Please refer to http://www.medicinalgenomics.com/product-literature/ for updated protocols and Material Safety Data Sheets (MSDS). Consult MSDS before using any new product.

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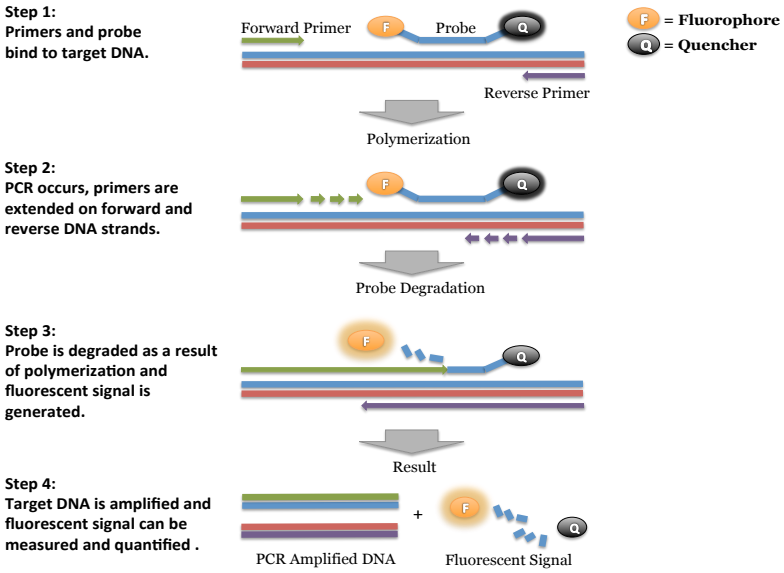
Introduction

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.

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.



Website: www.medicinalgenomics.com • Customer Support: 866.574.3582 • Support Email: support@medicinalgenomics.com

Kit Specifications

The qPCR Master Kit contains 200 reactions (Medicinal Genomics # 420200). 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 -20ºC upon arrival.

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

PathoSEEK™ Detection Assays and Positive Controls, ordered separately, store at -15ºC to -20ºC upon arrival.

For a full list of available qPCR assays and positive controls visit our website: www.medicinalgenomics.com/price-list/

Materials Supplied by the User

Consumables & Hardware

- Agilent AriaMx Real-Time PCR System G8830A Option 010 FAM, ROX, and HEX and Cy5 (Cy5 only if using Grim Reefer) (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 and cross contamination between wells.**
- 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, -20ºC
- 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 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.*

   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.

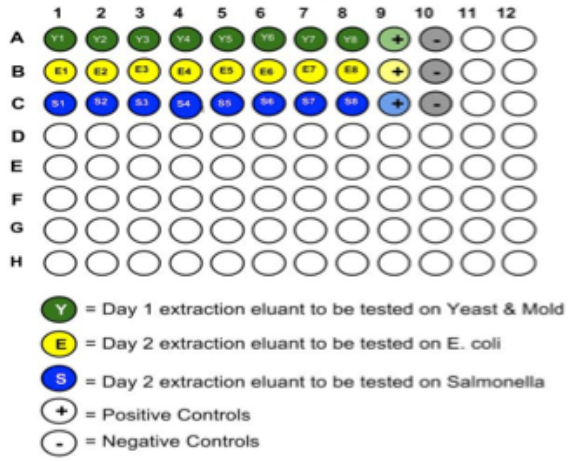
Reagents	1 Reaction	24 reactions (plus 1 excess rxn)	48 reactions (plus 2 excess rxn)
qPCR Master Mix	3.75µL	93.75µL	187.5µL
Assay Probe Mix (Assay Specific)	1µL	25µL	50µL
Grim Reefer Assay Probe Mix	0.5µL	12.5µL	25µL
Reaction Buffer	0.8µL	20µl	40µl
Water	7.7	192.5µL	385µL
Total	13.75µL	343.75µL	687.5µL

   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 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*

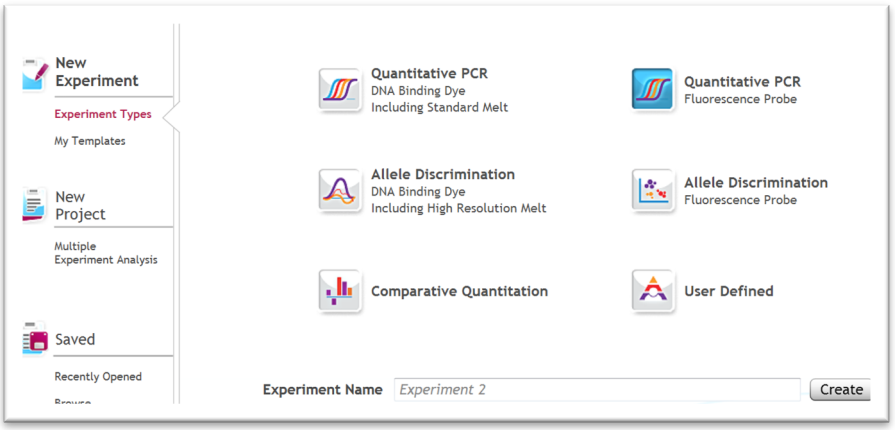
10. Seal the plate with strip caps or an adhesive seal.
11. Spin-down plate for at least 1 minute in plate microcentrifuge to bring well contents to bottom of wells and help to rid reaction of bubbles.
    Note: *Check for bubbles at the bottom of the wells (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the bottom of the wells, spin-down for another minute.*

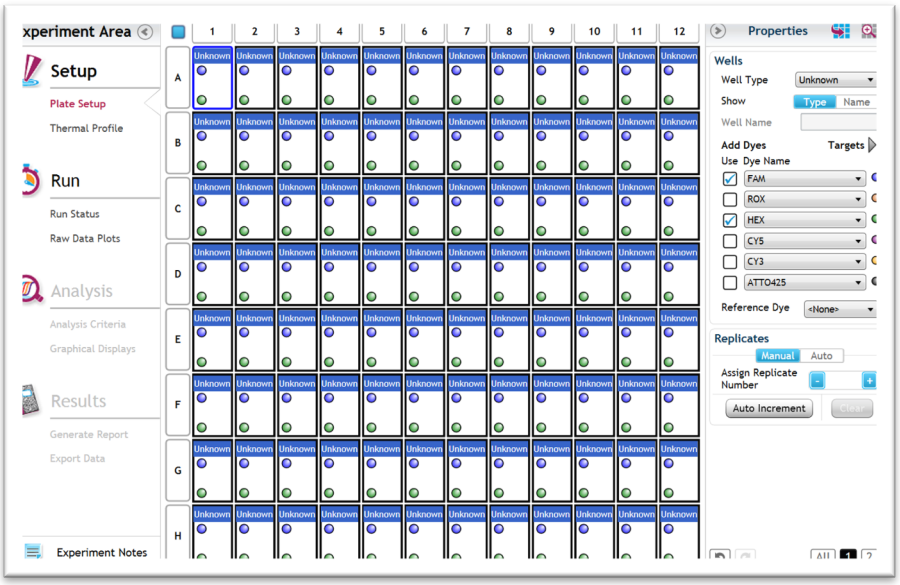
12. If using an adhesive seal, place the reusable compression pad (gray side down) on the plate directly lining up the holes in the pad with the wells of the plate.

13. Place the sealed plate with compression pad into the Agilent AriaMX instrument, positioning the A1 well in the top left corner
14. Close HOT TOP lid, close Main Lid.

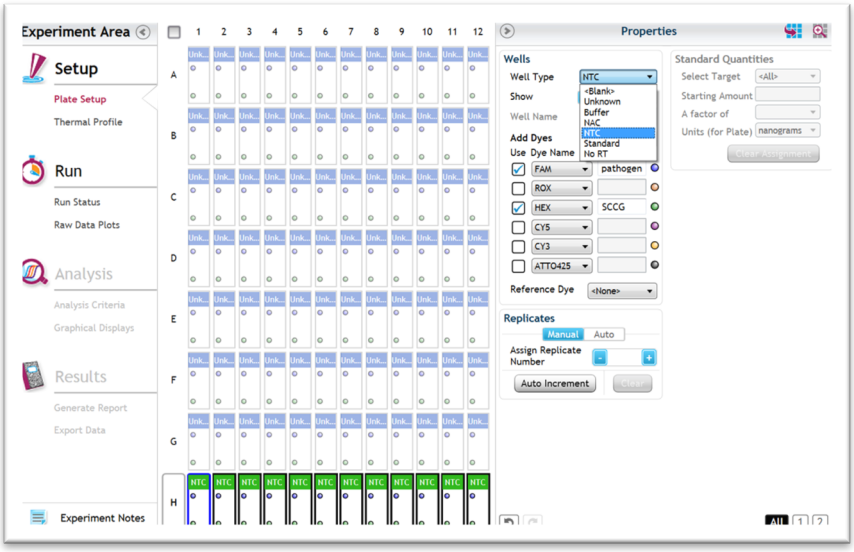
15. Create a New Experiment on the Agilent qPCR instrument.
    15.1. Select “Quantitative PCR” from Experiment Types. Under Setup>Plate Setup, select FAM, HEX, ROX and Cy5 under Add dyes. ROX is only necessary if running multiplexed assays, *E. coli*/Salmonella, *E. coli* (STEC)/Salmonella, Coliform/Entero or Pseudomonas/Staph. Cy5 is only necessary if using the Grim Reefer reagent.

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.





15.2. Change the well types to reflect your plate set up. Add Target names to include “pathogen name” for FAM or ROX and SCCG (single copy control gene) for HEX. Cy5 should be selected if running Grim Reefer.



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

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

15.4. Close the lid and click “Start Run”.
15.5. Save the experiment with the [User] and [date]
15.6. When run is complete, immediately dispose of the plate. Do not open 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 contaminates.

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 flourophore.

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 flourophore.