

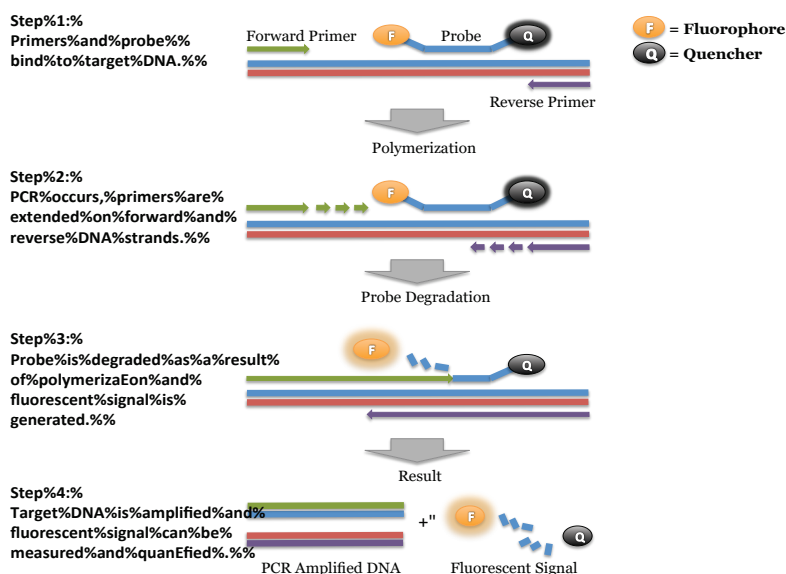
Introduction

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.

PathoSEEK™ microbial detection assays use a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of microbial species for 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 contains 200 reactions (Medicinal Genomics # 420002). Each PathoSEEK™ Detection Assay Probe Mix contains 200 reactions. Each PathoSEEK™ Positive Control contains 60 reactions.

Materials Supplied in the Kit

qPCR Master Kit, store at -15 to -20°C upon arrival [Medicinal Genomics #420002].

- Reaction Buffer (10x)
- Decontamination Enzyme (10 Units/μL) – (Not used in this protocol)
- qPCR Master Mix (5x)
- Nuclease Free Water

If performing Optional Grim Reefer Free DNA Removal Step the following reagents are also required:

- Grim Reefer Free DNA Removal Kit, Medicinal Genomics #420145 (Store at -20°C) (used in SenSATIVax SOP)
- Grim Reefer Free DNA Removal Control, Medicinal Genomics #420144 (Store at -20°C) (used in SenSATIVax SOP)
- Grim Reefer Free DNA Removal Assay, Medicinal Genomics #420143 (Store at -20°C) (used in this SOP)

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

- 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).
- 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.
- Remove positive control tubes needed from -20°C freezer. Allow tubes to thaw at room temperature. Once thawed, place tubes on ice.
- Before preparing the reaction, invert or vortex and spin-down the reagents.
 - Assay probe mix tubes, reaction buffer, positive controls and water – Vortex quickly followed by a pulse spin-down in a microcentrifuge.
 - qPCR Master Mix – Invert the tube 5 times (do not vortex), followed by a pulse spin-down in a microcentrifuge.
 - Return all reagents to the ice.

Note: Do not vortex the qPCR Master Mix at any point during the protocol.
- 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	3.75µL	93.75µL	187.5µL
Assay Probe Mix (Assay Specific)	1µL	25µL	50µL
Reaction Buffer	0.8µL	20µl	40µl
Water	8.2µL	205µL	410µL
Total	13.75µL	343.75µL	687.5µL

WITH GRIM REEFER:

In order to detect the GR positive control that was spiked into the sample during the DNA extraction using optional Grim Reefer, 0.5µL of GR Assay probe mix needs to be added per reaction. See the table below.

Reagents	1 Reaction	24 reactions (plus 1 excess rxn)	48 reactions (plus 2 excess rxn)
qPCR Master Mix	3.75µL	93.75µL	187.5µL
Assay Probe Mix (Assay Specific)	1µL	25µL	50µL
Grim Reefer Assay Probe Mix	0.5µL	12.5µL	25µL
Reaction Buffer	0.8µL	20µl	40µl
Water	7.7	192.5µL	385µL
Total	13.75µL	343.75µL	687.5µL

NOTE: The GR Assay is detected in the Cy5 Channel of the qPCR instrument so be sure to select the Cy5 channel when setting up the detection plate.

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.

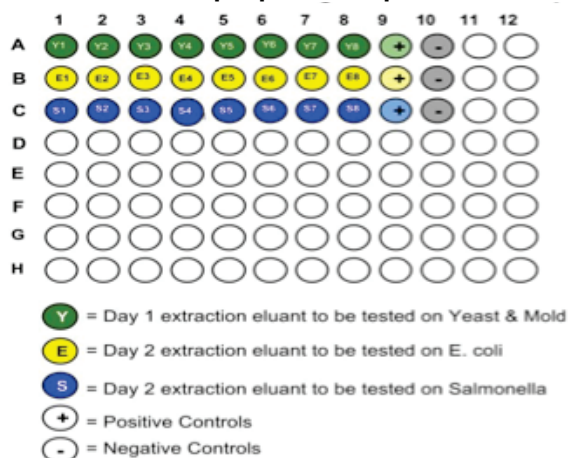
5.1.3. For the positive control(s), make a 1:10 dilution of each assay being run

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

Below is an example plate setup. This will vary depending on which assays are being tested.



6. Place the Extraction Plate on the magnet. 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 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 to not introduce bubbles during this mix.

Note: It may be helpful to label each of the corresponding column wells to accurately dispense the correct samples

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.

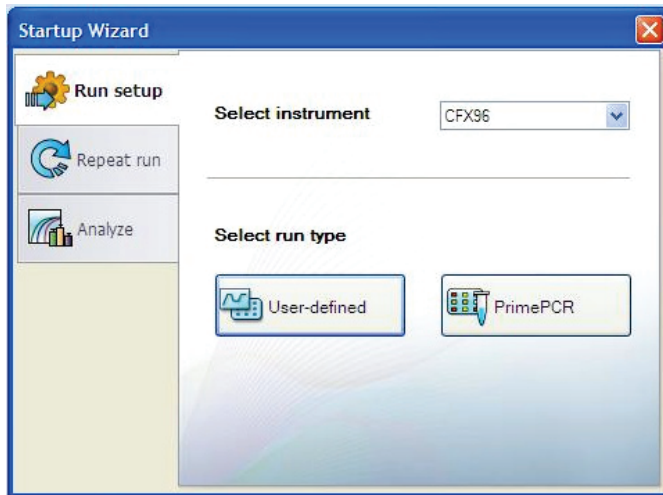
10.1. Spin-down for at least 1 minute in plate microcentrifuge.

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

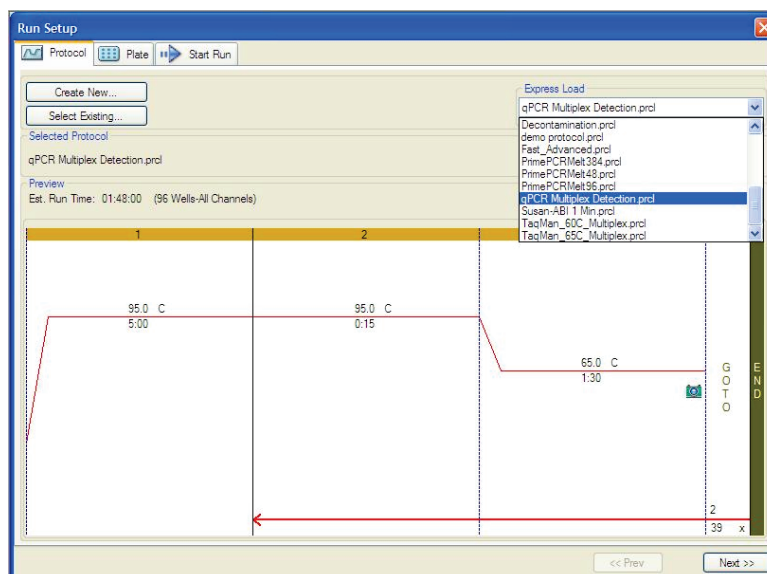
12. Start the qPCR Cycling program

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.

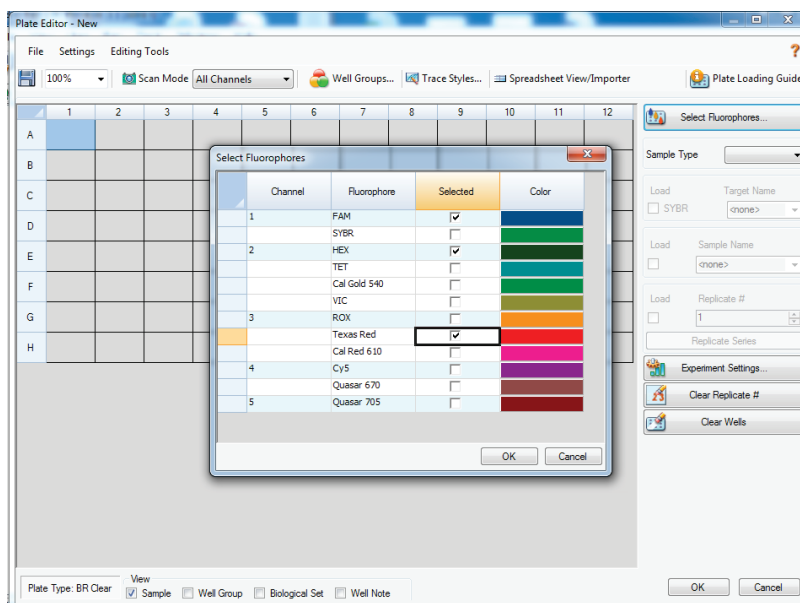


12.3. Design your plate under the plate tab in the Run Setup

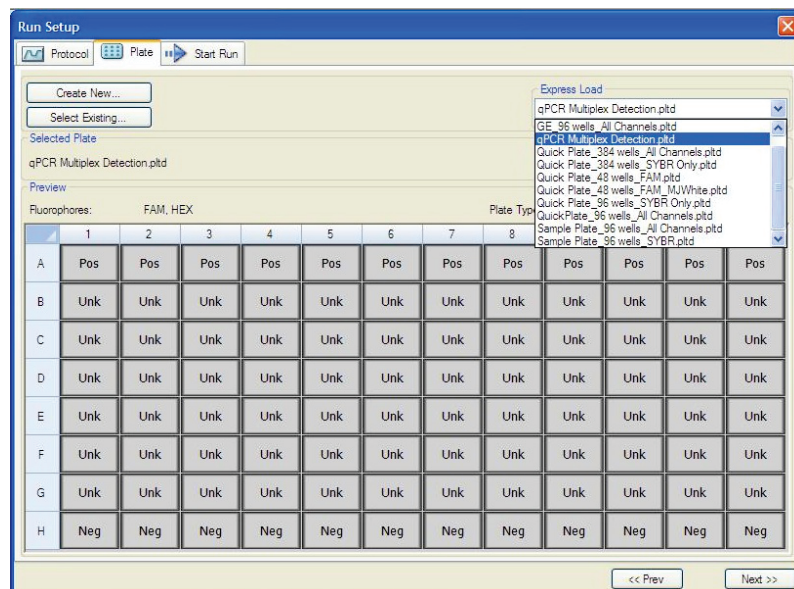
12.3.1. Select the qPCR Multiplex Detection from the dropdown menu. If not already present, click "Create New"

- 12.3.2. Plate editor window will appear. Choose FAM, HEX, Texas Red and Cy5 Fluorophores and click “OK”. Texas Red is only necessary if running multiplexed assays, *E. coli*/Salmonella, *E. coli* (STEC)/Salmonella or Total Coliform/Entero. Cy5 is only necessary if running optional Grim Reefer.

NOTE: If running singleplex assays: *E. coli*, *E. coli* (STEC), Salmonella, Pseudomonas, Staphylococcus, Entero or Coliform they will all be detected on the FAM channel.



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

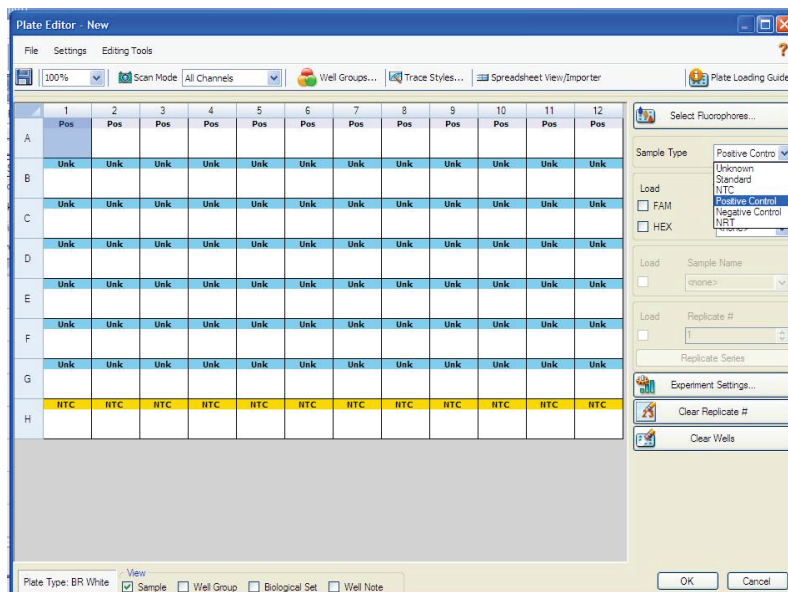


- 12.3.4. On the Plate Editor Screen, change the Sample Type to correlate with your specific plate setup.

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.

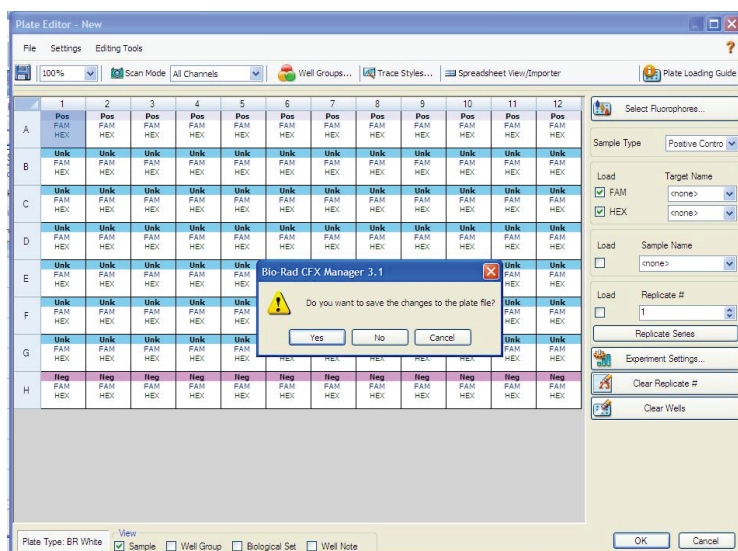
12.3.6.1. Highlight all the wells being used

12.3.6.2. If using a multiplex probe mix, Highlight the well locations and click on **FAM**, **HEX**, and **TEXAS RED**

12.3.6.3. If using Grim Reefer, select **Cy5**

12.3.7. When the plate is designed correctly click OK.

12.3.8. 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).

- 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.**

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.