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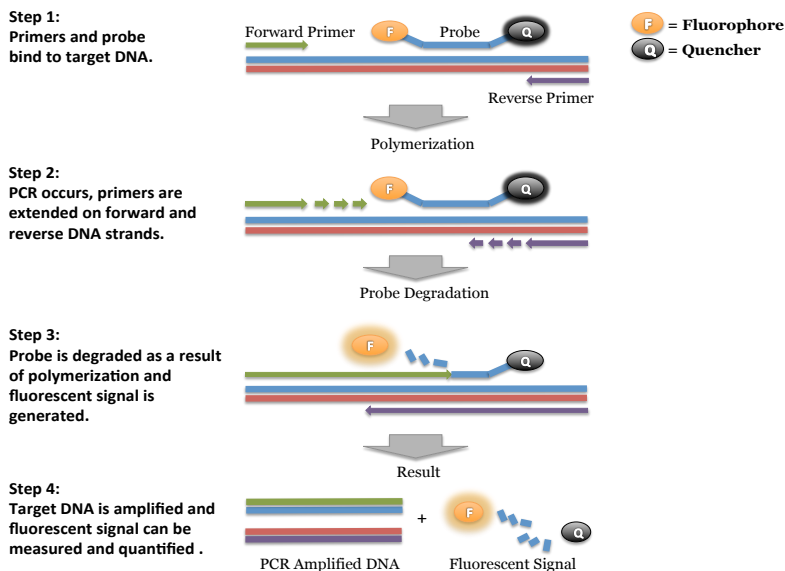
Introduction

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 microbial detection assay 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.

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 (CFX) [Medicinal Genomics #420147].
3. Aspergillus 5-plex control v2 [Medicinal Genomics #420330].

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

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

- Bio-Rad CFX96 Touch™ Real-Time System (contact Bio-Rad)
- Bio-Rad Personal PC (contact Bio-Rad)
- 96-well optical qPCR plates (Fisher Scientific 96-Well Armadillo PCR Plate, Fisher # AB2396 or Bio-Rad Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR Plates, Bio-Rad # HSP-9601)
- Adhesive optical seal for qPCR plates (Bio-Rad Microseal® # MSB-1001 or USA Scientific TempPlate® RT Optical Film # 2978-2100)
- 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 Bench-top 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 Bio-Rad CFX96 Touch™ Real-Time System and the Bio-Rad Personal PC).
 2. 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. 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.

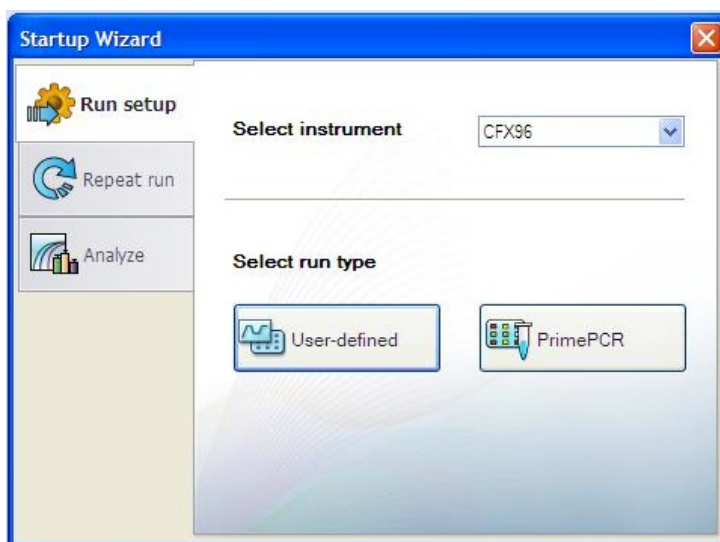
Note: It is best to add the largest volume reagent first, in this case water.

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).

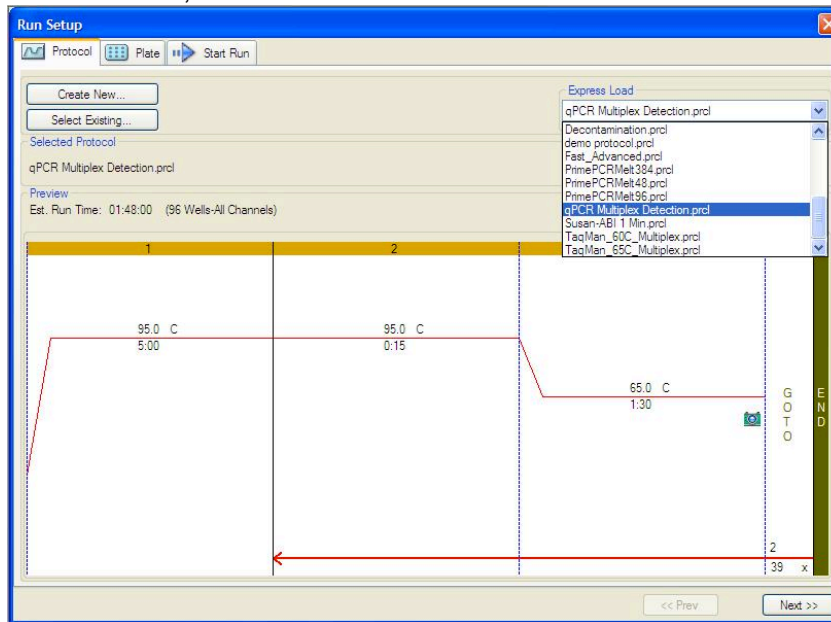
Note: It is best to add the largest volume reagent first, in this case the 9 µL water then the 1 µL of positive control, pipette mix well to ensure control DNA is in solution

6. Place the Extraction Plate on the magnet (optional). This is to ensure no magnetic beads are transferred into the qPCR reactions if indeed 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 Bio-Rad qPCR instrument, positioning the A1 well in the top left corner
12. Start the qPCR Cycling program
 - 12.1. Select User-Defined in the Startup Wizard under Run setup.



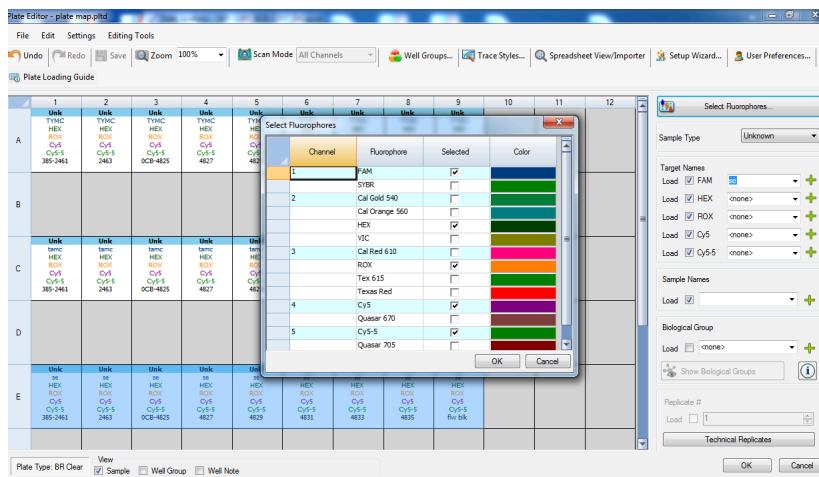
12.2. Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click “Next”.

- If not already pre-programmed, create a cycling program with the following specifications and save as “qPCR Multiplex Detection”
 - Hot start at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 65°C for 1 minute, 30 seconds.

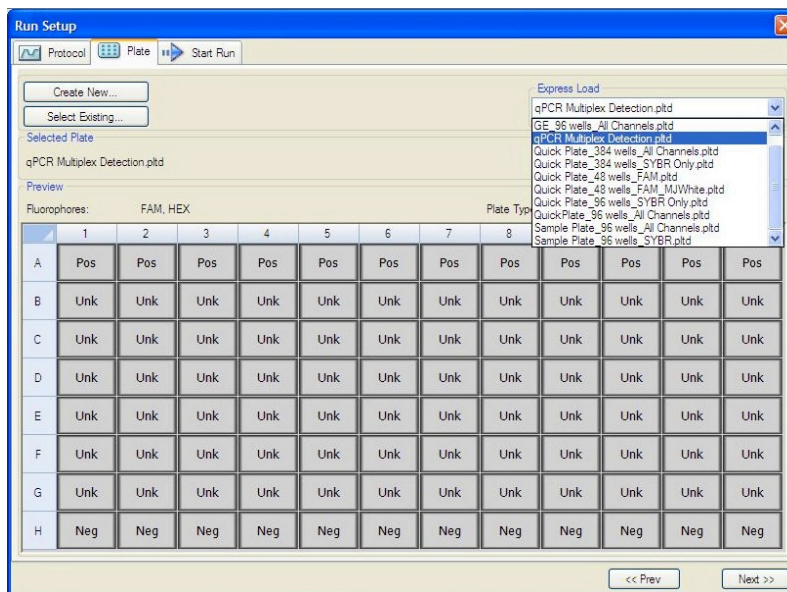


12.3. Design your plate under the plate tab in the Run Setup

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



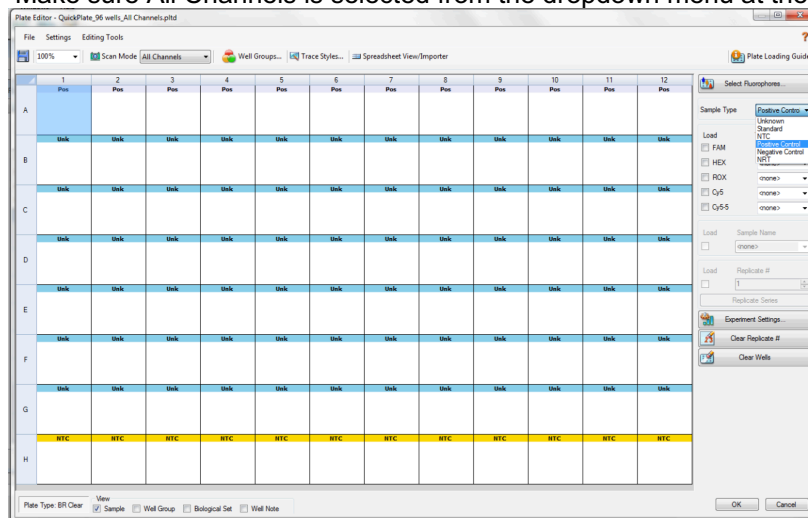
12.3.3. If plate layout previously saved, click “Edit Selected” to move to the Plate Editor Screen.



12.3.4. 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*

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

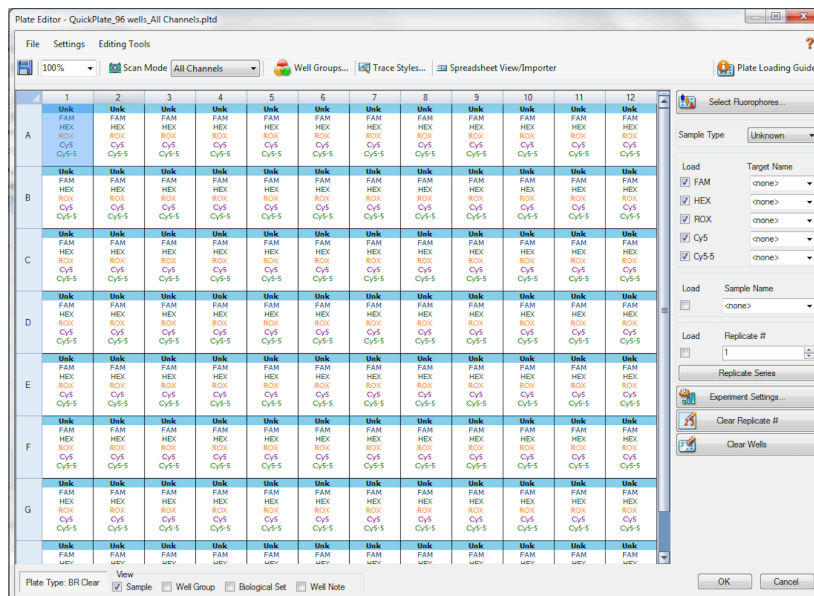


12.3.6. Attach the fluorophores to the wells being used.

12.3.6.1. Highlight all the wells being used

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

12.3.7. When the plate is designed correctly click OK.



Close the lid and click Start Run.

12.4. Close the lid and click Start Run.

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

12.6. When 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:

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	Cy5.5	> 40	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Control	≤35	FAM/ROX		

1. Presence / Absence Aspergillus 5 – Color Assay

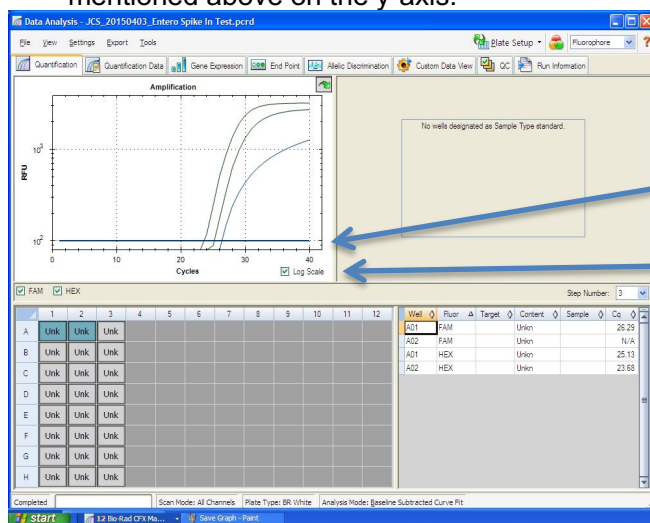
1.1. The Data Analysis window will open automatically when the run is complete.

1.2. Highlight the well of interest.

- The graph will appear above.
- The Cq values will appear to the right.

1.3. To analyze the results

- Start by turning the graph to Log Scale and manually moving the threshold to 10² for the all fluorophores.
 - To turn the graph to Log Scale, click on the box at the bottom right of the graph.
 - To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis.



Threshold Line

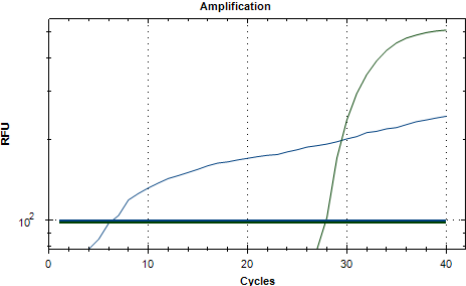
Log Scale

Controls

- Assay-specific Positive Control, on the FAM fluorophore, has a Cq value ≤ 35.
 - Visually confirm with the curve on the graph.
- Assay-specific Negative Control, on the FAM fluorophore, has no Cq value.
 - Visually confirm with the curve on the graph.

- Unknown Aspergillus Targets: The following fluorophores detect the following species of Aspergillus:
 - Aspergillus niger: ROX
 - Aspergillus flavus: Cy5
 - Aspergillus fumigatus: FAM
 - Aspergillus terreus: Cy5.5
 - 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 targets.
 - Any Cq value for the FAM, ROX, Cy5 or Cy5.5 fluorophores ≤ 40 .
 - 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.
 - An “absence” or passing result for the unknown Aspergillus targets.
 - No Cq value for the FAM, ROX, Cy5 or Cy5.5 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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