

PathoSEEK[®] 5-Color Aspergillus Multiplex Assay with SenSATIVAx[®] Extraction

User Guide

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 *Aspergillus* Multiplex Assays with SenSATIVAx® Extraction Protocol are designed to detect *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus terreus* in a single qPCR (Quantitative Polymerase Chain Reaction) in cannabis flower, cannabis concentrates, infused edibles and infused non-edibles.

Process Overview

The PathoSEEK® 5 – Color *Aspergillus* Multiplex microbial detection assays use a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of 4 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 set-up errors or failing experimental conditions. Below is a simplified depiction of the qPCR assays.

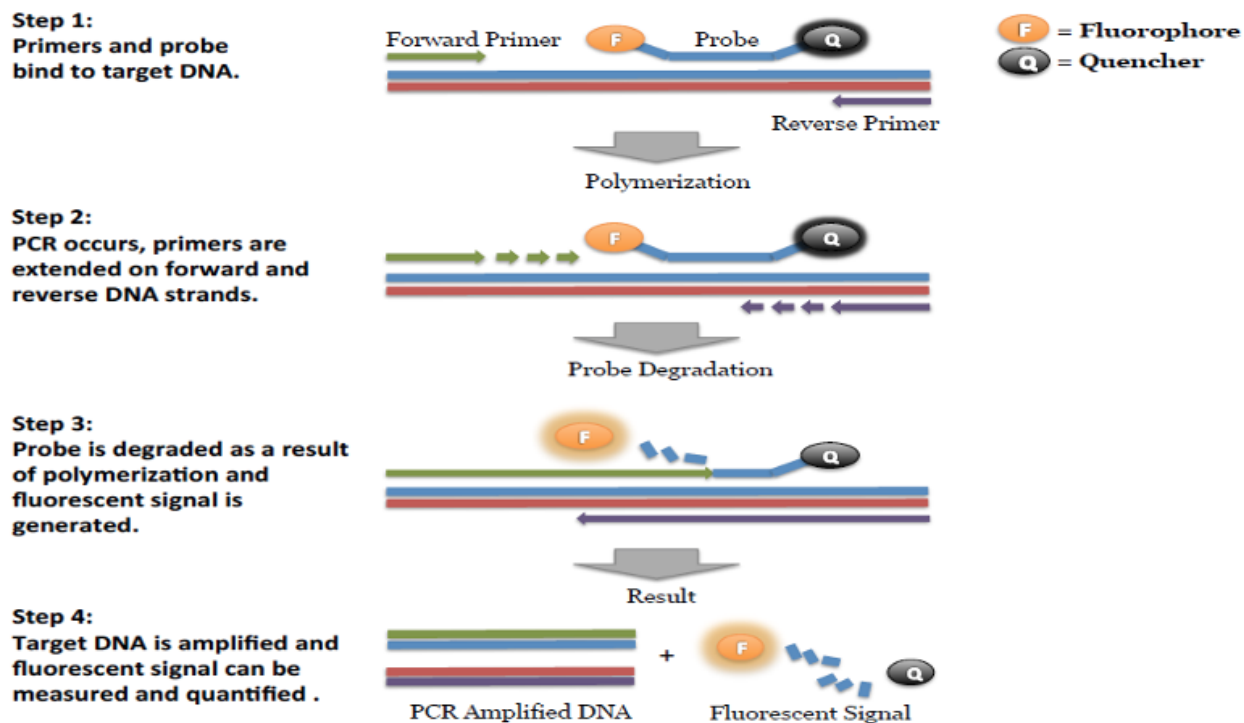


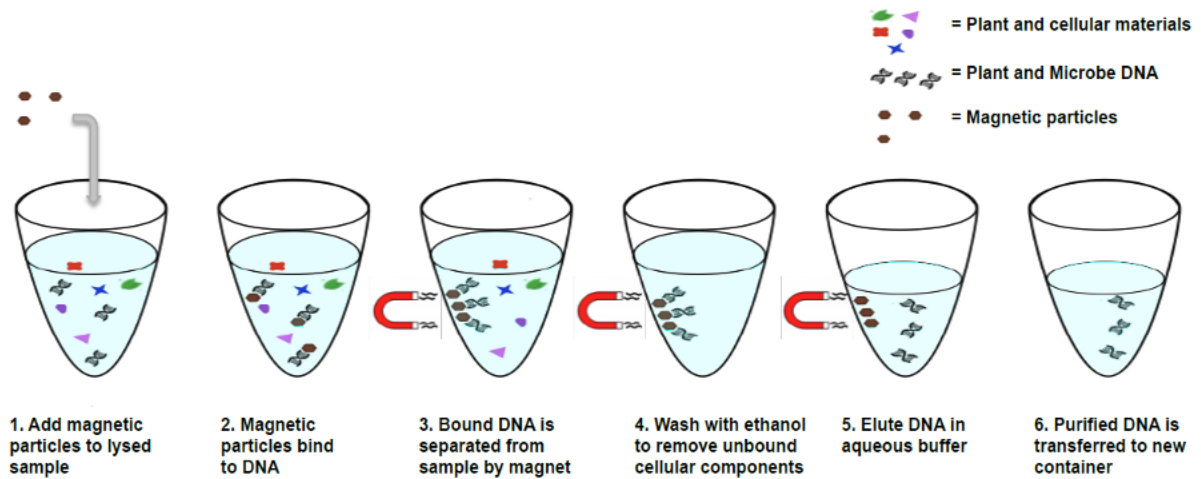
Figure 1: Overview of qPCR

Two multiplex assays are available for use, dependent on the thermocycler used for analysis.

The PathoSEEK 5 – Color Aspergillus Multiplex Assay for use on the AriaMx Real-Time PCR Thermocycler (Agilent) uses the ATTO 425 Fluorophore for detection of the *Aspergillus terreus*, while the PathoSEEK 5- Color Aspergillus Multiplex Assay for use on the CFX-96 (Bio-Rad) uses the Cy5.5 Fluorophore for the detection of *Aspergillus terreus*. SenSATIVax is a proprietary DNA isolation process that uses magnetic particles to isolate and purify both plant and microbial DNA from a raw, homogenized plant or MIP sample. The use of magnetic

particles affords 8 or 96 tip automation, enabling high throughput applications. DNA can be isolated from a single sample or a large batch in under 1 hour. Hands-on time is less than 45 minutes.

Figure 2: SenSATIVAx Extraction Protocol



Limit of Detection

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

Materials and Methods

Test Kit Name: PathoSEEK® 5-Color Aspergillus Multiplex Assay with SenSATIVAx® Extraction

Test Kit Information

1. *PathoSEEK 5-Color Aspergillus Multiplex Assay for (AriaMX)*
 - a. *P/N 420148*
2. *PathoSEEK 5-Color Aspergillus Multiplex Assay for (CFX96)*
 - a. *P/N 420147.*
3. *SenSATIVAx Flower/Leaf DNA Extraction Kit.*
 - a. *P/N 420001.*
4. *SenSATIVAx MIP/Extract DNA Extraction Kit.*
 - a. *P/N 420004.*
5. *PathoSEEK Aspergillus Multiplex Positive Control.*
 - a. *P/N 420330*
6. *Medicinal Genomics qPCR Master Kit v3*
 - a. *P/N 420201.*

Test Kit Components

1. *PathoSEEK 5-Color Aspergillus Multiplex Assay for (AriaMX).*— P/N 420148 (200 tests).

- a. *Assay* — 1 tube. Store kit at -15 to -20°C. Expires 2 Years from Date of Manufacture.
2. *PathoSEEK 5-Color Aspergillus Multiplex Assay for (CFX96).*— P/N 420147 (200 tests).
 - a. *Assay* — 1 tube. Store kit at -15 to -20°C. Expires 2 Years from Date of Manufacture.
3. *SenSATIVAx Flower/Leaf DNA Extraction.*— P/N 420001 (200 tests).
 - a. *MGC Lysis Buffer.*— 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture
 - b. *MGC Binding Buffer.*—1 bottle. Store at 2-8°C. Expires 1 Year from Date of Manufacture
 - c. *MGC Elution Buffer.*—1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture
4. *SenSATIVAx MIP/Extract DNA Extraction.*— P/N 420004 (200 tests).
 - a. *Binding Buffer* — 1 bottle. Store at 2-8°C. Expires 1 Year from Date of Manufacture
 - b. *Elution Buffer* — 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture
 - c. *Solution A* — 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture

- d. *Solution B* — 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture
5. *PathoSEEK Aspergillus Multiplex Positive Control*.— P/N 420330. (50 reactions)
- a. Control - 1 tube. Store at -15 to -20°C. Expires 2 Years from Date of Manufacture
6. *qPCR Master Kit v3*— P/N 420201. Kit. Store at -15 to -20°C. Expires 2 Years from Date of Manufacture
- (1) *Reaction Buffer (10x)*.— 1 tube.
- (2) *Nuclease Free Water*.—2 tubes.
- (3) *qPCR Master Mix* - 1 tube.

Supplies, Reagents, and Equipment

(a) Agilent AriaMx Real-Time PCR System G8830A—Containing the following Optical

Channels: FAM, ROX, HEX, Cy5 and ATTO 425.

- (1) Agilent HP Notebook PC option 650 or similar
- (2) 96 Well Optical qPCR plate.— Agilent #401490, Thermo Scientific #AB0800 or #AB2800, USA Scientific Eppendorf Twin-tec #4095-2401
- (3) Adhesive optical seal for qPCR plates.— Agilent #401492; USA Scientific TempPlate RT Optical Film #2978-2100.
- (4) Optical Strip Caps.—Agilent #401425.

Note: If using adhesive seals instead of strip caps, use Applied Biosystems MicroAmp Optical Film Compression Pad, Fisher Scientific, #43-126-39 to prevent evaporation and cross contamination between wells.

(b) Bio-Rad CFX96 Touch™ Real-Time System.

(1) Bio-Rad Personal PC

(2) 96 Well Optical qPCR plate.— Bio-Rad #HSP-96601 or Fisher Scientific #AB2396

(3) Adhesive optical seal for qPCR plates.— Bio-Rad #MSB-1001; USA Scientific TempPlate RT Optical Film #2978-2100.

(c) Adjustable, variable volume pipettes (single or multichannel).—P10, P20, P50, P200, P300 and P1000.

(d) Adjustable, variable volume filter pipettes tips.—For P10, P20, P50, P200, P300 and P1000.

(e) Crushed ice.

(f) 96 Well PCR Cryogenic Rack.—VWR #89004-570.

(g) 1.5 µL Tube Benchtop Cryogenic Rack.— VWR #89004-558 or equivalent.

(h) Freezer.—Capable of maintaining -20°C.

(i) Table Top Mini Plate Centrifuge.—Fisher Scientific #14-100-143 or equivalent.

(j) Table Top Mini Centrifuge.—VWR #10067-588, #2631-0006 or equivalent.

(k) Vortex-Genie Pulse.—Scientific Industries, SKU: SI-0236 or equivalent.

(l) High Speed centrifuge.— to accommodate 1.5mL tubes such as Eppendorf model 5425 or similar with ability to spin up to speeds of 19,300 RCF.

- (m)** Filter Bags.—Whirl Pak #B01385WA
- (n)** Beaker or Solo Cup. (optional)
- (o)** Tryptic Soy Broth.—Medicinal Genomics #420205. Store at 2-8°C.
- (p)** 1.5 mL Eppendorf Tubes.
- (q)** 15 mL or 50 mL conical tubes.
- (r)** 96 Well Plate Magnet.—MGC P/N 420202
- (s)** 96 Well Extraction Plate.— Perkin Elmer P/N 6008290
- (t)** Eppendorf Tube Rack.
- (u)** Scientific Scale.—Capable of measuring to milligrams.
- (v)** Refrigerator.—Capable of maintaining 2–8°C.
- (w)** Incubator.—Capable of maintaining $37 \pm 2^{\circ}\text{C}$, VWR #97025-630 or equivalent.
- (x)** 25mL Serological Pipette.—VWR 89130-890 or 89130-900 or equivalent.
- (y)** 10% bleach.
- (z)** 70% Ethanol.— MGC P/N 420030
- (aa)** Chloroform (Ethanol as preservative/ Certified ACS) - Fisher Scientific, C298

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

(a) *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.

(b) 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.

(c) 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.

(d) Hazard Statement: Chloroform

- (1) Harmful if inhaled or swallowed.
- (2) Do not breathe vapor or mist. Do not ingest. Avoid contact with eyes, skin and clothing. Use only with adequate ventilation, which may require a chemical fume hood.
- (3) Keep the container tightly closed and sealed until ready for use. Wash thoroughly after handling.
- (4) Please refer to the Manufacturer Safety Data Sheet (SDS) for more information and proper disposal



(e) Hazard Statement: 70% Ethanol

- (1) Highly flammable liquid and vapor May cause respiratory irritation
- (2) May cause drowsiness or dizziness Causes damage to organs
- (3) May cause damage to organs through prolonged or repeated exposure
- (4) Please refer to the Safety Data Sheet (SDS) for more information and proper disposal



Intended User

The PathoSEEK® 5-Color Aspergillus Multiplex Assay with SenSATIVAx® Extraction 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

(a) Aliquot Tryptic Soy Broth (TSB).

Note: TSB is a very good growth medium for microbes. Due to this, it is best to pour the approximate amount of TSB into another sterile tube or container so as to not contaminate the whole bottle. Return it to the 2-8°C refrigerator immediately after use.

(b) Wipe down the workspace with a 10% bleach solution, including the bench top and all equipment being used.

(c) Remove the MGC Binding Buffer and TSB from the 2-8°C refrigerator (it should come to room temperature, 20-28°C, before use).

(d) Prepare consumables. Label all the filter bags with “[sample name] [date]”.

(e) Prepare consumables. Label all the 1.5mL centrifuge tubes needed with [sample name]

(f) Label extraction plate with date, and if transferring eluted DNA to new plate.

- (g) 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.
- (h) *Cannabis flower, n grams* —Weigh flower sample material into one side of the mesh liner inside the Whirl-Pak bag. Add $9 \times n$ mL of TSB 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 $37 \pm 1^\circ\text{C}$.
- (i) *MIP and concentrates, n grams.* — Weigh the MIP matrix into a 15 mL conical tube, 50 mL conical tube, or Whirl-Pak bag depending on MIP volume. Add $2.4 \times n$ mL of TSB to each test portion. Vortex or homogenize sample and TSB. Incubate for 24 – 48 h at $37 \pm 1^\circ\text{C}$.
- (j) If processing multiple samples, be sure to change gloves between each, to ensure there is no cross contamination between samples during the weighing process. See flower example in Figure 3.

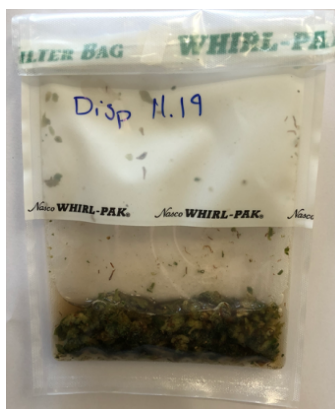


Figure 3: Homogenized cannabis flower and TSB.

DNA Extraction

SenSATIVAx for Flower/Leaf DNA Extraction

- a) Aspirate **1 mL** from the side of the filter bag, free of plant debris, and dispense into the 1.5mL tube.
- b) Add 50 μ L of MGC Lysis buffer and vortex for 10 seconds then let incubate on the bench for 2 minutes.
- c) After 2 minute incubation, spin for at least 1-3 minutes in a bench top mini centrifuge.

Note: The supernatant should be translucent at this point. If the sample is still opaque (cloudy) spin for longer. This is important for removing cellular debris. See Figure 4.

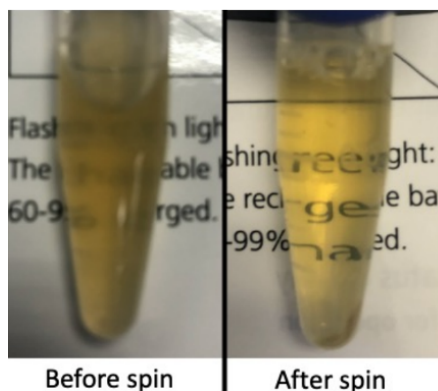


Figure 4: Example of translucent lysate after spinning.

- d) Remove the 200 μ L of supernatant from the 1.5ml tube containing the centrifuged sample, being careful not to disturb the pellet at the bottom of the tube. Place the 200 μ L in a labeled 96 well extraction plate labeled with Extraction Plate Day1 [date]”

Note: Pellet size will vary depending on trichome density.

e) Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer for at least 30 seconds.

f) Add 200µL of MGC Binding Buffer to each sample, and pipette tip mix 15 times.

Note: Be careful to avoid adding too many bubbles by pipetting gently when tip mixing. This is extremely important as to not contaminate the wells in proximity.

g) Incubate the plate on the bench for at least 5 minutes.

h) Place the extraction plate onto the 96 well plate magnet plate for at least 5 minutes.

i) After the 5 min incubation, remove as much of the 400µL of the supernatant as possible.

Be careful not to disturb or aspirate the beads. See Figure 5.

(1) Add 400µL of 70% ethanol (EtOH) with the extraction plate still on the magnet plate.

(2) Wait at least 30 seconds and remove all the EtOH.

Note: Place the pipet tip at the bottom center of the well to remove all liquid.

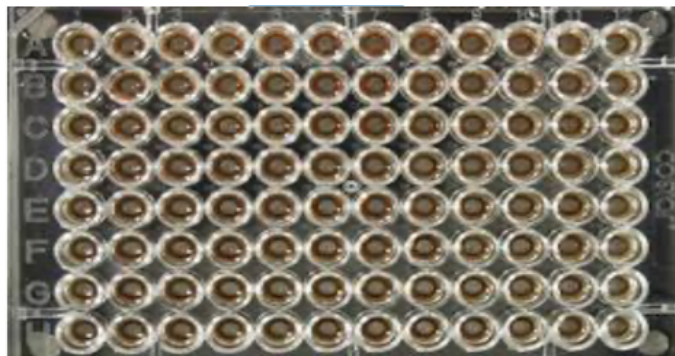


Figure 5: Extraction plate during wash step on magnetic plate.

j) Repeat 400 µL 70% EtOH wash with the extraction plate still on the magnet plate. Wait at least 30 seconds and remove all the EtOH.

Note: If EtOH still remains in the wells, go back in with a smaller pipet tip to remove the excess. Leftover EtOH can inhibit qPCR efficiency.

k) After all the EtOH has been removed, let the beads dry at room temperature. Allow the beads to dry for up to 15 minutes. Necessary drying time will vary based on complete removal of the second ethanol wash, as well as lab environment. Visually inspect beads for residual ethanol before the elution step.

Note: It is important to NOT allow the beads to dry for an extended period of time. Over-drying can cause a reduction in DNA yield.

l) Remove the extraction plate from the magnet plate and add 25 µL of MGC Elution Buffer.

(1) Tip mix approximately 15 times or until the beads are completely re-suspended.

Note: The re-suspensions may appear varied in their appearance, but the result will be the same. See Figure 6.

(2) Incubate the plate for at least 1 minute on the bench, before returning the plate to the magnetic plate.

(3) Let the plate sit on the magnet for at least 1 minute before transferring the eluant to a new extraction plate labeled with “Final Extract [date]”.

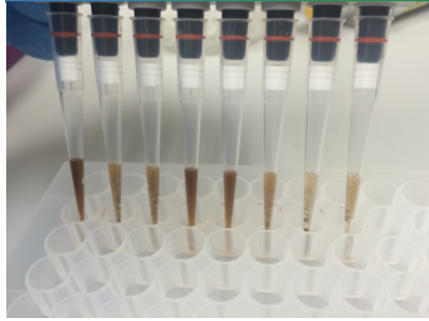


Figure 6: Multichannel pipette tips showing magnetic beads resuspended in elution buffer.

m) Seal the plate with the adhesive seal, making sure to completely seal the plate wells using a pen or flat object to slide back and forth along the seal. Store at -20°C until ready to perform the qPCR protocol.

SenSATIVAx[®] for MIP/Extracts DNA Extraction

a) Prepare a SCCG positive control dilution of 1:5,000 (internal control)

(1) Label a new 1.5mL Eppendorf tube (SCCG 1:50), add 1µl of SCCG positive control into 49µl of dH₂O. Vortex to mix thoroughly and quick spin tube. Label another 1.5mL Eppendorf tube (SCCG 1:5,000), add 99µl of dH₂O, then add 1µl of the SCCG 1:50 dilution. Vortex to mix thoroughly and quick spin tube. This will result in a 1:5,000 dilution of SCCG.

Note: It's easy to mis-pipette when trying to pipette only 1 µL of liquid. Visually check your pipette tip after aspirating 1 µL to ensure it is in the tip before adding it to the tube for dilutions 1 and 2.

(2) Place on ice until use.

Note: The 100 µL dilution prepared above is enough diluted SCCG for approximately 10 extractions. If more extractions are going to be prepared at the same time, the initial 1:50 dilution can be used to make multiple 1:5,000 dilutions of SCCG.

b) Add initial sample weight x 4.6 mL SenSATIVAx Solution A to conical tube with enriched sample/TSB. Vortex the sample vigorously until homogenized.

c) Transfer 1 mL of the homogenized sample into a 1.5 mL tube.

d) Add 10µL of the SCCG internal control (1:5,000) to 1.5 mL tube and vortex to mix well.

e) Centrifuge for 10 minutes at 19,300 RCF using a high-speed bench top centrifuge.

(1) If no bench top centrifuge is available, centrifuge for 15 minutes using a mini centrifuge.

Note: Some matrices will require the use of a high-speed centrifuge due to the presence of certain substances such as gelatin that hinder phase separation.

f) Transfer 600 ul of the solution to a new tube. Push pipet tip through the top solid layer (if one exists), without disturbing the pellet on the bottom to aspirate the sample.

g) Add 600 µL chloroform and vortex vigorously until solution turns a milky white color throughout

Note: This may require longer vortexing for some matrices

Caution: ALWAYS WEAR GLOVES WHEN HANDLING CHLOROFORM



Fig 7. Example of a milky sample with chloroform in it.

- h)** Centrifuge for 5 minutes at 19,300 RCF using a bench top centrifuge
- i)** If no bench top centrifuge is available, centrifuge for 15 minutes using a mini centrifuge.

NOTE: If there is still any color in your aqueous layer (top layer) after centrifugation another chloroform wash may help give you a strong internal control signal (HEX) for every assay. Transfer 300 μ L of the top layer to a new 1.5 mL tube and add 300 μ L chloroform, vortex and centrifuge again.

- j)** Transfer 100 μ L of aqueous layer (TOP LAYER) from Step 7 to a well of the labeled 96 well extraction plate. Be careful not to disturb the lower chloroform layer.
- k)** Add 100 μ L of SenSATIVAx Solution B to the 100ul sample in the 96 well extraction plate and mix by pipetting up and down 3 times..
- l)** Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer for at least 30 seconds.
- m)** Add 200 μ L of MGC Binding Buffer to each sample, and pipette tip mix 15 times.

Note: Be careful to avoid adding too many bubbles by pipetting gently when tip mixing. This is extremely important as to not contaminate the wells in proximity.

- n)** Incubate the plate on the bench for at least 5 minutes.
- o)** Place the extraction plate onto the 96 well plate magnet plate for at least 5 minutes.
- p)** After the 5 min incubation, remove as much of the 400µL of the supernatant as possible.

Be careful not to disturb or aspirate the beads.

(1) Add 400µL of 70% ethanol (EtOH) with the extraction plate still on the magnet plate.

(2) Wait at least 30 seconds and remove all the EtOH.

Note: Place the pipet tip at the bottom center of the well to remove all liquid.

- q)** Repeat 400 µL 70% EtOH wash with the extraction plate still on the magnet plate. Wait at least 30 seconds and remove all the EtOH.

Note: If EtOH still remains in the wells, go back in with a smaller pipet tip to remove the excess. Leftover EtOH can inhibit qPCR efficiency.

- r)** After all the EtOH has been removed, let the beads dry at room temperature. Allow the beads to dry for up to 15 minutes. Necessary drying time will vary based on complete removal of the second ethanol wash, as well as lab environment. Visually inspect beads for residual ethanol before the elution step.

Note: It is important to NOT allow the beads to dry for an extended period of time.

Over-drying can cause a reduction in DNA yield.

s) Remove the extraction plate from the magnet plate and add 25 µL of MGC Elution Buffer.

- (1) Tip mix approximately 15 times or until the beads are completely re-suspended.

Note: The re-suspensions may appear varied in their appearance, but the result will be the same.

- (2) Incubate the plate for at least 1 minute on the bench, before returning the plate to the magnetic plate.

- (3) Let the plate sit on the magnet for at least 1 minute before transferring the eluant to a new extraction plate labeled with “Final Extract [date]”.

t) Seal the plate with the adhesive seal, making sure to completely seal the plate wells using a pen or flat object to slide back and forth along the seal. Store at -20°C until ready to perform the qPCR protocol.

Real-Time Quantitative PCR (qPCR) Setup Protocol

a) Remove qPCR reagents including qPCR Master Mix, water, reaction buffer and assay probe mixes to be used from the -20°C freezer. Place qPCR master mix on ice or leave at -20°C until ready to use. Allow remaining tubes to thaw at room temperature. Once thawed, immediately place tubes on ice.

b) Before preparing the reaction, invert or vortex and spin-down the reagents.

(1) Assay probe mix tubes, reaction buffer, positive controls and water – Vortex quickly followed by a pulse spin-down in a microcentrifuge.

(2) qPCR Master Mix – Invert the tube 5 times (do not vortex), followed by a pulse spin-down in a microcentrifuge.

(3) Return all reagents to the ice.

Note: Do not vortex the qPCR Master Mix at any point during the protocol.

c) Make a separate master mix in a 1.5mL tube for each assay type being run. All probe mixes contain the internal plant control, SCCG probe mix, and the probe for the microbial targets. Label each tube with [Assay Name] MM. Always prepare enough master mix for 1 or 2 additional reactions over the total number of tests to account for pipetting and dead volumes.

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

Table 2: PCR Reagent Volumes

| Reagents | 1 Reaction | 24 Reactions (Plus 1 excess rxn) | 48 Reactions (Plus 2 excess rxn) |
|-------------------------|------------|-------------------------------------|-------------------------------------|
| qPCR Master Mix | 3.75 µL | 93.75 µL | 187.5 µL |
| Assay Probe Mix | 1 µL | 25 µL | 50 µL |
| Reaction Buffer | 0.8 µL | 20 µL | 40 µL |
| Water | 8.2 µL | 205 µL | 410 µL |
| Total Assay Probe MM | 13.75 µL | 343.75 µL | 687.5 µL |

d) Once combined, gently tip mix or invert the tube 5 times to combine the assay master mix.

(1) Pulse spin-down tube in microcentrifuge.

(2) Place qPCR Master Mix tubes on ice until used.

(3) For the positive control, make a 1:10 dilution

i. Add 1 µL of Positive Control to 9 µL nuclease free water (found in the kit), 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.

e) Place the Extraction Plate on the magnet. This is to ensure no magnetic beads are transferred into the qPCR reactions if there are some left over from the extraction elution process.

f) Use a 96-well optical qPCR plate and label the plate “qPCR Plate_ [date]”.

g) Carefully remove the seal from the Extraction Plate. If frozen, let the DNA 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. Keep the extraction plate on the magnet when aspirating the 5 µL.

(1) 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

h) Add 13.75 µL of specific Assay Probe MM 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.

- i)** Seal the plate with strip caps or an adhesive seal.
- j)** 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 bottom of the wells, spin-down for another minute.

- k)** For the Agilent Aria: 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 holes in the plate.
- l)** Place the sealed plate onto the PCR instrument, positioning the A1 well in the top left corner.
- m)** Follow the software specific instructions to initiate the run.

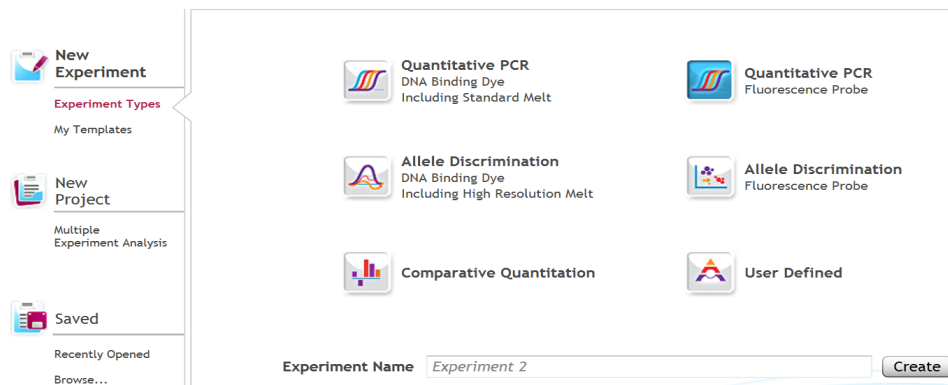
Running the Agilent AriaMX

The following species will be detected on the following Fluorophores:

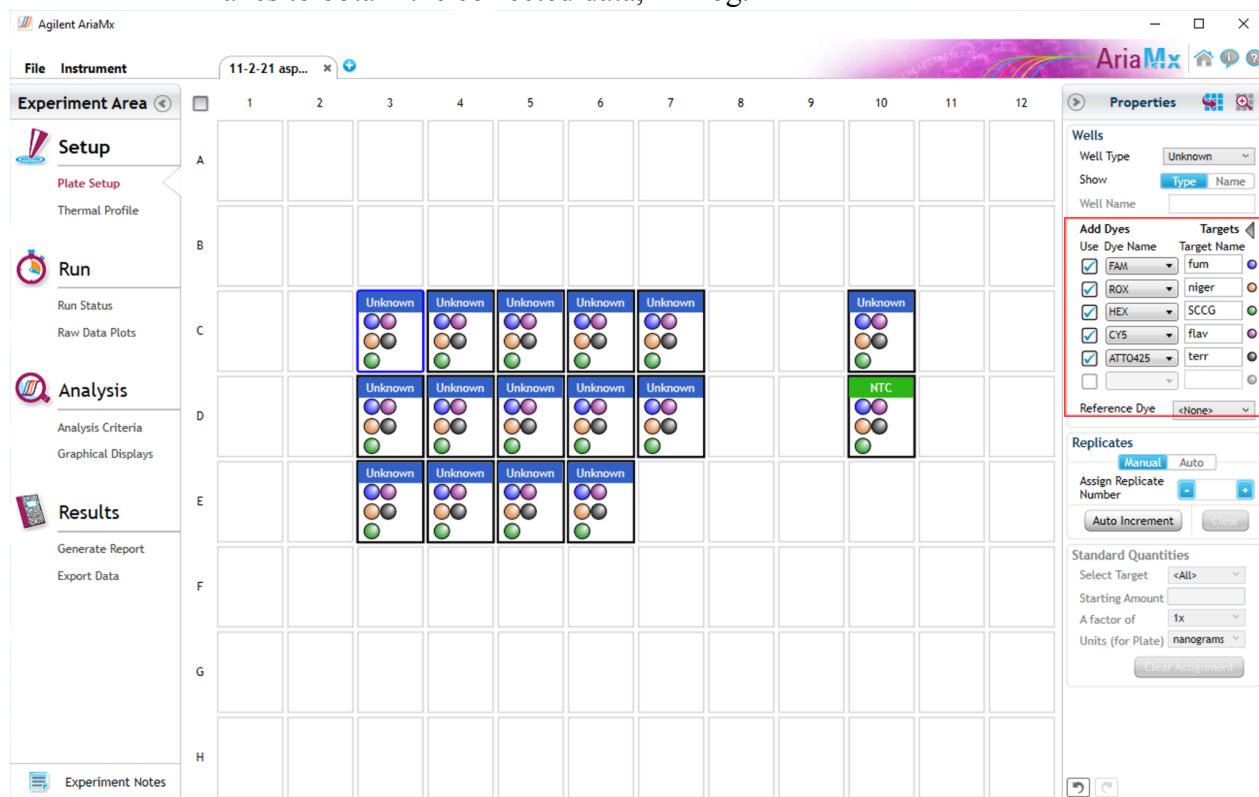
- Aspergillus niger: ROX
- Aspergillus flavus: Cy5
- Aspergillus fumigatus: FAM
- Aspergillus terreus: ATTO 425
- Cannabis DNA: HEX

a) Create a New Experiment on the Agilent qPCR instrument.

i) Select “Quantitative PCR: Fluorescence Probe” from Experiment Types.



- ii) Under Setup > Plate Setup, highlight **only** wells that contain reactions and select 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.

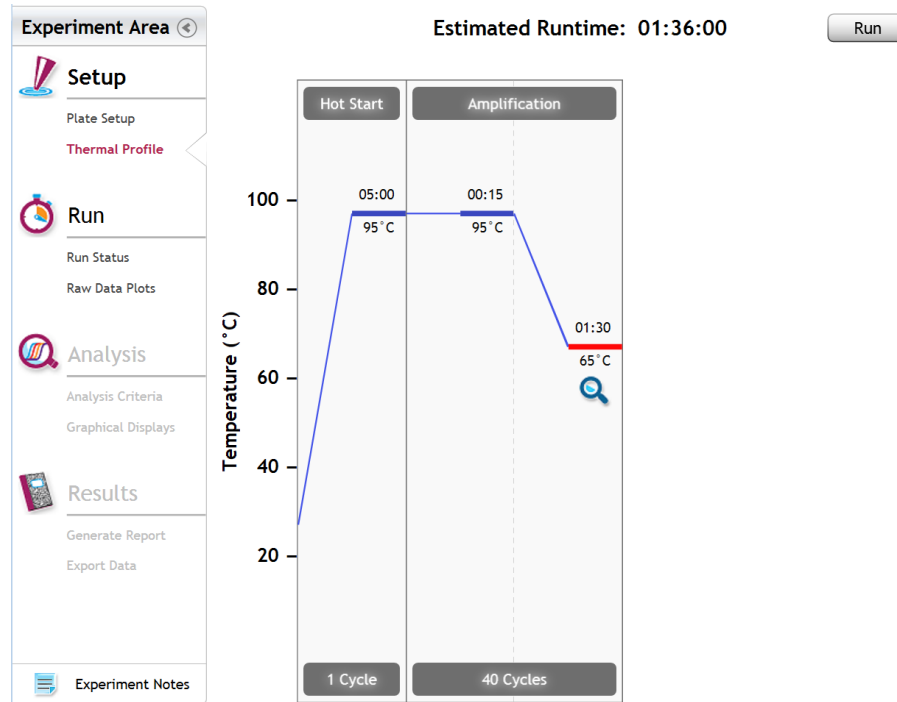


- b) Change the well types to reflect your plate set up. All wells should be set to Unknown except the negative control can be set to NTC well type. Add Target names to the dyes under **Targets**:

- i) FAM : Aspergillus fumigatus
- ii) ROX : Aspergillus niger
- iii) Cy5 : Aspergillus flavus
- iv) ATTO 425 : Aspergillus terreus
- v) HEX : Single Copy Cannabis Gene (SCCG)

c) Under Setup > Thermal Profile, create the following PCR thermal profile

(1) Hot start at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 65°C for 90 seconds.



d) Close the lid and click “Start Run”

e) Save the experiment with the [User] and [date]

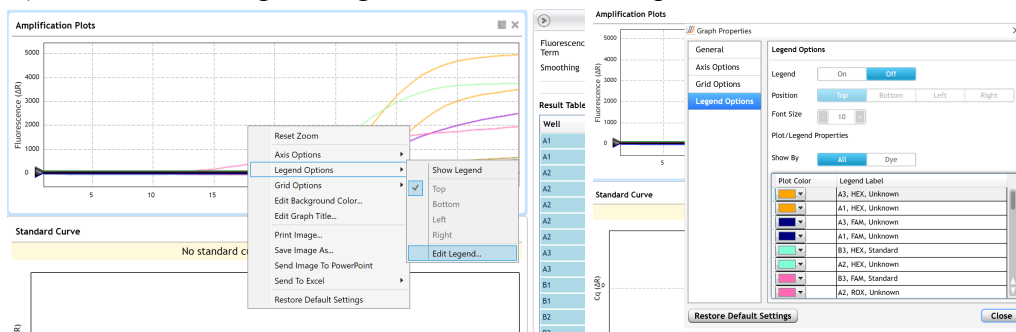
f) When the qPCR run is complete, immediately dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

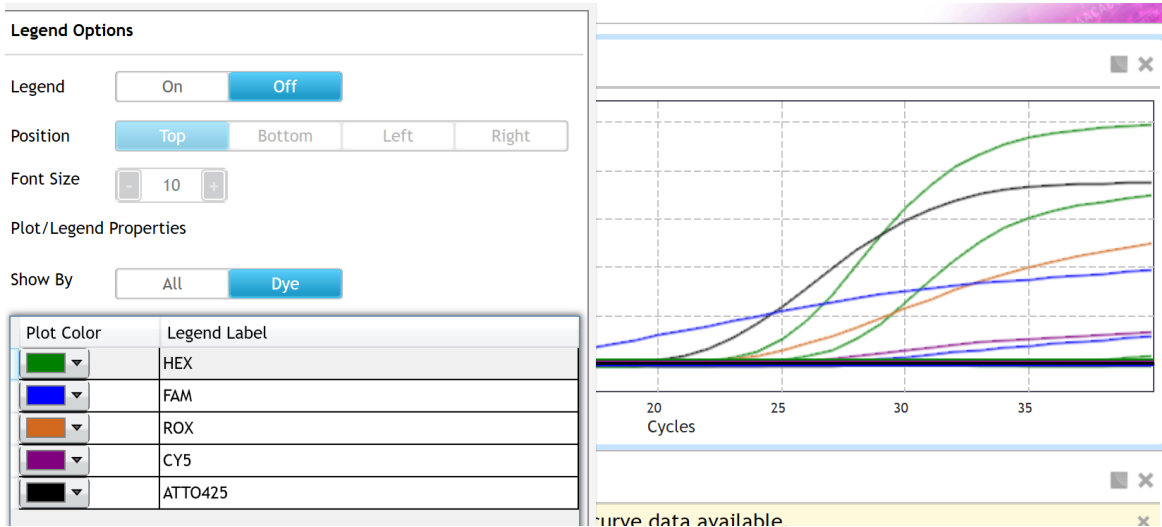
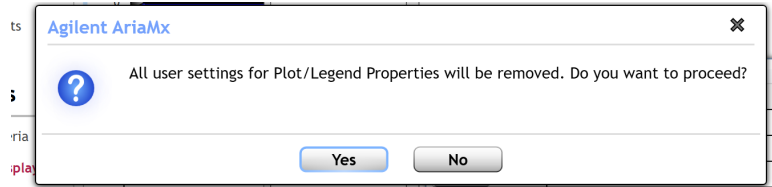
PathoSEEK® Aspergillus 5-color Data Analysis Quick Reference Table:

Table 1: All Matrices

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

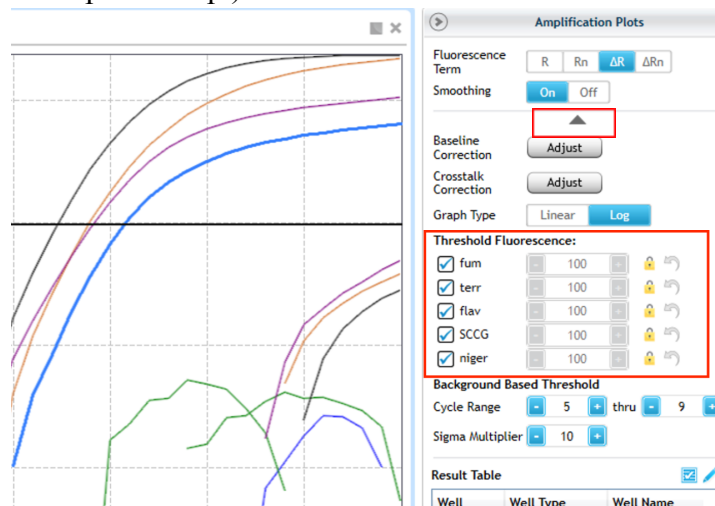
- a) Open the Data Analysis window when the run is complete.
- b) Highlight the wells of interest in the Analysis Criteria under Analysis, then select Graphical Display
 - i) Amplification plots will be available for viewing
 - ii) The Cq values will appear to the right in the table
 - iii) Right click inside the graph, select Edit Legend under Legend Options
 - iv) Change “All” to “Dye”
 - v) All user settings for Plot/Legend Properties will be removed. Do you want to proceed? Select “Yes”.
 - vi) This will assign a single color to each fluorophore.





c) To analyze the results

- 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
- Manually adjust thresholds to 100 RFU for the HEX, FAM, ROX, Cy5 and ATTO425 fluorophores. (which will have the target name because you've added them in the plate setup.)



- ii) A “presence” or failing result for the unknown Aspergillus target(s).
 - 1) Any Cq value for the FAM, ROX, Cy5 or ATTO 425 Fluorophores ≤ 40 .
 - (a) 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.
 - (i) 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.

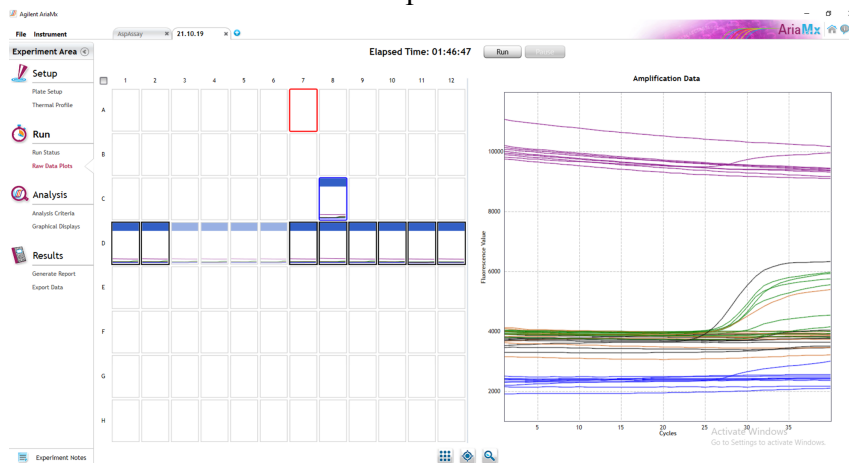


Figure: Raw data (R) plots for which the cycle quantification (Cq) will be generated.

- (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.)

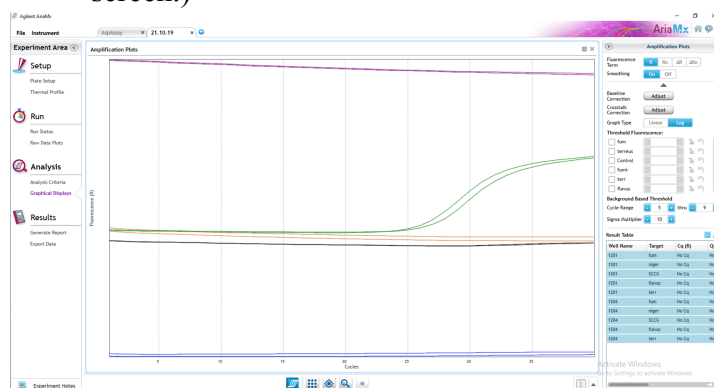
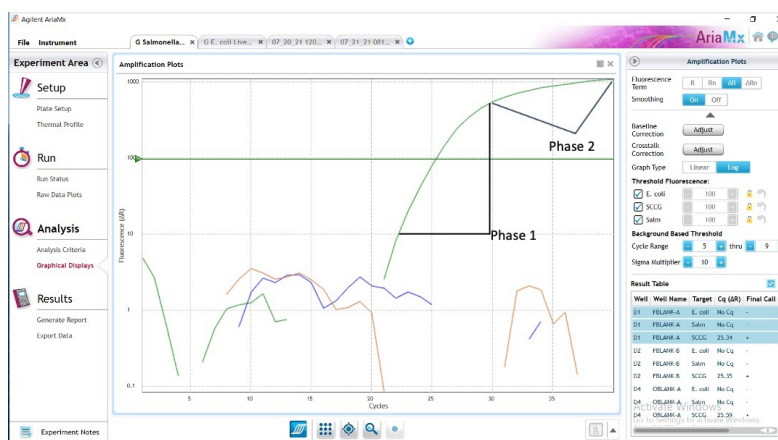


Figure: Example of good baseline with sigmoidal shaped curve increase in the internal control fluorescence.

- (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.



- 2) An “absence” or passing result for the unknown Aspergillus target.
 - (a) No Cq value for the FAM or ROX Fluorophores.
 - (b) Visually confirm that there is no curve on the graph.

Running the BioRad CFX96

The following targets will be detected on the following fluorophores:

- Aspergillus niger: ROX
- Aspergillus flavus: Cy5
- Aspergillus fumigatus: FAM
- Aspergillus terreus: Cy5.5
- Cannabis DNA: HEX

a) Start the qPCR Cycling program

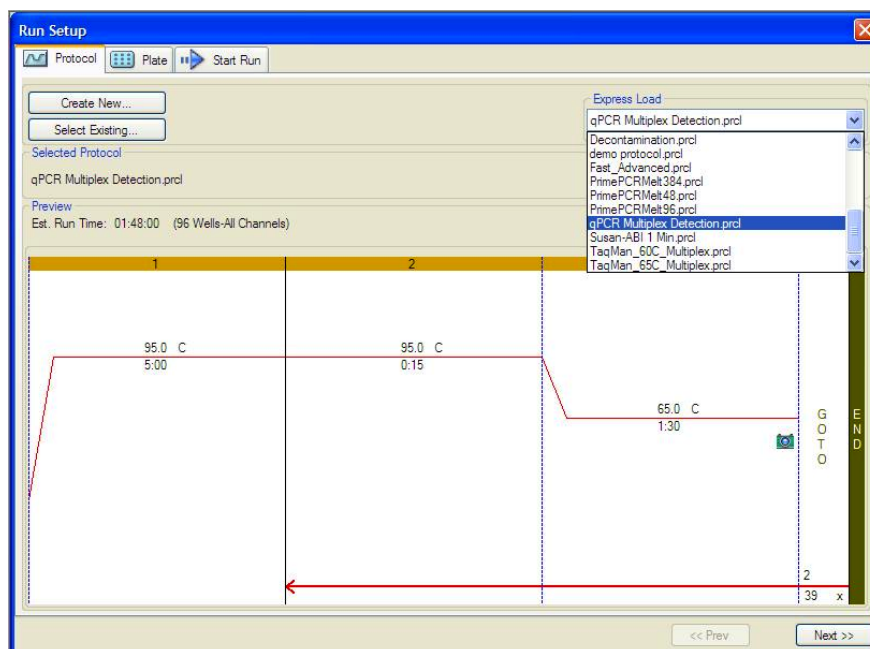
(1) Select User-Defined in the Startup Wizard under Run setup



b) Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click “Next”.

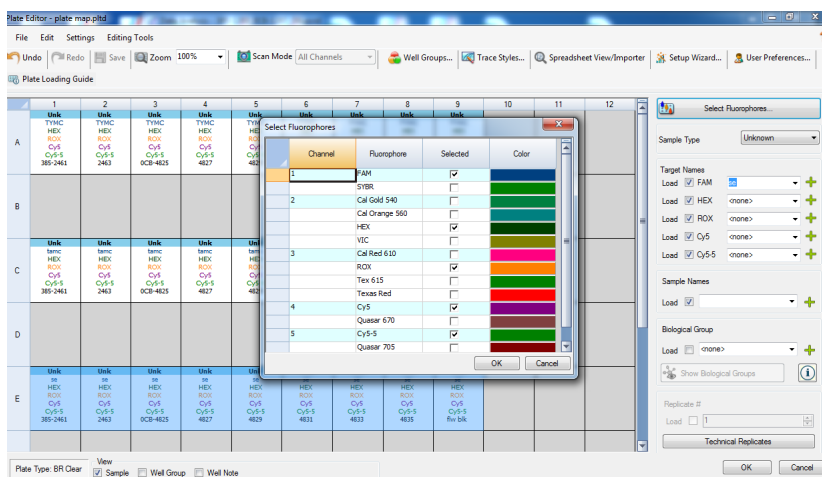
(1) If not already pre-programmed, create a cycling program with the following specifications and save as “qPCR Multiplex Detection”

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

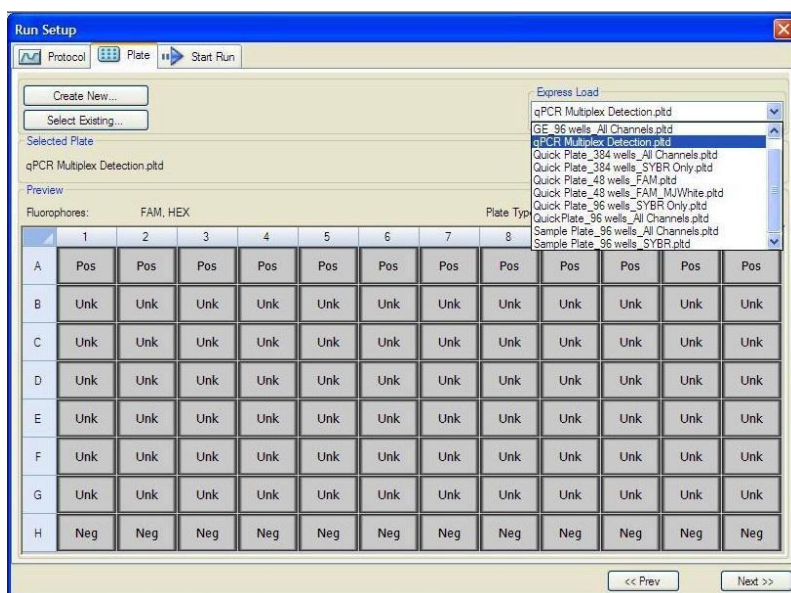


c) Design your plate under the plate tab in the Run Setup

- (1) Select the qPCR Multiplex Detection from the dropdown menu. If not already present, click “Create New”
- (2) Plate editor window will appear. Choose FAM, HEX, ROX, Cy5 and Cy5.5 Fluorophores and click “OK”.



- (3) If plate layout was previously saved, click “Edit Selected” to move to the Plate Editor Screen.

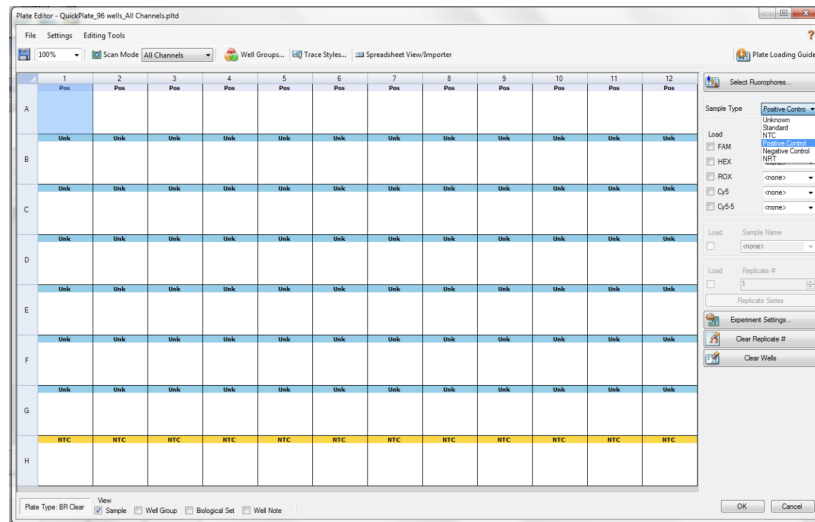


- (4) On the Plate Editor Screen, change the Sample Type to correlate with your specific plate setup.

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*

- (5) Make sure All Channels is selected from the dropdown menu at the top.



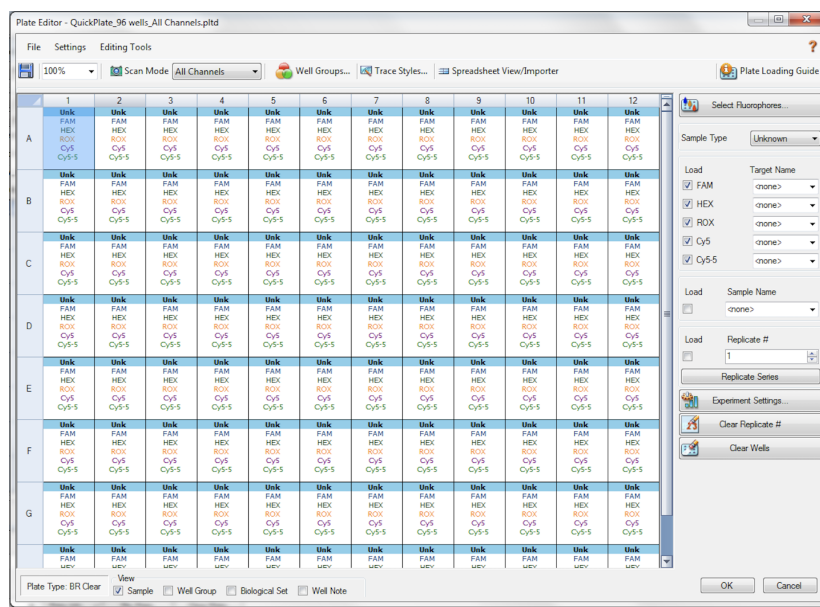
(6) Attach the fluorophores to the wells being used.

i. Highlight all the wells being used

ii. For Aspergillus 5-Color Assay, **Highlight the well locations and click on FAM, HEX, ROX, Cy5 and Cy5.5**

(7) When the plate is designed correctly, click OK.

(8) 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 fine).

(9) Close the lid and click Start Run.

(10) Save the experiment with the [User] and [date]

(11) 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.**

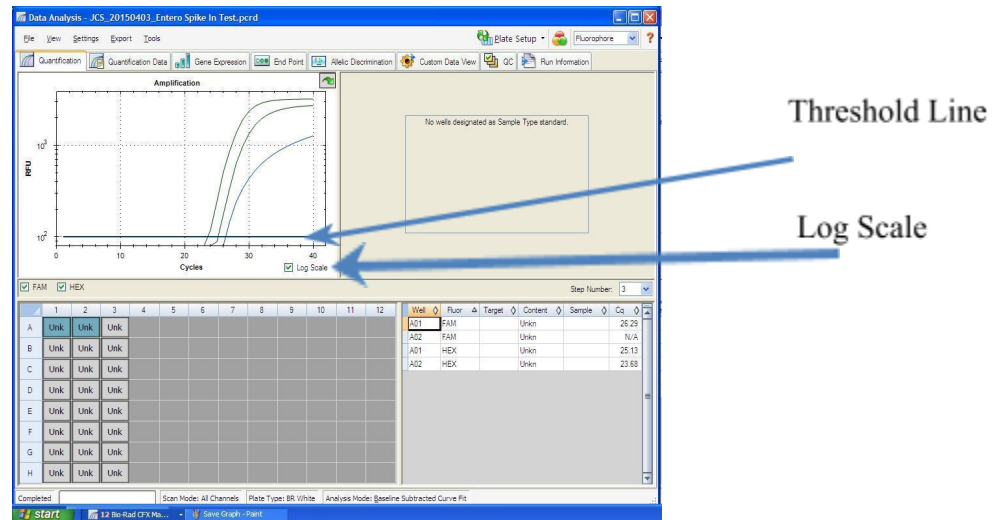
Data Analysis BioRad CFX96

PathoSEEK® Aspergillus 5-color Data Analysis Quick Reference Table:

Table 1: All Matrices

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

- a)** The Data Analysis window will open automatically when the run is complete.
- b)** Highlight the well of interest.
 - (1) The graph will appear above.
 - (2) The Cq values will appear to the right.
- c)** To analyze the results
 - (1) Start by turning the graph to Log Scale and manually moving the threshold to 10² 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.



(2) Controls

i. Assay-specific Positive Control, on the FAM, ROX, Cy5, and Cy5.5 fluorophores have a Cq value ≤ 35 .

a. Visually confirm with the curve on the graph.

ii. Assay-specific Negative Control, on the FAM, ROX, Cy5, and Cy5.5 fluorophores have no Cq value.

a. Visually confirm with the curve on the graph.

(3) Unknown Aspergillus Targets: The following fluorophores detect the following species of Aspergillus:

i. Aspergillus niger: ROX

ii. Aspergillus flavus: Cy5

iii. Aspergillus fumigatus: FAM

iv. Aspergillus terreus: Cy5.5

v. 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

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

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

b. 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.

- vii. An “absence” or passing result for the unknown *Aspergillus* targets.
 - a. No C_q value for the FAM, ROX, Cy5 or Cy5.5 fluorophores.
 - b. Visually confirm no curve on the graph.

Confirmation of Presumptive Positives

All positive results should be confirmed following AOAC SMPR 2019.001 or the laboratory's confirmation protocol and state and/or regional requirements.

1) From the primary enrichment, perform an isolation streak to a fungal specific agar (Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar [SAB-DEX], etc.). Incubate at $25 \pm 2^{\circ}\text{C}$ for 5-7 day.

a) Confirm the presence of *Aspergillus* using morphological characteristics of the colony growth and identify the typical conidial head of *Aspergillus* using a compound microscope.

2) Alternatively, confirm a positive result following state and/or regional requirements.

AOAC Performance Tested MethodSM Applicability Claim

The PathoSEEK® 5-Color *Aspergillus* Multiplex Assay with SenSATIVAx® Extraction has been validated according to the AOAC Performance Tested MethodSM Program for the detection of *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus terreus* in dried cannabis flower (>0.3% THC, 10 g), cannabis concentrates (5 g) and THC - infused chocolate (25 g) on the AriaMx and CFX-96 instruments. The validation study met the requirements as set forth in the AOAC Standard Method Performance RequirementsSM 2019.001 for the detection of *Aspergillus* in cannabis and cannabis infused products.

Troubleshooting Guide

| Symptom | Reason | Solution |
|---|---|--|
| Internal control (SCCG Primer) failure | Extraction Failure | Repeat SenSATIVAx™ and PathoSEEK™ by following the protocol. |
| | Residual ethanol in elution | Ethanol is an inhibitor to PCR. Return to the SenSATIVAx™ protocol and repeat all steps. |
| | Mix up in Reaction Setup | Repeat the qPCR by following the protocol. |
| | Missing Fluorophore on plate set up | In the Data Analysis window click on View/Edit Plate Setup from the Settings drop down. All wells should have both FAM and HEX. Once completed and the window is closed the analysis should automatically update. |
| Internal Control (SCCG) Positive result on positive or negative control samples or 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 | Plant DNA 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 extraction area | Designate separate benches, pipettes etc. for extractions and qPCR setup |
| Positive Negative Control | Small Cq value <15 | Visually confirm that there is an amplification curve. If not, this is a low level background and is to be expected. |
| | 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 | Repeat the qPCR by following the protocol. |
| Total run failure | Excessive vortex of the qPCR Master Mix | 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 Fluorophore.

The **Internal Control** is added to every sample reaction where a Cq is expected. It ensures the effectiveness and efficiency of each reaction. The internal control targets plant DNA, or more specifically, a Single Copy Control Gene (SCCG), using the HEX Fluorophore.

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

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

The results may vary based on laboratory conditions. Altitude and humidity are among factors known to affect the growth of bacterial and fungal species. All thresholds were determined based on the results using the BIO-RAD CFX96 Touch® Real-Time PCR Detection System. It is recommended that thresholds be calibrated for each specific laboratory setting.

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