PathoSEEK® Aspergillus Multiplex Detection Assays with MaGiC Lysis Kit

User Guide v3

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

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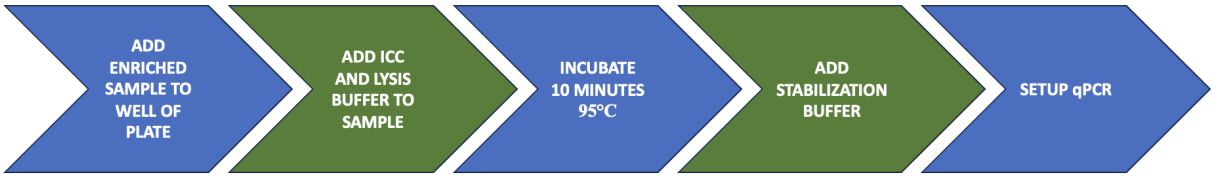
Introduction

Current regulations require cannabis flower and cannabis products to be free of select species of *Aspergillus*. The PathoSEEK® 5-Color and 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 and cannabis concentrates.

Process Overview

The PathoSEEK 5-Color and 2-Color Aspergillus Multiplex Detection Assays use a multiplexing strategy with an internal control (IC) that is introduced as the lysis step to ensure accurate detection of four species of *Aspergillus* as well as the internal control 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 a simplified depiction of the MaGiC lysis in Figure 1 and qPCR assays in Figure 2.

Figure 1: Overview of MaGiC Lysis



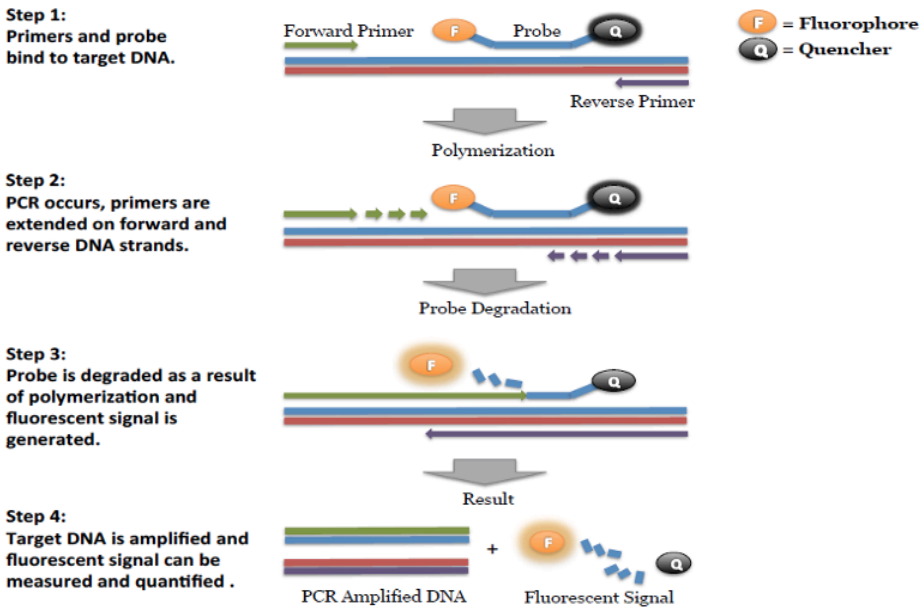


Figure 2: Overview of qPCR

The PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2 for use on the AriaMx Real-Time PCR Thermal cycler (Agilent) uses the ATTO 425 Fluorophore for detection of the *Aspergillus terreus*, while the PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2, for use on the CFX-96 (Bio-Rad), uses the Cy5.5 Fluorophore for the detection of *Aspergillus terreus*. The PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 is compatible with qPCR instruments that support HEX and FAM and has been validated on the BMS Mic, Agilent AriaMX, and Bio-RAD CFX96.

Limit of Detection

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

Kit Components

PathoSEEK 5-Color Aspergillus Multiplex - AriaMx with MaGiC Lysis Kit - **P/N 420518** (contains sufficient reagents for 200 reactions). Please note some components are stored at different temperatures.

Component Name	Qty Provided	Storage Conditions
MaGiC Lysis Reagent	2 Bottles (12 mL each)	RT (20–28°C)
MaGiC Stabilization Buffer	1 Bottle (24 mL)	RT (20–28°C)
PathoSEEK Amplification Mix *Includes 2 tubes nuclease free water for resuspension*	4 Vials (50 rxns each)	RT (20–28°C) / -15 to -20 ºC*
PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2	1 Tube (200 µL)	-15 to -20 ºC

Note: 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 -15 to -20 ºC for up to 3 months.

PathoSEEK 5-Color Aspergillus Multiplex - CFX with MaGiC Lysis Kit- **P/N 420519** (contains sufficient reagents for 200 reactions). Please note some components are stored at different temperatures.

Component Name	Qty Provided	Storage Conditions
MaGiC Lysis Reagent	2 Bottles (12 mL each)	RT (20–28°C)
MaGiC Stabilization Buffer	1 Bottle (24 mL)	RT (20–28°C)
PathoSEEK Amplification Mix *Includes 2 tubes nuclease free water for resuspension*	4 Vials (50 rxns each)	RT (20–28°C) / -15 to -20 ºC*
PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2	1 Tube (200 µL)	-15 to -20 ºC

Note: 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 -15 to -20 ºC for up to 3 months.

PathoSEEK 2-Color Aspergillus Multiplex with MaGiC Lysis Kit, **P/N 420523** (contains sufficient reagents for 200 reactions). Please note some components are stored at different temperatures.

Component Name	Qty Provided	Storage Conditions
MaGiC Lysis Reagent	2 Bottles (12 mL each)	RT (20–28°C)
MaGiC Stabilization Buffer	1 Bottle (24 mL)	RT (20–28°C)
PathoSEEK Amplification Mix *Includes 2 tubes nuclease free water for resuspension*	4 Vials (50 rxns/each)	RT (20–28°C)/ -15 to -20 ºC*
PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2	1 Tube (200 µL)	-15 to -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 -15 to -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	-15 to -20 ºC
420337	Internal Control	1 Tube (50 µL)	-15 to -20 ºC
420330	PathoSEEK Aspergillus Multiplex Positive Control v3	1 Tube (50 µL)	-15 to -20 ºC
420184	PCR Grade Water	500 mL Bottle	2-25 °C

Additional **Optional** Reagents Not in Kit:

Item P/N	Item Name	Qty Provided	Storage Conditions
420145	Grim Reefer Free DNA Removal Enzyme and Buffer	100 Reactions	-15 to -20 ºC
	Grim Reefer Enzyme	1 Bottle (2.5 mL)	-15 to -20 ºC
	Grim Reefer Buffer	1 Bottle (12.75 mL)	-15 to -20 ºC
420150	Grim Reefer Deactivation Buffer	1 Tube (2.4 mL)	20 to 28ºC

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**
    - Software version 3.1.2306.0602
    - 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.
    - Software version 10.0.26100
    - Bio-Rad supplied or lab supplied Windows PC
- Bio Molecular Systems Mic 4-Channel PCR Instrument - **Medicinal Genomics P/N 420241**
    - Latest Version of BMS Workbench Software - can be found on the BMS Westsite, https://biomolecularsystems.com/media-downloads/myra-downloads-2/

    - 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
- Plate centrifuge - USA Sci. Part # 4062-0572 - Eppendorf 5430 centrifuge w/ rotary knobs, non-refrigerated, with Rotor A-2-MTP swing bucket rotor
- 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
- Beaker or Solo Cup. (optional))
- 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
- 25 mL 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 Aspergillus 5- Color and 2- Color Multiplex Assays with MaGiC Lysis Kit 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. To prepare 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)
        1. This solution is stable for one month at 4°C
2. Wipe down the workspace with a 10% bleach solution, including the bench top and all equipment being used.
3. If not already at room temperature, remove PDB + Chloramphenicol from the 2-8 °C refrigerator, the PDB should come to room temperature, 20–28 °C, before use.
4. Prepare consumables. Label all the filter bags or conical tubes with "[sample name] [date]".
5. Label lysis plate with date.
6. 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.
7. *Dried 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.
    a. Incubate a full 48 hours if Grim Reefer Free DNA Removal steps will be performed.
8. *Concentrates* ***n*** *grams* —Weigh concentrate into a 15 mL conical tube, 50 mL conical tube, or Whirl-Pak bag depending on the concentrate test portion size. Add 9 x *n* mL of PDB with chloramphenicol to each test portion. Homogenize the test portion until thoroughly mixed in PDB + chloramphenicol. Incubate for 48 - 54 h at 37 ± 2 °C.
9. 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. See flower example in Figure 3.

Figure 3: Homogenized cannabis flower and PDB + Chloramphenicol.

MaGiC Lysis

Matrices: Dried Cannabis Flower, Infused Cannabis Products, and Cannabis Concentrates

1. Dilute IC to 1:50,000
    a. Dilute stock IC to 1:100.
        i. Ensure stock IC is fully thawed and vortexed, and then pulse spin down in a mini centrifuge before use.
        ii. Add 2 µL of stock IC to 198 uL of nuclease free water. Vortex well and pulse spin down in a mini centrifuge.
    b. Serially dilute the 1:100 dilution of IC an additional 1:100 to make a 1:10,000 dilution.
        i. Add 2 µL of 1:100 IC to 198 µL of nuclease free water. Vortex and pulse spin down in a mini centrifuge. This is the 1:10,000 dilution IC.
    c. Dilute the 1:10,000 IC by 1:5, which results in the final dilution of 1:50,000.
        i. Add 20 µL of the 1:10,000 IC to 80 µL of nuclease free water. Vortex and pulse spin down in a mini centrifuge. Final dilution is 1:50,000 IC.
2. Remove samples from the incubator (contained in the Whirl Pak Bag, 15 or 50 mL conical tube).
    a. Flower sample: Mix thoroughly by hand manipulating and/or squeezing the contents in Whirl Pak bag for the flower enrichment for 1 minute.

b. **Infused Products:** Mix thoroughly by hand manipulating and/or squeezing the contents in Whirl Pak bag for the food enrichment for 1 minute. Vortex conical tube thoroughly for 30 seconds.

c. **Concentrates:** Vortex conical tube thoroughly for 30 seconds.

Note: Aspergillus growth tends to clump together, this post enrichment homogenization and/or vortexing will aid in spreading cells throughout the media in the Whirl Pak bag or tubes.

3. Remove 50 µL of enriched test portion and transfer into the well of a 96 well PCR plate.

OPTIONAL: Grim Reefer Free DNA Removal

4. If performing Grim Reefer Free DNA Removal (to be used in 48 incubation protocols ***ONLY***), proceed with the following steps; if not, skip to step 5.
    a. Add 10 µL of Grim Reefer Buffer to each sample being tested, using a fresh pipette tip for each transfer.
    b. Add 2 µL of Grim Reefer Enzyme to each sample being tested, using a fresh pipette tip for each transfer. .
    c. Pipette tip mix thoroughly using a fresh pipette tip for each transfer. Tip mix 15 times to ensure the sample is mixed with the Grim Reefer Buffer and Grim Reefer Enzyme.
    d. Seal plate and incubate on a thermal cycler at 37℃ for 10 minutes.
    e. Remove plate from thermal cycler and spin plate in plate centrifuge.
    f. Carefully remove the seal and add 10 µL of Grim Reefer Deactivation Buffer to each sample. Pipette tip mix thoroughly using a fresh pipette tip for each transfer. Tip mix 15 times to ensure the sample is mixed with the Grim Reefer Deactivation Buffer.
    g. Proceed to step 5.
5. For Infused Products and Concentrate samples, Spike IC into Samples (Optional for flower samples)
    a. Add 5 µL of 1:50,000 dilution of IC to the 50 µl of each concentrate sample.
    b. OPTIONAL: Add 5 uL of the 1:50,000 dilution of IC to the 50 µl of each flower sample.
6. Add 100 µL of MaGiC Lysis Buffer to all wells being tested, using a fresh pipette tip for each transfer. Tip mix 15 times to ensure the sample is mixed with the lysis buffer.

7. Seal the top of the plate and briefly spin down in a centrifuge.
8. Place plate on the thermal cycler and run MaGiC Lysis program
    a. Incubate 95 °C for 10 minutes, then 4 °C for 5 min (if 4°C is not an option, set to 25 °C for 10 min).

       Note: When using the Agilent AriaMX, a compression pad should be placed over the adhesive seal to prevent evaporation from the plate and seal.
9. Remove plate with lysed samples from the thermal cycler and briefly spin the plate down in centrifuge to remove evaporation from the plate seal. Carefully remove the plate seal.
10. Tip mix 5 times and then transfer 50 µL of lysed sample to a new plate.
11. Add 100 µL of MaGiC Stabilization buffer to each sample well using a new pipette tip for each sample. Pipette mix 5 times. The samples are now ready for qPCR set up, see Real - Time Quantitative PCR (qPCR) Setup Protocol with PathoSEEK Amplification Mix.
12. Seal the plate with sample lysates with a plate seal and store in -20 °C freezer if not proceeding directly to qPCR setup.
    a. Sample lysates with stabilization buffer added are stable at -20 ºC for 1 month.
    b. Thaw frozen samples at room temperature. Sample lysates which have been frozen must be tip mixed 15 times prior to setting up qPCR or performing dilutions.

Real-Time Quantitative PCR (qPCR) Setup Protocol

1. Remove PathoSEEK Amplification Mix, PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2, PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2, or the PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2, and Aspergillus Multiplex Positive Control v3 from the -20 ºC freezer.
    a. If lyophilized Amplification Mix has not been previously rehydrated, rehydrate with 550 µl of Nuclease Free Water. Swirl or pipette tip mix. After resuspension, store remainder Amplification Mix at -15 to -20 °C when not in use.
    b. Allow all frozen reagents to defrost at room temperature (20-28 °C). Once defrosted, place tubes on ice.
2. Before preparing the Master Mix, invert or vortex and pulse spin down the reagents in a mini centrifuge

a. 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2, 5-Color Aspergillus Multiplex (CFX) Detection Assay v2, or the PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 (probe mix) – vortex tube quickly followed by a pulse spin down in a minicentrifuge.
b. Aspergillus Multiplex Positive Control v3 tube – vortex tube quickly followed by a pulse spin down in a minicentrifuge.
c. PathoSEEK Amplification Mix – Invert the bottle 5-10 times to mix or briefly vortex.
d. Return all reagents to the ice.

3. Prepare Master Mix in a 1.5 mL tube (the detection assay also includes the probe for the IC). Label the tube as "Master Mix". See Table 1 (PathoSEEK Amplification Master Mix Reagent Volumes).

   Always prepare enough Master Mix for an additional one or two reactions to account for pipetting and dead volumes. Be sure to include 2 extra reactions for the qPCR positive and negative controls. For example, if testing 10 samples, you would need to make enough Master Mix for 13 or 14 reactions, which would account for 1 or 2 excess.

Table 1: PathoSEEK Amplification Master Mix Reagent Volumes

Reagent	**Volume for 1 qPCR Reaction**
PathoSEEK Amplification Mix	10 µL
5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2 or 5-Color Aspergillus Multiplex (CFX) Detection Assay v2 or 2-Color Aspergillus Multiplex Detection Assay v2	1 µL
Nuclease Free Water	4 µL
Total	15 µL

4. Once the Master Mix is combined gently cap the tube and vortex to mix.
   a. Pulse spin down tube in minicentrifuge.
   b. Place the Master Mix tube on ice until used.
5. For the negative control, use nuclease free water that was used to rehydrate your Amplification Mix.

6. For the positive control, use the Aspergillus Multiplex Positive Control v3 Positive Control reagent. Dilute the positive control 1:10.
    a. Add 1 µL of Positive Control to 9 µL nuclease free water (found in the kit), vortex to mix and pulse spin down the tube in mini centrifuge

       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.
7. For PCR reaction use a 96-well optical qPCR plate or optically clear qPCR tubes.
8. Transfer lysed samples into qPCR plate wells or tubes
    a. Carefully remove the seal from the Extraction Plate.

       Note: If lysed samples were frozen, let the DNA thaw completely and spin the plate in centrifuge to avoid cross contamination between samples. Tip mix thawed samples wells before transferring to the qPCR plate or tubes.
    b. Pipette transfers 5 µL of each sample lysate into the corresponding qPCR tube or well on the qPCR plate.
    c. Add 5 µL of the diluted Positive Control to the corresponding positive control plate well or tube.
    d. Add 5 µL of nuclease free water to the corresponding negative control plate well or tube.

       Note: ALWAYS use a fresh tip for every liquid transfer of sample, positive, or negative control into the qPCR plate
9. Add 15 µL of Master Mix to each corresponding sample well, positive control well, and negative control well in the qPCR plate or tubes. Gently tip mix a few times after each addition of aster Mix. Be careful not to introduce bubbles during this mix. Use a fresh tip for each transfer of Mater Mix to each well
10. Seal the plate with strip caps or an adhesive seal, or seal qPCR tubes with strip caps.
11. For the Agilent AriaMX or Bio-Rad CFX, spin down qPCR plate or tubes for at least 1 minute in plate (or tube) centrifuge to bring well contents to the bottom of wells (or tubes) and help to get rid of reaction bubbles.

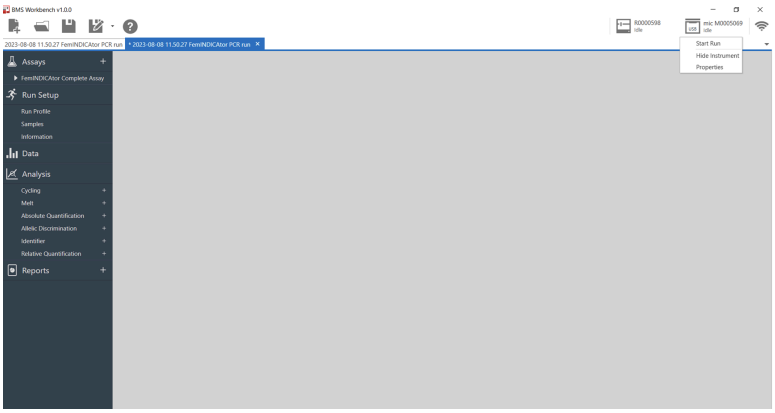
    Note: Check for bubbles in the wells or tubes (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the wells (or tubes), spin down for another minute in plate centrifuge.

12. For the Agilent Aria MX: If using an adhesive seal; place the reusable compression pad (gray side down) on the plate directly lining up the holes in the pad with the wells in the plate.
13. Place the sealed plate or tubes onto the PCR instrument.
14. Follow the software specific instructions for each qPCR platform to initiate the run.

Running the BioMolecular Systems Mic

(PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 ONLY)

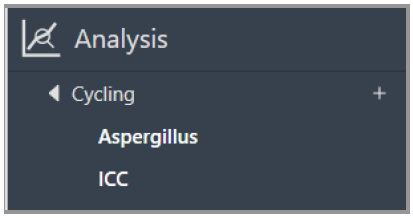
1. Open the BMS Workbench software and create a new file.
2. Select qPCR Run.
3. Select the appropriate template by clicking the "+" sign next to assays or ensure that the appropriate thermal cycling conditions are entered: Hot start at 95 ºC for 5 minutes, followed by 40 cycles of 95 ºC for 15 seconds 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.



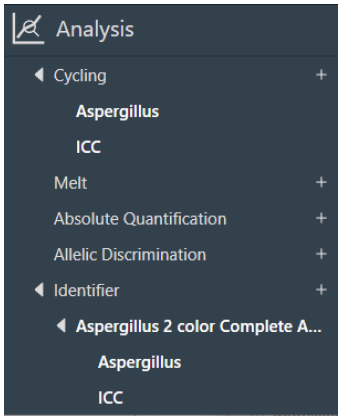
5. A pop-up will appear asking for the reaction volume, which will be auto-filled with the correct volume based on the template chosen. Close the lid and the instrument will start.
   Note: If the cover isn't closed, the program will not start.
6. Let the Mic run to completion before analyzing the data.

Data Analysis with BioMolecular System MIC

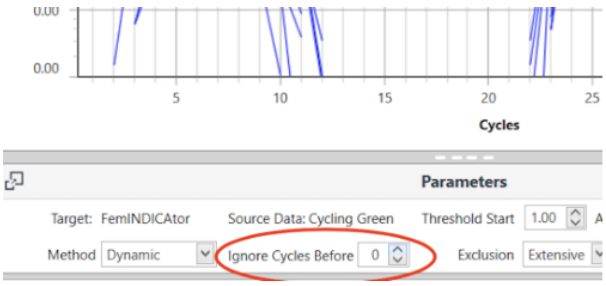
1. When the run has completed, click on the "+" sign next to the "Cycling" tab and select IC (Internal Control). The selection will appear under the "Cycling" tab. Next, select Aspergillus 2-Color Assay.



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, see Figure 4

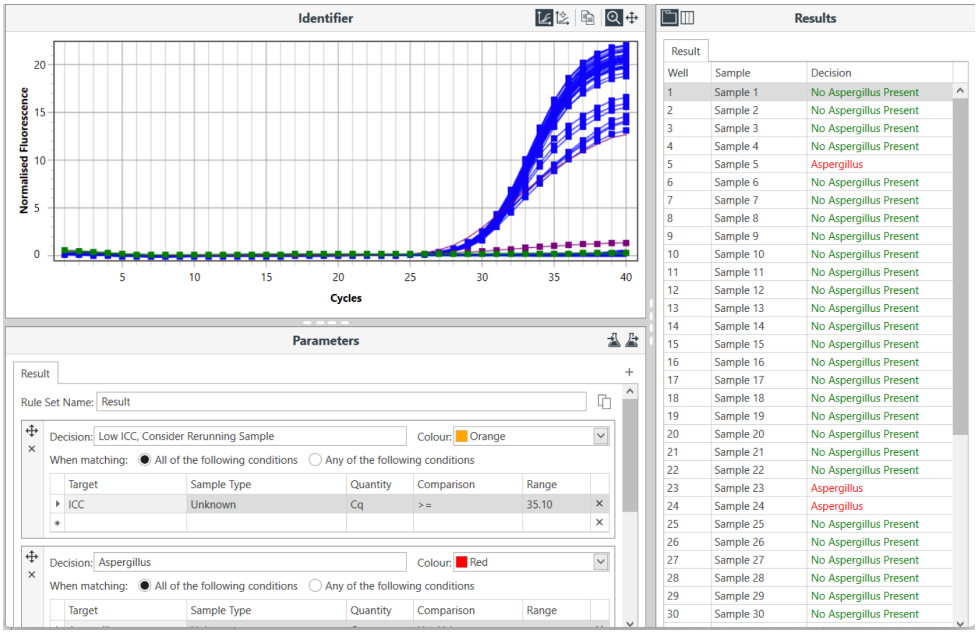


Figure 4. Bio Molecular Systems Mic: PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 Data Analysis:

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

Running the Agilent AriaMX

The following species will be detected on the following Fluorophores:

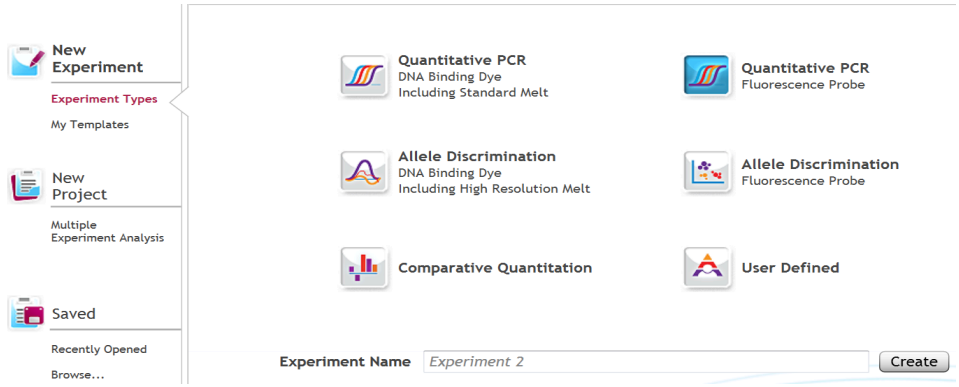
PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2

Target	Optical Channel
Aspergillus niger, flavus, fumigatus and terreus	FAM
Internal Control	HEX

PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2

Target	Optical Channel
Aspergillus niger	ROX
Aspergillus flavus	Cy5
Aspergillus fumigatus	FAM
Aspergillus terreus	ATTO425
Internal Control	HEX

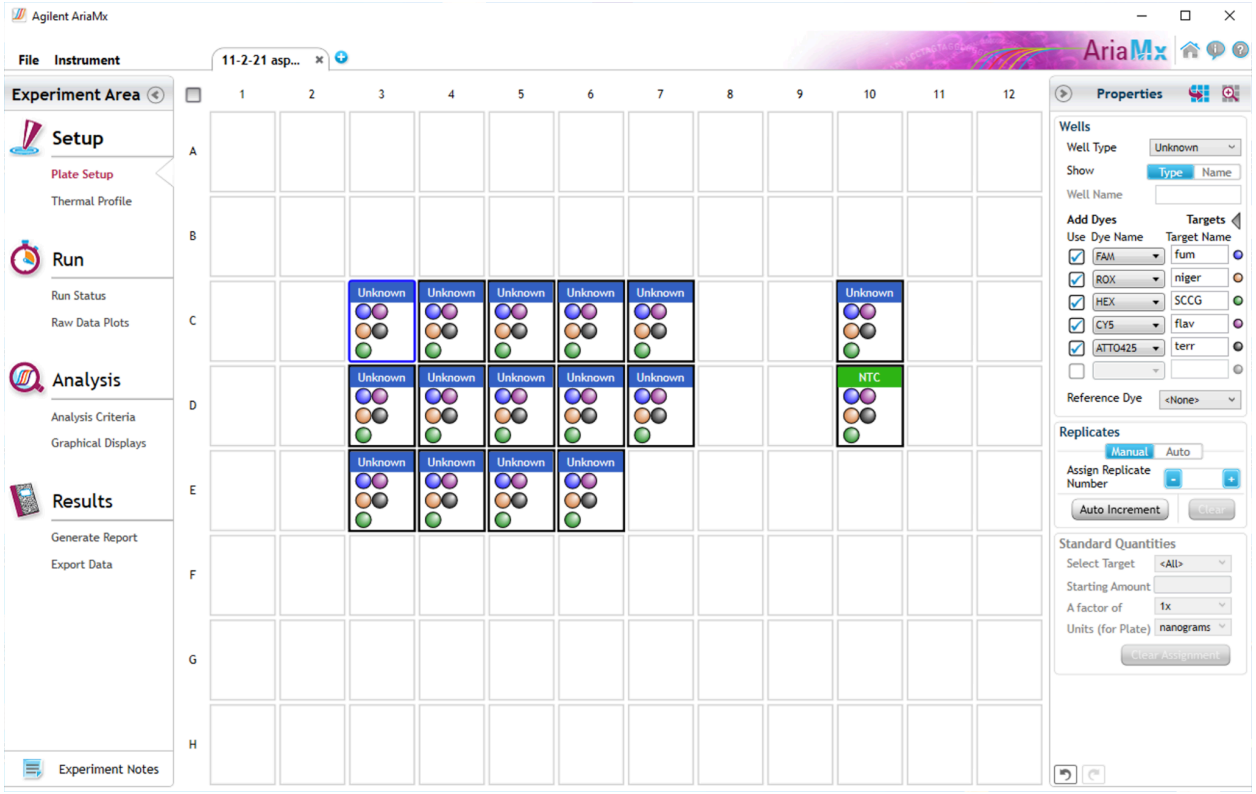
1. Create a New Experiment on the Agilent qPCR instrument.
    a. Select "Quantitative PCR: Fluorescence Probe" from Experiment Types.



    b. Under Setup > Plate Setup, highlight **only** wells that contain reactions and select necessary fluorophores:
        i. PathoSEEK Aspergillus 5 - Color Detection Assay v2: FAM, HEX, ROX, Cy5, and ATTO 425 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.

ii. PathoSEEK Aspergillus 2 - Color Detection Assay v2: 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 set up. All wells should be set to Unknown except 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. PathoSEEK Aspergillus 5 - Color Detection Assay v2

      i. FAM : *Aspergillus fumigatus*

      ii. ROX : *Aspergillus niger*

      iii. Cy5 : *Aspergillus flavus*

      iv. ATTO 425 : *Aspergillus terreus*

      v. HEX : Internal Control

   b. PathoSEEK Aspergillus 2 - Color Detection Assay v2

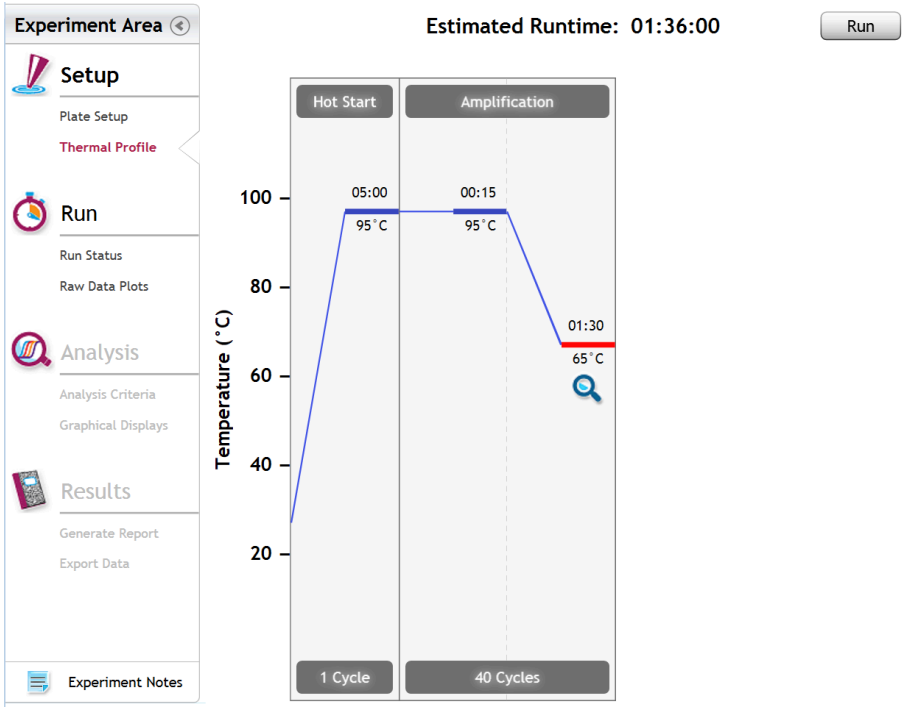
i. FAM : *Aspergillus niger, flavus, fumigatus* and *terreus*

ii. HEX : Internal Control

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.

   Note: A compression pad should be placed on top of the plate with an adhesive seal to prevent cross contamination.



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 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2 Data Analysis

Table 2: **Agilent AriaMx**: PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2 Data Analysis

PathoSEEK™ Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Aspergillus niger	≤ 40	ROX	No Cq	Presence/Absence
Aspergillus flavus	≤ 40	Cy5	No Cq	Presence/Absence
Aspergillus fumigatus	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus terreus	≤ 40	ATTO 425	No Cq	Presence/Absence
Internal Control (IC)*	≤35	HEX	*Internal cannabis control verifies the presence or absence of cannabis DNA	
Assay Positive Control	≤35	FAM/ROX/Cy5/ATTO 425		

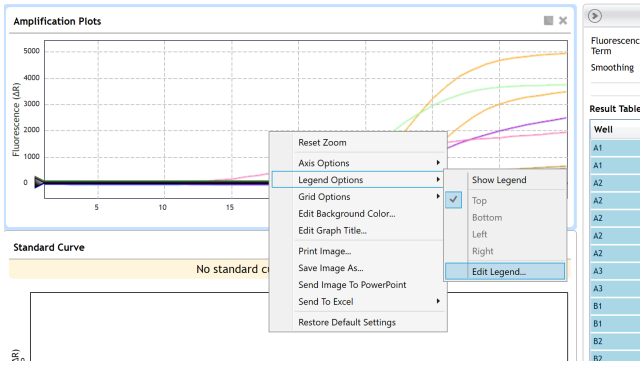
PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 Data Analysis

Table 3: **Agilent AriaMx**: PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 Data Analysis

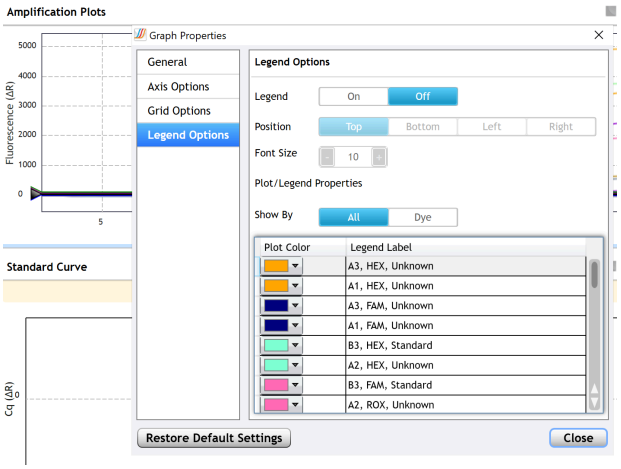
PathoSEEK™ Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Aspergillus niger, flavus, fumigatus, terreus	≤ 40	FAM	No Cq	Presence/Absence
Internal Control (IC)*	≤35	HEX	*Internal cannabis control verifies the presence or absence of cannabis DNA	
Assay Positive Control	≤35	FAM		

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 Display
    1. Amplification plots will be available for viewing
    2. The Cq values will appear to the right in the table

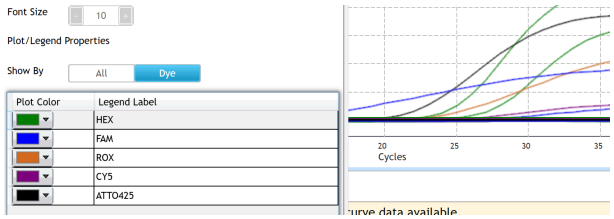
3. Right click inside the graph, select Edit Legend under Legend Options



4. Change "All" to "Dye"

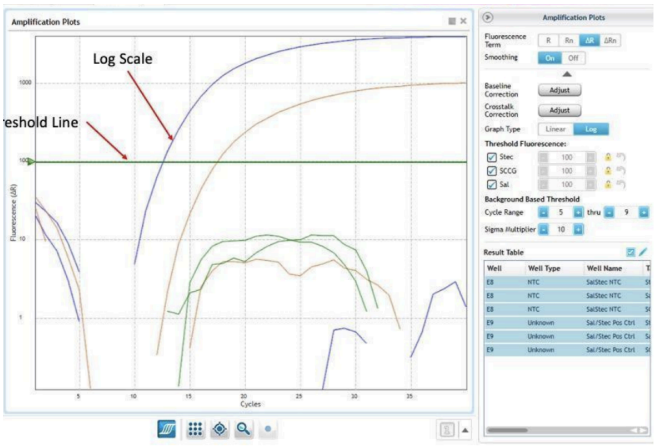


5. All user settings for Plot/Legend Properties will be removed. Do you want to proceed? Select "Yes".
6. This will assign a single color to each fluorophore.

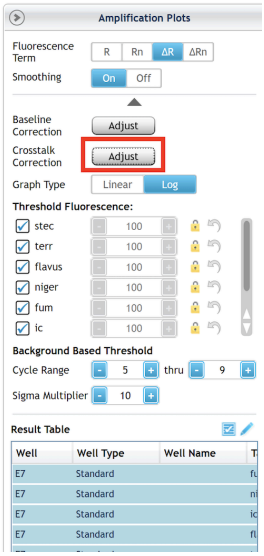


3. To analyze the results

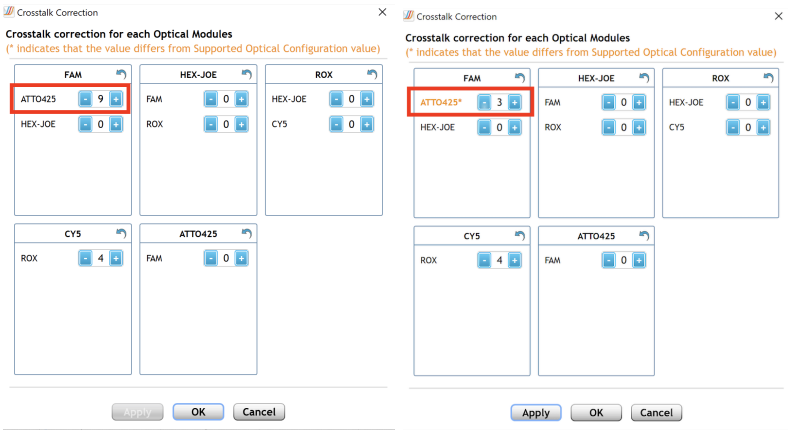
1. Start by turning the graph to Log Scale with a right click on the chart, select Axis options, enable y-axis log scale. Expand the amplification plots settings by clicking on the triangle.



4. Adjust Crosstalk Correction
    1. In the Graphical Displays tab click “Adjust” next to Crosstalk Correction.

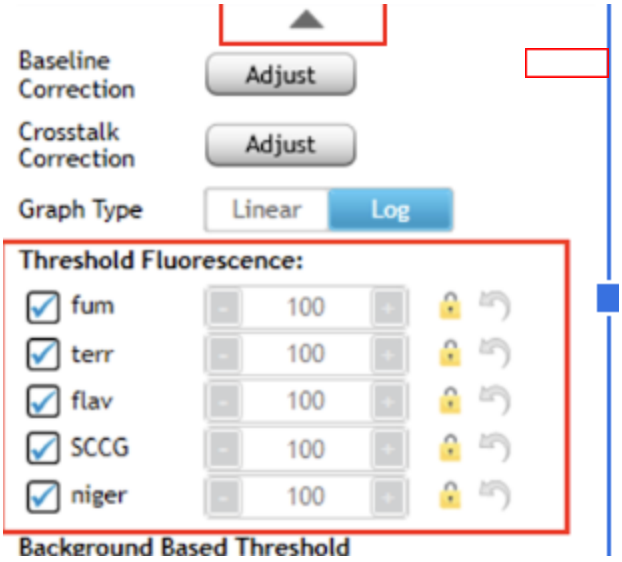


    2. In next window locate the FAM Dye Box and change the ATTO425 setting from 9 to 3:



3. Click “Apply” then click “OK”.

5. Manually adjust thresholds to 100 RFU
    1. PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2 - adjust and lock thresholds to 100 RFU for the HEX, FAM, ROX, Cy5, and ATTO425 fluorophores. (Target names will have previously been identified.)
    2. PathoSEEK 2- Color Aspergillus Multiplex Detection Assay v2 - adjust and lock thresholds to 100 RFU for the FAM and HEX fluorophores. (Target names will have previously been identified.)



6. Controls

1. Positive Control using PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2
    a. Amplification on the FAM, ROX, Cy5, and ATTO 425 Fluorophores at a Cq value of ≤ 35 should be observed.
        i. Visually confirm with the curve on the graph.
2. Positive Control using PathoSEEK 2- Color Aspergillus Multiplex Detection Assay v2
    a. Amplification on the FAM Fluorophore at a Cq value of ≤ 35 should be observed.
        i. Visually confirm with the curve on the graph.
3. Negative Control
    a. PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2 - Amplification on the FAM, ROX, Cy5, and ATTO 425 Fluorophores should not be observed or have no Cq Value.
    b. PathoSEEK 2- Color Aspergillus Multiplex Detection Assay v2 - Amplification on the FAM Fluorophore should not be observed or have no Cq Value.
    c. Visually confirm with the curve on the graph.

7. Unknown Aspergillus Targets
    1. PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2
        a. A "presence" or failing result for the samples.
            i. Any Cq value for the FAM, ROX, Cy5, or ATTO 425 Fluorophores ≤ 40.
    2. PathoSEEK 2- Color Aspergillus Multiplex Detection Assay v2
        a. A "presence" or failing result for the samples.
            i. Any Cq value for the FAM Fluorophore ≤ 40.

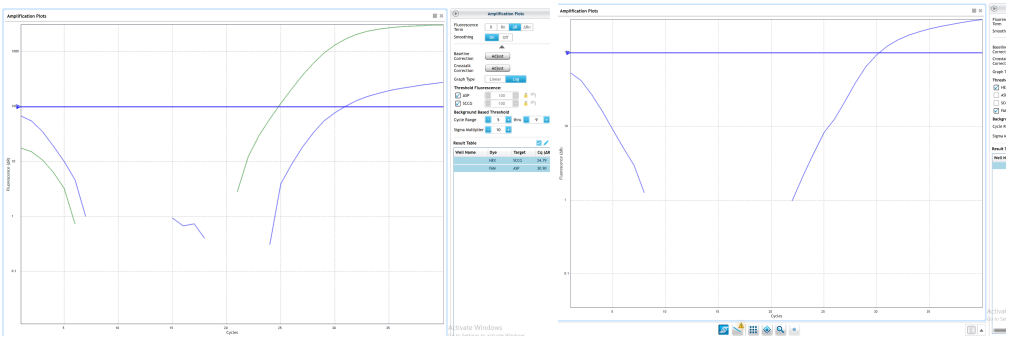


Figure 5: Example of a sample where Aspergillus was detected

3. 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.
    a. Review the raw data for each sample: 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.

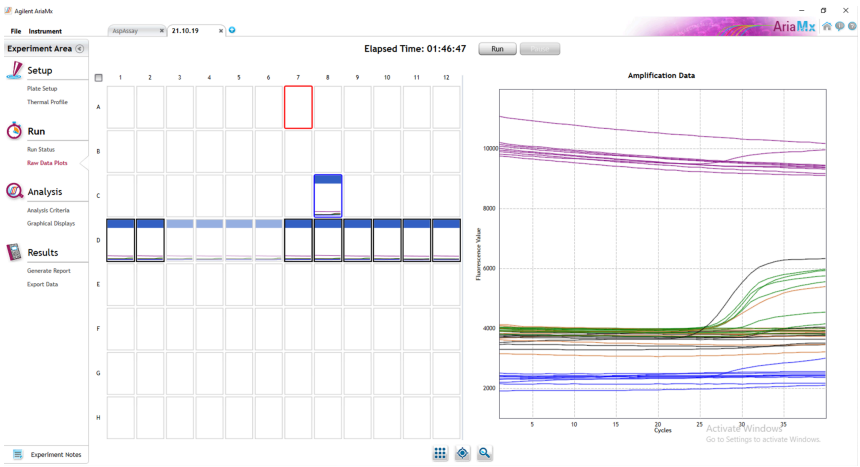


Figure 6: Raw data (R) plots for which the cycle quantification (Cq) will be generated.

    b. 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.)

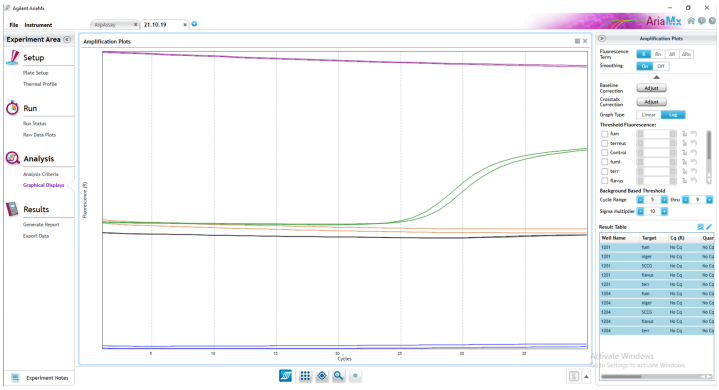


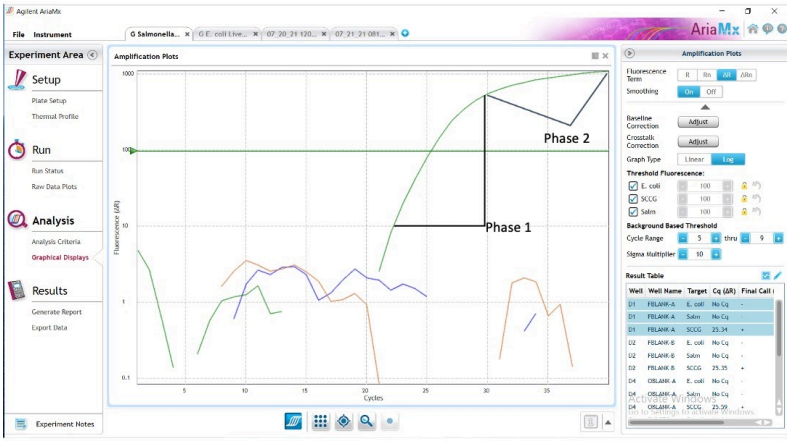
Figure 7: Example of good baseline with sigmoidal shaped curve increase in the internal control fluorescence.

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

   i. 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.

   ii. Phase 1 shows exponential growth in the fluorescence, in a span of around 5 cycles.

   iii. Phase 2 is a plateau where the amplification signal growth ends but remains level.



4. An “absence” or passing result for the samples.
    a. Internal Control (IC), on the HEX fluorophore, has a Cq value ≤ 35 for all sample types.
        i. Visually confirm with the curve on the graph
        ii. If HEX Cq value is >35, dilute the lysed sample 1:2 or 1:10 with nuclease free water and rerun qPCR.

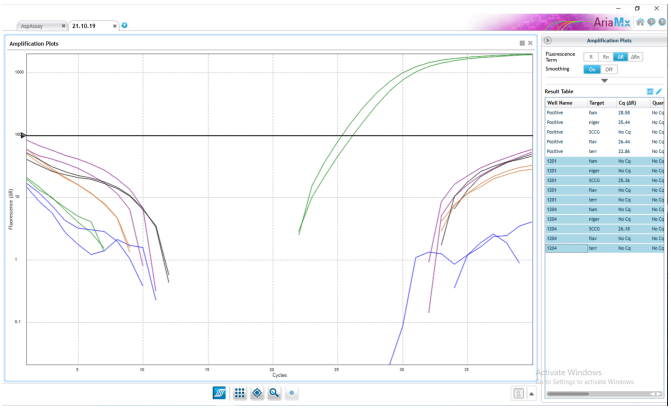


Figure 8: Example of samples where Aspergillus was not detected

    b. PathoSEEK 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2 - No Cq value for the FAM, ROX, Cy5, and ATTO425 Fluorophores should be observed.
        i. Visually confirm that there is no curve on the graph.
    c. PathoSEEK 2 - Color Aspergillus Multiplex Detection Assay v2 - No Cq value for the FAM Fluorophore should be observed

Running the BioRad CFX96

The following targets will be detected on the following fluorophores:

PathoSEEK 2 - Color Aspergillus Multiplex Detection Assay v2

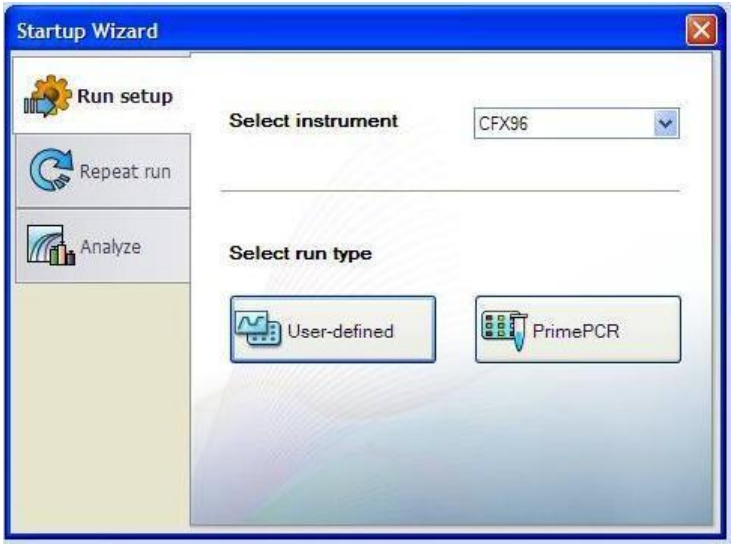
Target	Optical Channel
Aspergillus niger, flavus, fumigatus and terreus	FAM
Internal Control	HEX

PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2

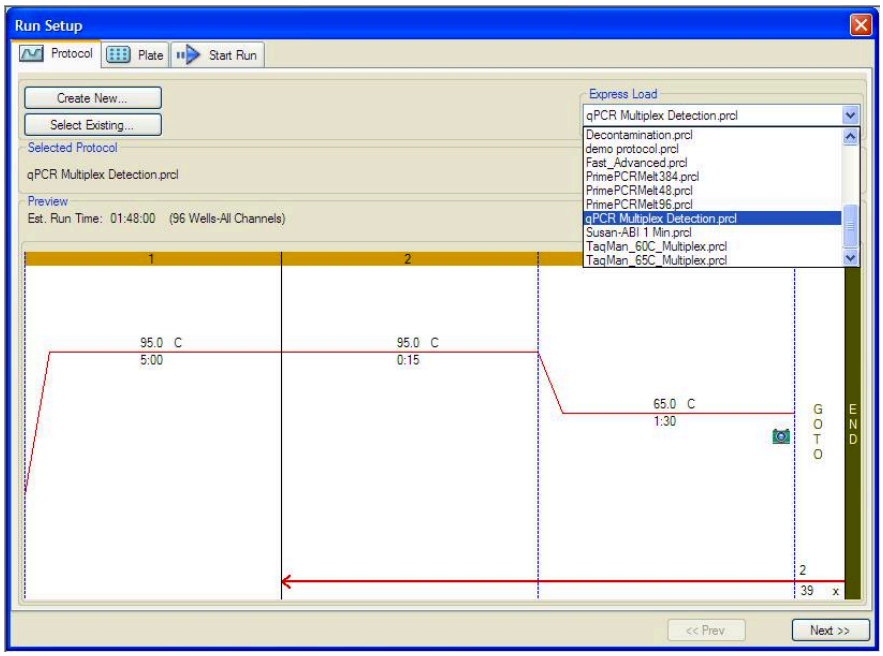
Target	Optical Channel
Aspergillus niger	ROX
Aspergillus flavus	Cy5
Aspergillus fumigatus	FAM
Aspergillus terreus	Cy5.5
Internal Control	HEX

1. Start the qPCR Cycling program

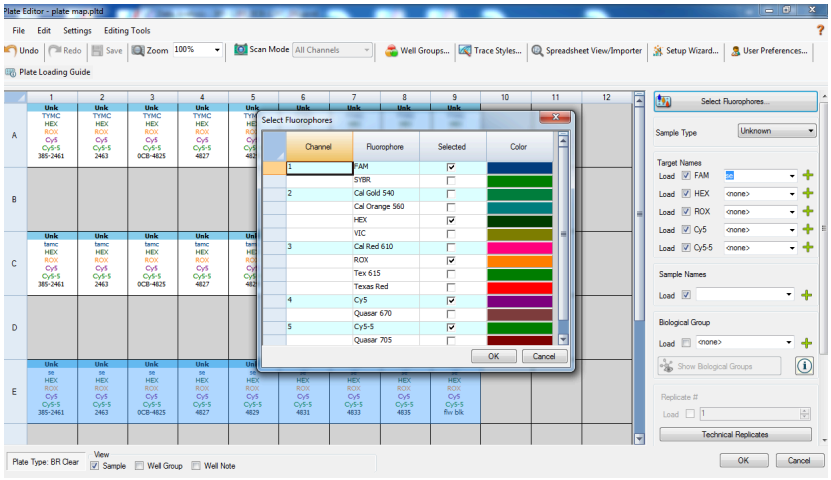
    a. Select User-Defined in the Startup Wizard under Run setup



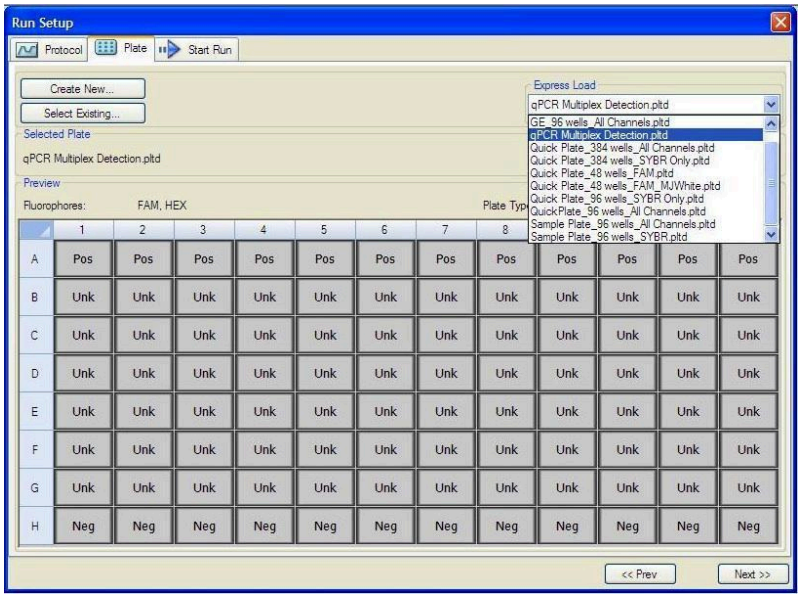
2. Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click "Next".
    a. If not already pre-programmed, create a cycling program with the following specifications and save as "qPCR Multiplex Detection"
    b. Hot start at 95ºC for 5 minutes, followed by 40 cycles of 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. The plate editor window will appear.
        i. PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2 - Choose FAM, HEX, ROX, Cy5, and Cy5.5 Fluorophores and click "OK".
        ii. PathoSEEK 2 - Color Aspergillus Multiplex Detection Assay v2 - Choose FAM and HEX Fluorophores and click "OK".



c. If the 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.

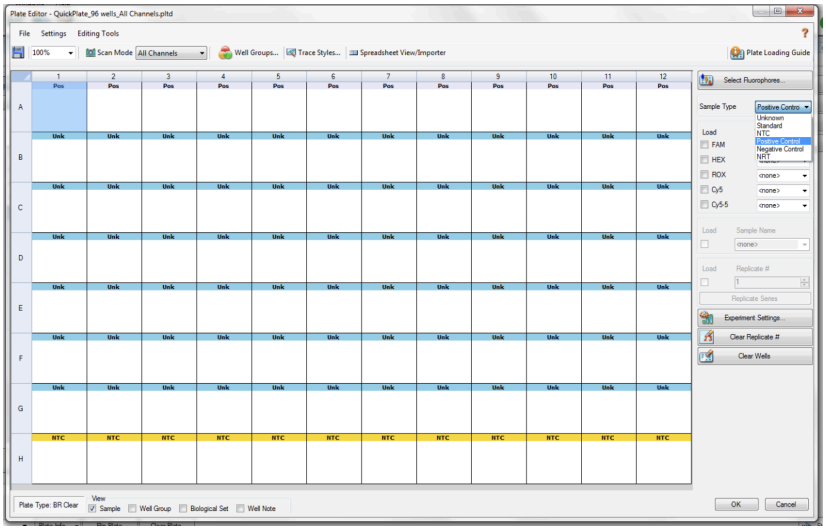
NOTE: To select the Sample Type, highlight the wells you would like to define, then choose from the dropdown menu one of three types:

Unknown

Positive Control

Negative Control

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



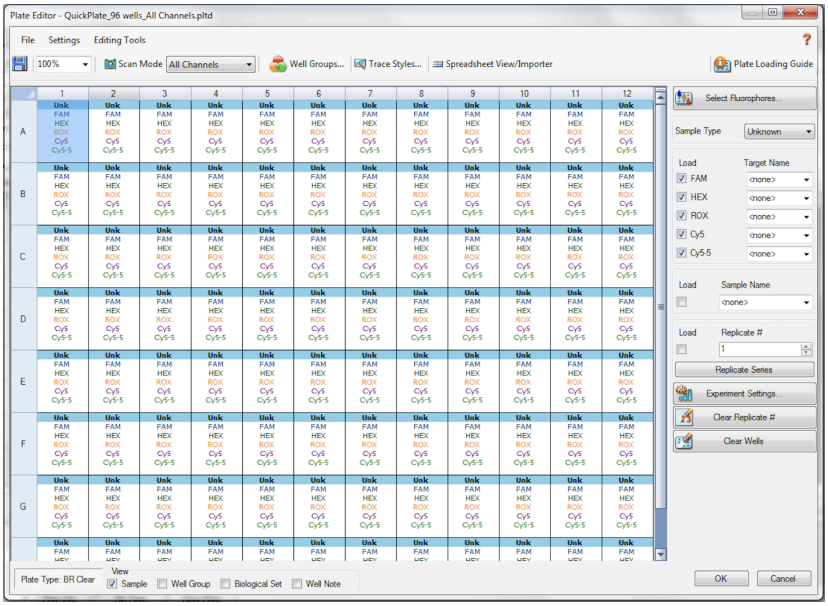
f. Attach the fluorophores to the wells being used.

i. Highlight all the wells being used

1. For PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2, Highlight the well locations and click on FAM, HEX, ROX, Cy5 and Cy5.5
2. For PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2, Highlight the well locations and click on FAM and HEX.

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

4. 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).

5. Close the lid and click Start Run.
6. Save the experiment with the [User] and [date].
7. 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 5-Color Aspergillus Multiplex (CFX) Detection Assay v2 Data Analysis

Table 4: **Bio-Rad CFX96**: PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2 Data Analysis:

PathoSEEK™ Assay	**Cq Value**	**Fluor**	**Negative Control (Cq)**	**CFU threshold (CFU/g)**
Aspergillus niger	≤ 40	ROX	No Cq	Presence/Absence
Aspergillus flavus	≤ 40	Cy5	No Cq	Presence/Absence
Aspergillus fumigatus	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus terreus	≤ 40	Cy5.5	No Cq	Presence/Absence
Internal Control (IC)*	≤35	HEX	*Internal cannabis control verifies the presence or absence of cannabis DNA	
Assay Positive Control	≤35	FAM/ROX/Cy5/Cy5.5		

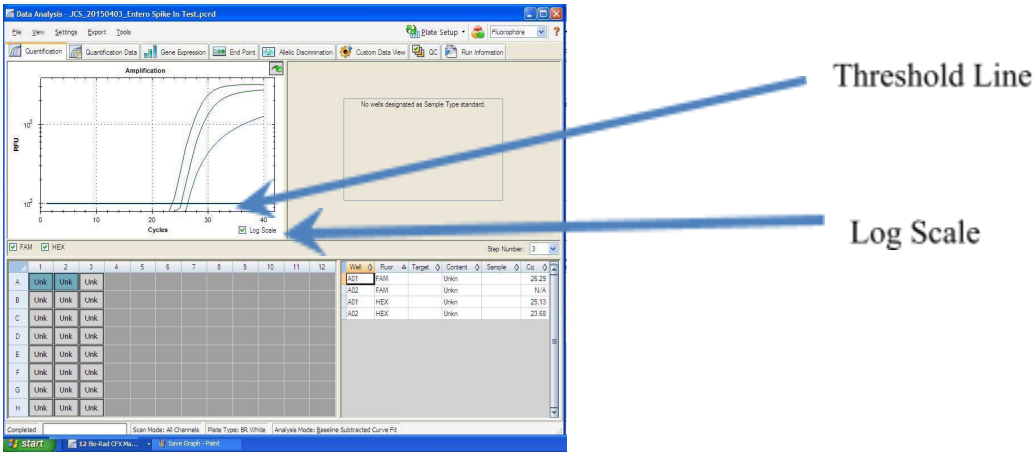
PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 Data Analysis

Table 5: **Bio-Rad CFX96**: PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 Data Analysis:

PathoSEEK™ Assay	**Cq Value**	**Fluor**	**Negative Control (Cq)**	**CFU threshold (CFU/g)**
Aspergillus niger, flavus, fumigatus, terreus	≤ 40	FAM	No Cq	Presence/Absence
Internal Control (IC)*	≤35	HEX	*Internal cannabis control verifies the presence or absence of cannabis 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.
        iii. 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.



4. Controls
    a. Positive Control
        i. PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2 - Amplification on the FAM, ROX, Cy5, and Cy5.5 Fluorophores at a Cq value of ≤ 35 should be observed.
        ii. PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 - Amplification on the FAM Fluorophore at a Cq value of ≤ 35 should be observed.
        iii. Visually confirm with the curve on the graph.

    b. Negative Control
        i. PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2 - Amplification on the FAM, ROX, Cy5, and Cy5.5 Fluorophores should not be observed or have no Cq Value.

ii. PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 - Amplification on the FAM Fluorophore should not be observed or have no Cq Value.

iii. Visually confirm with the curve on the graph.

5. Unknown Aspergillus Samples

   a. PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2

      i. The following fluorophores detect the following species of Aspergillus:

         1. *Aspergillus niger*: ROX
         2. *Aspergillus flavus*: Cy5
         3. *Aspergillus fumigatus*: FAM
         4. *Aspergillus terreus*: Cy5.5

   b. PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2

      i. The following fluorophores detect the following species of Aspergillus:

         1. *Aspergillus niger, flavus, fumigatus and terreus*: FAM

   c. A "presence" or failing result for the samples.

      i. PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2

         1. Any Cq value for the FAM, ROX, Cy5, or Cy5.5 fluorophores ≤ 40.

      ii. PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2

         1. Any Cq value for the FAM fluorophore ≤ 40

      iii. 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.

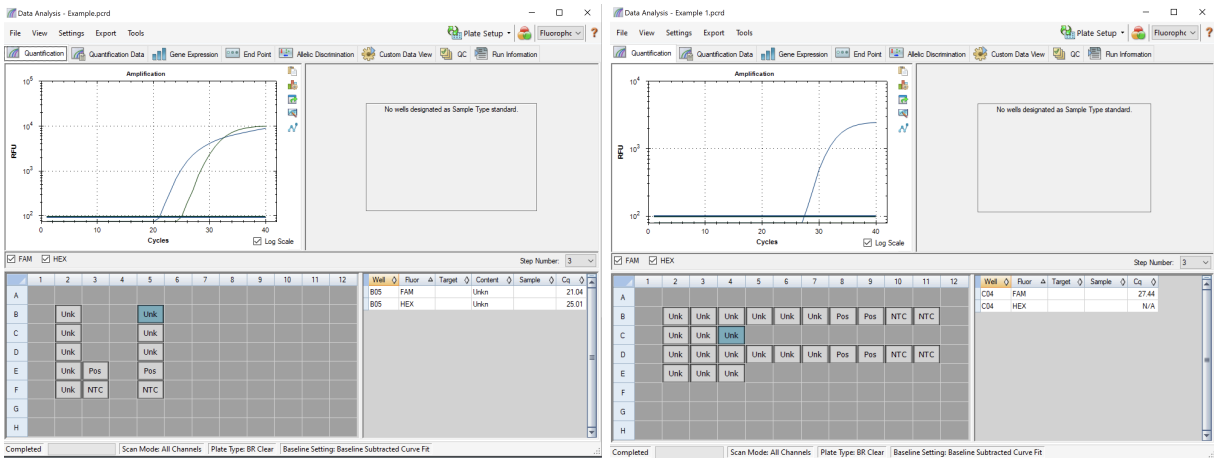


Figure 9: Example of a sample where Aspergillus was detected.

d. An "absence" or passing result for the samples

i. Internal Control (IC), on the HEX fluorophore, has a Cq value ≤ 35 for all sample types.

a. Visually confirm with the curve on the graph

b. If HEX Cq value is >35, dilute the lysed sample 1:2 or 1:10 with nuclease free water and rerun qPCR.

ii. PathoSEEK 5-Color Aspergillus Multiplex (CFX) Detection Assay v2 - No Cq value for the FAM, ROX, Cy5, and Cy5.5 Fluorophores should be observed

a. Visually confirm no curve on the graph.

iii. PathoSEEK 2-Color Aspergillus Multiplex Detection Assay v2 - No Cq value for the FAM Fluorophore should be observed

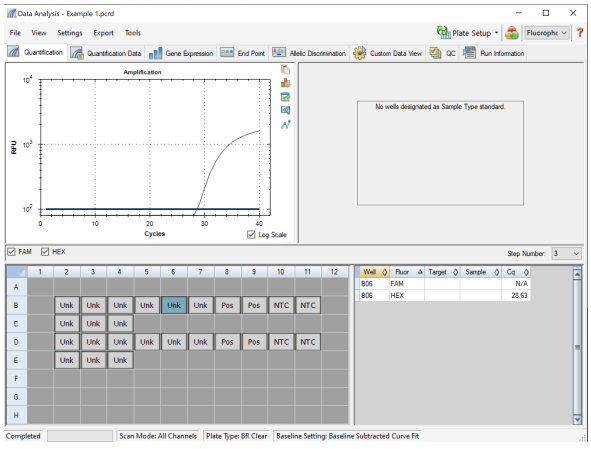


Figure 10: Example of a sample in which Aspergillus was not detected.

Troubleshooting Guide

Symptom	Reason	Solution
Internal control failure	IC 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.
	Optional IC spike not performed for flower samples for Aspergillus Assays	The IC spike into flower is not mandatory when running the Aspergillus MaGiC lysis process. However if samples are low quality, it may be necessary.
	qPCR inhibition	Dilute MaGiC Lysates 1:2 or 1:10 with nuclease free water and re-perform qPCR. If dilution is not successful, repeat MaGiC Lysis.
Amplification of the Internal control is not expected in the assay positive or negative control wells. No Cq or a Cq of more than 35 is acceptable. Any Cq lower than 35 constitutes a rerun	Cannabis DNA or IC 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.
Background Amplification	Unclear	This is usually seen with a very low Cq reading (<15), the curve is usually missing the exponential growth phase, but rather a gradual increase of fluorescence signal. This is usually a negative result, but should be repeated.

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 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 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	First User Guide with MaGiC Lysis replacing SenSATIVAx DNA Isolation
v2	November 2024	• Merge 5-Color and 2-Color User Guides. • Add option for Grim Reefer Free DNA Removal for 48 Hour Enrichment.
v3	May 2025	• Amplification Mix packaging update. • Grim Reefer Deactivation Buffer UOM correction. • Added adjusting ATTO425 crosstalk correction setting to Aria Data Analysis. • Removed special instructions for preparing chocolate matrix • Update to plate centrifuge recommendation to have swinging bucket rotor.

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.