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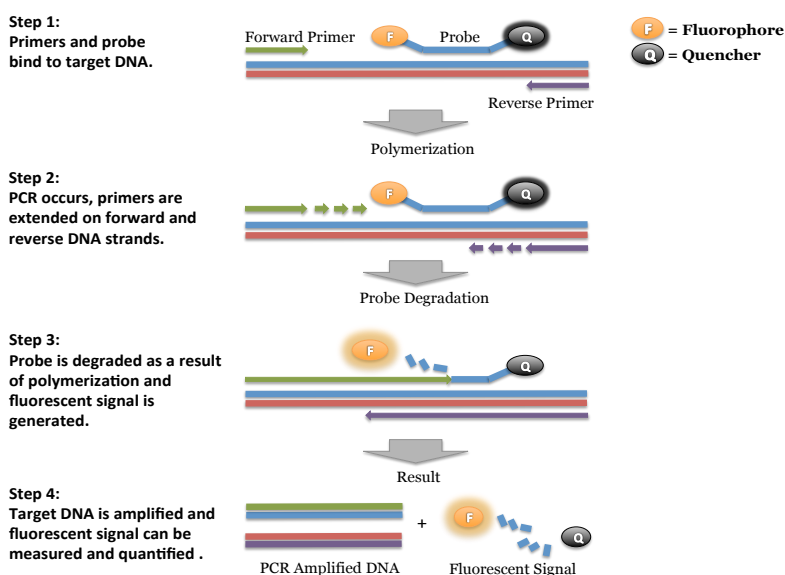
Introduction

PathoSEEK™ Microbial Safety Testing Platform utilizes a novel, contamination-free, PCR-based assay and provides an internal plant DNA control for every reaction. It is a simple two-step protocol, which is flexible and automation compatible.

The PathoSEEK™ 5 – Color Aspergillus Multiplex microbial detection assay uses 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.

Process Overview

The PathoSEEK process includes Real-time quantitative PCR assays using a multiplex system of primers to detect potential pathogens within the plant, extract or MIP (Marijuana Infused Product) sample. Below is a simplified depiction of the qPCR assays. The forward and reverse primers have universal primer tails to enable potential Next Generation Sequencing of resulting products.



Kit Specifications

The qPCR Master Kit v3 contains 200 reactions (Medicinal Genomics # 420201). Each PathoSEEK™ Detection Assay Probe Mix contains 200 reactions. Each PathoSEEK™ Positive Control contains 60 reactions.

Materials Needed from Medicinal Genomics

1. qPCR Master Kit v3, store at -15 to -20°C upon arrival [Medicinal Genomics #420201].
 - Reaction Buffer (10x)
 - Decontamination Enzyme (10 Units/μL) – (Not used in this protocol)
 - qPCR Master Mix (5x)
 - Nuclease Free Water
2. Aspergillus 5-Plex Assay (AriaMX) [Medicinal Genomics #420148].
3. Aspergillus 5-plex control v2 [Medicinal Genomics #420330].

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

NOTE:

SenSATIVax Extraction Elution Volume = **25 uL** for Aspergillus 5 – color assay
Using DNA from a 50 uL elution will adversely affect the sensitivity of the assay

Materials Supplied by the User

Consumables & Hardware

- Agilent AriaMx Real-Time PCR System G8830A Option 010 with the following Optical Channels: FAM, ROX, HEX, Cy5 and ATTO 425 (Contact Agilent)
- Agilent HP 650 Notebook PC option 650 (Contact Agilent)
- 96 well optical qPCR plates (Agilent AriaMx 96 well plates, Agilent # 401490, 401491 or Fisher Scientific 96-Well Armadillo PCR Plate, Fisher # AB2396)
- Adhesive optical seal for qPCR plates (Agilent adhesive plate seals, Agilent # 401492 or USA Scientific TempPlate® RT Optical Film # 2978-2100) or Agilent Optical Strip Caps #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**
- Multi-channel pipette P50 or P20 (optional)
- Single channel pipette P10, P20 and P200
- Filtered pipette tips for P10, P20, P50, and P200
- Crushed ice or cold racks (96 well PCR Cryogenic Rack, VWR #89004-570 and 1.5µL Tube Benchtop Cryogenic Racks, VWR #89004-558 or similar)
- Freezer, -20°C
- Table top mini plate centrifuge (Fisher Scientific #14-100-143 or similar)



- Table top mini tube centrifuge (VWR® Mini Centrifuge #10067-588 or 6-place personal microcentrifuge for 1.5/2.0 ml tubes # 2631-0006, or similar)



- Table top Vortex Genie (Scientific Industries #SI-0236 or Similar)



Reagents

- 10% bleach

Real-Time Quantitative PCR (qPCR) Protocol

1. Using the 10% bleach solution, wipe down the workspace, including the bench top and all equipment being used (except the Agilent AriaMX Instrument).
 2. Remove qPCR reagents including qPCR Master Mix, water, reaction buffer and assay probe mix to be used from the -20°C freezer. Place qPCR master mix on ice. Allow remaining tubes to thaw at room temperature. Once thawed, immediately place tubes on ice.
 3. Remove positive control tubes needed from -20°C freezer. Allow tubes to thaw at room temperature. Once thawed, place tubes on ice.
 4. Before preparing the reaction, invert or vortex and spin-down the reagents.
 - 4.1. Assay probe mix tubes, reaction buffer, positive controls and water – Vortex quickly followed by a pulse spin-down in a microcentrifuge.
 - 4.2. qPCR Master Mix – Invert the tube 5 times (**do not vortex**), followed by a pulse spin-down in a microcentrifuge.
 - 4.3. Return all reagents to the ice.
- Note: Do not vortex the qPCR Master Mix at any point during the protocol.*
5. 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.

NO GRIM REEFER:

Reagents	1 Reaction	24 reactions (plus 1 excess rxn)	48 reactions (plus 2 excess rxn)
qPCR Master Mix v3	3.75µL	93.75µL	187.5µL
Aspergillus 5 – color Assay Probe Mix	1µL	25µL	50µL
Reaction Buffer	0.8µL	20µl	40µl
Water	8.2µL	205µL	410µL
Total	13.75µL	343.75µL	687.5µL

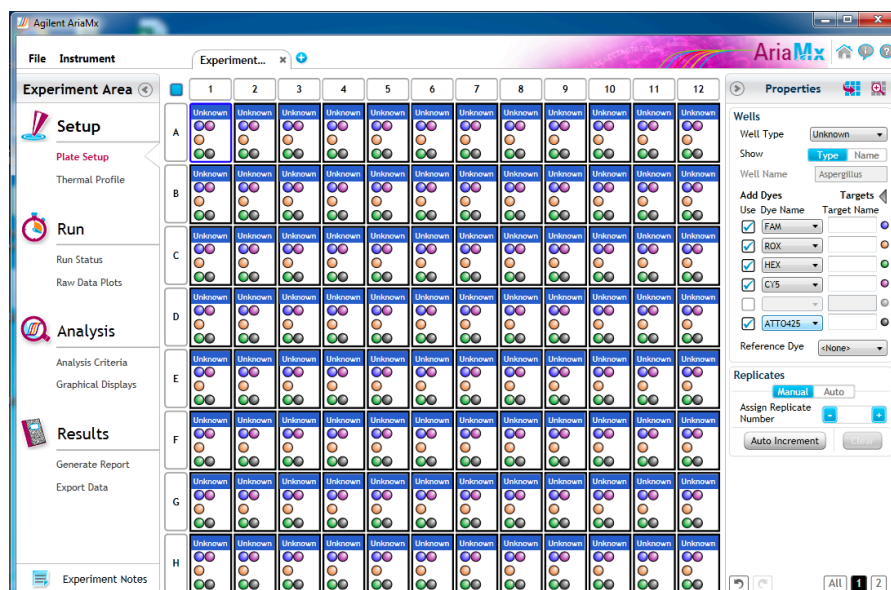
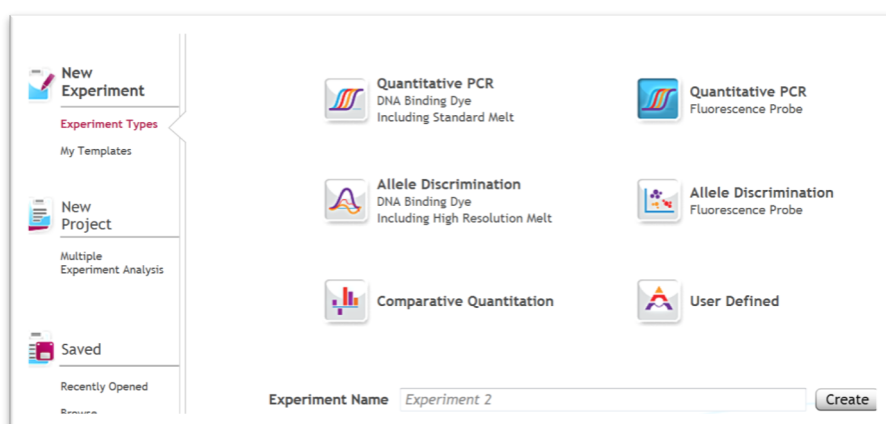
- 5.1. Once combined gently tip mix or invert the tube 5 times to combine the assay master mix.
 - 5.1.1. Pulse spin-down tube in microcentrifuge.
 - 5.1.2. Place MM tubes on ice until used.
 - 5.1.3. For the positive control(s), make a 1:10 dilution of each assay being run
 - 5.1.3.1. 1µL of Positive Control dilute with 9µL of water (found in the kit)
 - 5.1.3.2. 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 well to ensure control DNA is in solution

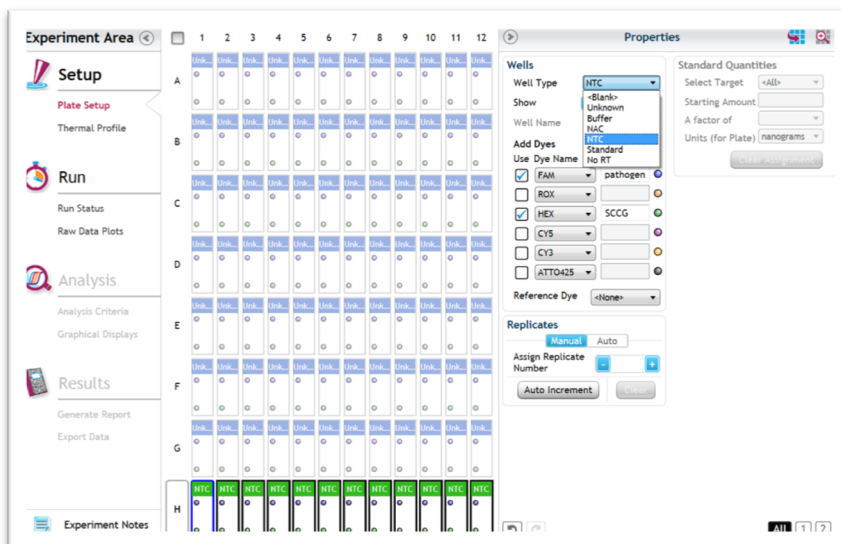
6. Place the Extraction Plate on the magnet (optional). This is to ensure no magnetic beads are transferred into the qPCR reactions if there are some left over from the extraction elution process.
 7. Use a new 96-well optical qPCR plate and label the plate “qPCR Plate_[date]”.
 8. Carefully remove the seal from the Extraction Plate and transfer 5µL of each sample into the corresponding well on the qPCR plate. Keep the extraction plate on the magnet when aspirating the 5µL.
 - 8.1. Add 5µL of the diluted Positive Controls to their corresponding wells. Then add 5µL of water to the corresponding Negative Control wells.
- Note:** ALWAYS use a fresh tip for every liquid transfer into the qPCR plate

9. Add 13.75µL of Aspergillus 5 - Color Assay Probe MM to each 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 to not introduce bubbles during this mix.
10. 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.
 - 10.1. Spin-down for at least 1 minute in plate microcentrifuge.

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.
11. Place the sealed plate onto the Agilent AriaMX instrument, positioning the A1 well in the top left corner
12. Create a New Experiment on the Agilent qPCR instrument.
 - 12.1. Select “Quantitative PCR” from Experiment Types. Under Setup>Plate Setup, highlight wells that contain reactions and select FAM, HEX, ROX, Cy5 and ATTO 425 under Add Dyes.



Change the well types to reflect your plate set up. If desired, add Target names to include “pathogen name” for FAM ROX, Cy5, ATTO 425 and SCCG (single copy control gene) for HEX.



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

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



12.3. Close the lid and click “Start Run”.

12.4. Save the experiment with the [User] and [date]

12.5. When 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

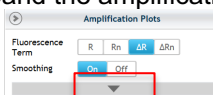
PathoSEEK™ Aspergillus 5-color Data Analysis Quick Reference Table:

Table 1: All Matrices – No Decontamination Step

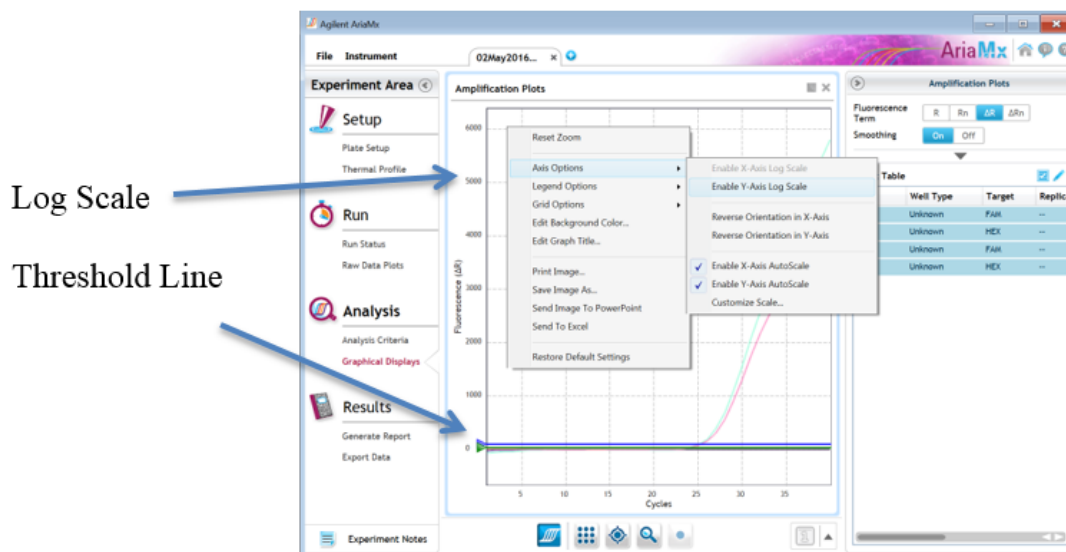
PathoSEEK™ Assay	Cq Value (High CFU count)	Fluor	Negative Control (Cq)	CFU threshold (CFU/g) (This may vary by state)
<i>Aspergillus niger</i>	≤ 40	ROX	> 40	Presence/Absence
<i>Aspergillus flavus</i>	≤ 40	Cy5	> 40	Presence/Absence
<i>Aspergillus fumigatus</i>	≤ 40	FAM	> 40	Presence/Absence
<i>Aspergillus terreus</i>	≤ 40	ATTO 425	> 40	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Control	≤35	FAM/ROX		

1. 5 - Color Aspergillus Assay Analysis

- 1.1. Open the Data Analysis window when the run is complete.
- 1.2. Highlight the wells of interest in the Analysis Criteria under Analysis, then select Graphical Display
 - Amplification plots will be available for viewing
 - The Cq values will appear to the right in the table
- 1.3. 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

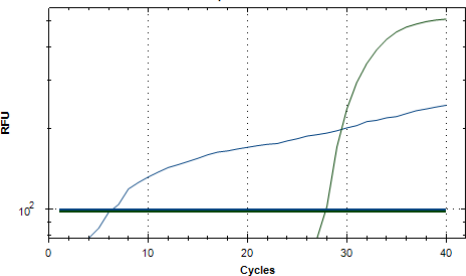


- Manually adjust thresholds to 100 RFU for the HEX, FAM, ROX, Cy5 and ATTO 425 fluorophores.



- Controls
 - Positive Control, on the FAM, ROX, Cy5 and ATTO 425 Fluorophores, have a Cq values ≤ 35 .
 - Visually confirm with the curve on the graph.
 - Negative Control, on the FAM, ROX, Cy5 and ATTO 425 Fluorophores, have no Cq value.
 - Visually confirm with the curve on the graph.
- Unknown Aspergillus Targets
 - Internal Control, on the HEX Fluorophore, has a Cq value ≤ 35 for flower samples, ≤ 40 for all other matrices.
 - Visually confirm with the curve on the graph
 - A “presence” or failing result for the unknown Aspergillus target(s).
 - Any Cq value for the FAM, ROX, Cy5 or ATTO 425 Fluorophores ≤ 40 .
 - Visually confirm with the curve on the graph. It is very important to confirm with the amplification curve when a presence result occurs. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15. (See troubleshooting guide below for more details.)
 - An “absence” or passing result for the unknown Aspergillus target.
 - No Cq value for the FAM, ROX, Cy5 or ATTO 425 Fluorophores.
 - Visually confirm no curve on the graph.

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 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 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.
<p>Background Amplification</p> 	Unclear	<p>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 florescent signal. This is usually a negative result, but should be repeated.</p>

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 is targeting a Single Copy Control Gene or SCCG, using the HEX fluorophore.

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.

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