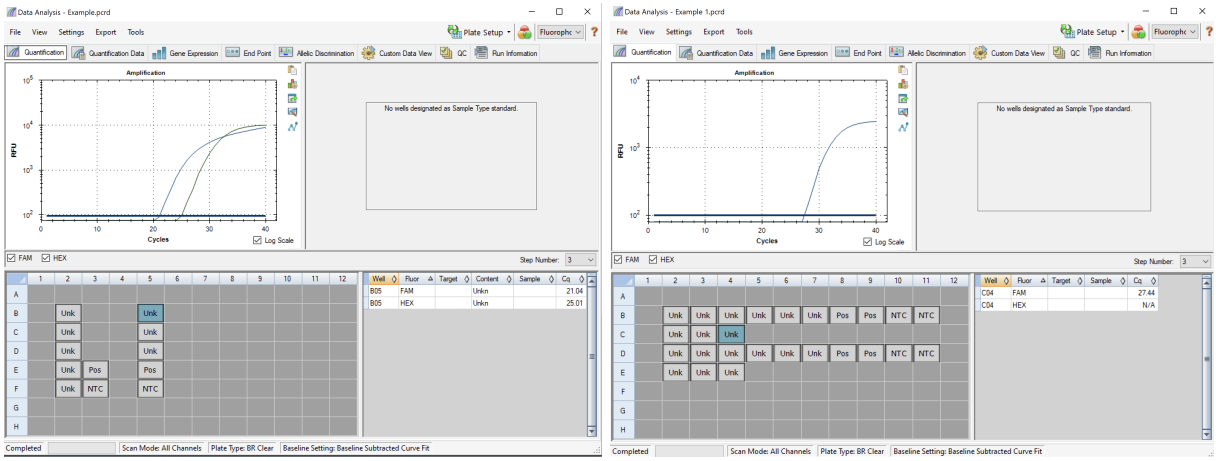


Figure: Example of a sample where Aspergillus was detected.

2. An “absence” or passing result for the unknown Aspergillus targets.

a. No Cq value for the FAM fluorophore.

i. Visually confirm no curve on the graph.

3. Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, < 40 for all other matrices.

a. Visually confirm with the curve on the graph

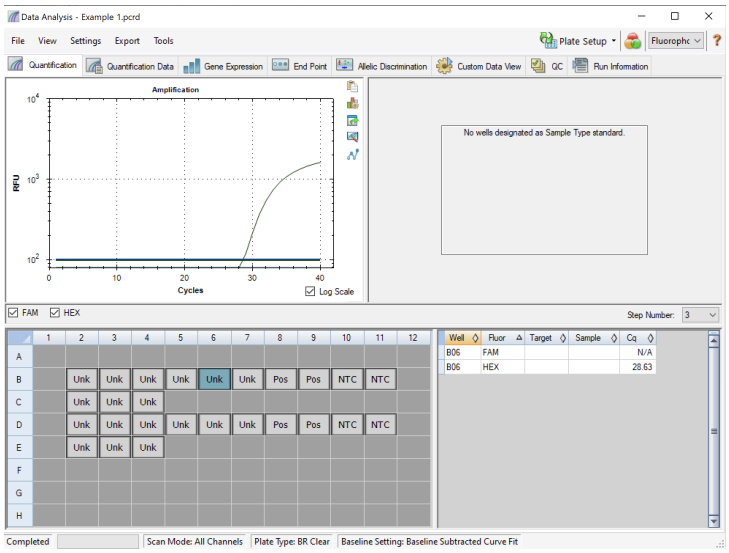
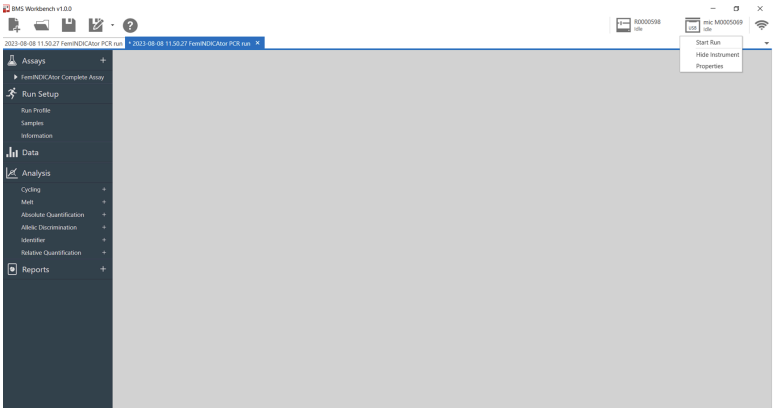


Figure: Example of a sample where Aspergillus was not detected.

Running the BioMolecular Systems MIC

1. Open the BMS Workbench software and create a new file.
2. Select qPCR Run.
3. Select the appropriate template by clicking the "+" sign next to assays or ensure that the appropriate thermal cycling conditions are entered: Hot start at 95 ºC for 5 minutes, followed by 40 cycles of 95 ºC for 15 seconds and 65 ºC for 90 seconds.
4. Click on the Mic icon in the upper right-hand corner, and select "Start run" from the menu that appears.



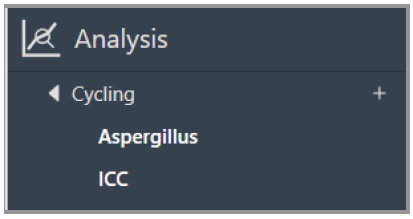
5. A pop-up will appear asking for the reaction volume, which will be auto-filled with the correct volume based on the template chosen. Close the lid and the instrument will start.

   Note: If the cover isn't closed, the program will not start.

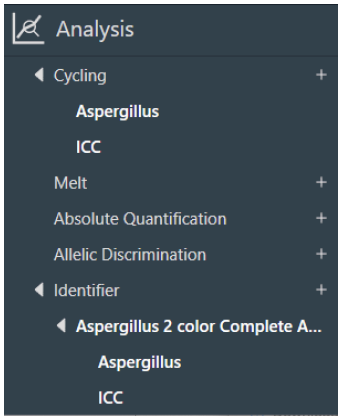
6. Let the Mic run to completion before analyzing the data.

Data Analysis with BioMolecular System MIC

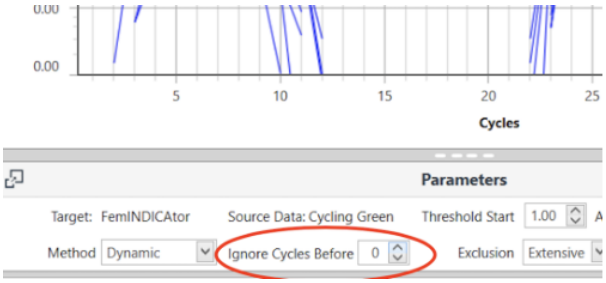
1. When the run has completed, click on the "+" sign next to the "Cycling" tab and select ICC (Internal Cannabis Control). The selection will appear under the "Cycling" tab. Next, select Aspergillus 2-Color Assay.



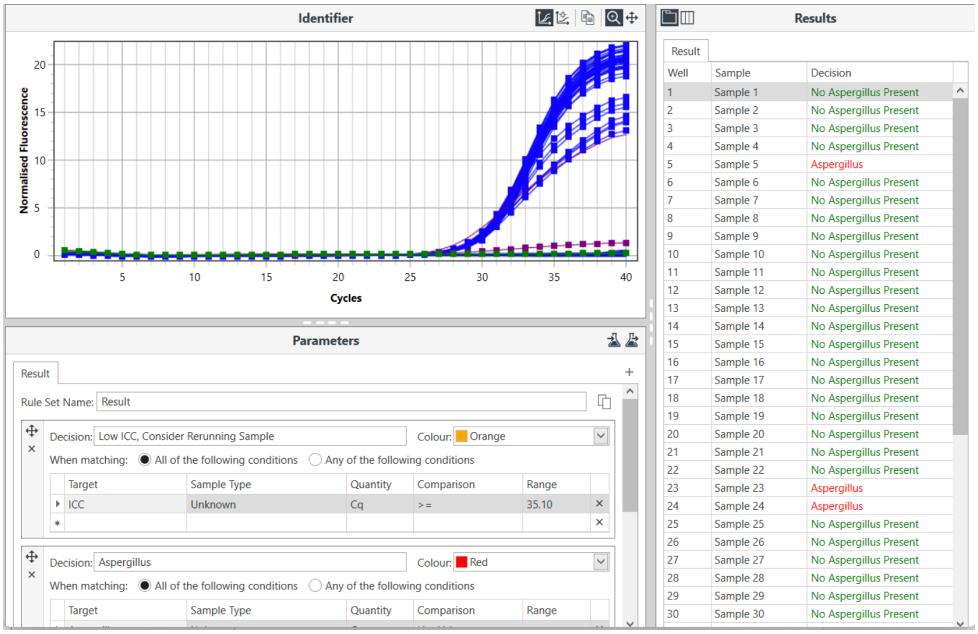
2. When these have been expanded, select the "+" sign next to the "Identifier" tab and select the available report.



3. If the automatic calls are blank, then one of the cycles is out of threshold. To fix this, find "Ignore Cycles Before" in the "Parameters" section, and increase its value one whole number at a time until a gray bar appears on the graph. This may have to be done with one, or all of the filters.



4. To review an automatic report, click the "+" sign next to the "Identifier" tab then select the relevant "Complete Assay". This feature will call the samples tested as detect, non-detect, or inconclusive based on the qPCR data. These results will be displayed on the right side of the screen.



5. Data may be exported by selecting the “Report” tab, then clicking the Export icon.

Troubleshooting Guide

Symptom	Reason	Solution
Internal control failure	ICC not spiked into sample/MaGiC lysis when applicable	Repeat MaGiC Lysis and PathoSEEK by following the protocol.
	Sample not added	Repeat MaGiC Lysis and PathoSEEK by following the protocol.
	Mix up in Reaction Setup	Repeat the qPCR by following the protocol.
	Missing Fluorophore on plate set up	Check plate setup in qPCR platform data file and ensure the correct fluorophores were chosen for the assay being run.
	qPCR inhibition	Dilute MaGiC Lysates 1:10 with nuclease free water and re-perform qPCR
Internal Control Positive result on assay positive or negative control reactions or reactions containing samples that do not contain plant DNA No Cq or a Cq of more than 35 is acceptable. Any Cq lower than 35 constitutes a rerun	Cannabis DNA or ICC contamination in a reagent	Troubleshoot which reagent was contaminated; use new reagents, thoroughly clean all pipettes and bench areas with 10% bleach solution.
	qPCR bench too close to MaGiC lysis area	Designate separate benches, pipettes etc. for lysis and qPCR setup
Positive Negative Control	Small Cq value <15	Visually confirm that there is a true amplification curve. If not, this is a low level background and can be ignored
	Contamination	Repeat the qPCR by following the protocol.
	Insufficient pre-setup bleaching	Wipe down the lab workspace and all equipment with 10% Bleach. Repeat qPCR.
Negative Positive Control	Mix up in Reaction Setup	Ensure correct well location was chosen for assay positive control. If yes, repeat the qPCR by following the protocol.

Glossary and Definitions

Deoxyribonucleic acid (DNA) is a molecule that encodes the genetic instructions used in the development and functioning of all known living organisms.

Polymerase Chain Reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

A **fluorophore** is a fluorescent chemical compound that can re-emit light upon light excitation.

The **Negative Controls** are the reactions where no Cq is expected. It helps to ensure that all Assay-specific reactions are clean of contaminants.

The assay-specific **Positive Controls** are the reactions where a Cq is expected. It helps ensure that all Assay-specific reactions are working correctly. The Assay specific Positive Control is targeting the pathogen using the FAM, ROX and Cy5 Fluorophores.

Amplification of the **Internal Cannabis Control** or the microbial target of interest is expected in every reaction containing DNA isolated from a cannabis sample. It ensures the DNA isolation procedure was successful or the presence of microbial contamination. The internal cannabis control targets the cannabis genome, using the HEX Fluorophore.

MIP is short for Marijuana Infused Product. A MIP is cannabis plant material or concentrate mixed into a consumable.

Revision History

Version	Date	Description
v1	August 2024	PathoSEEK® Aspergillus 2-Color v2 and Aspergillus Singleplex assays with MaGiC Product Launch

DISCLAIMER

This test was developed, and its performance characteristics determined by Medicinal Genomics Company, for laboratory use. Any deviations from this protocol are not supported by MGC.

This test has not been validated on remediated (irradiated, ozone treated, acid treated, hydrogen peroxide treated, etc.) samples. Samples that have undergone remediation may cause discordant results between plating methods and PathoSEEK methods. When remediated samples produce a result above the action limit on qPCR, we recommend confirming viability with an approved plating method.

Results may vary based on laboratory conditions. Altitude and humidity are among factors known to affect the growth of bacterial and fungal species.