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Introduction

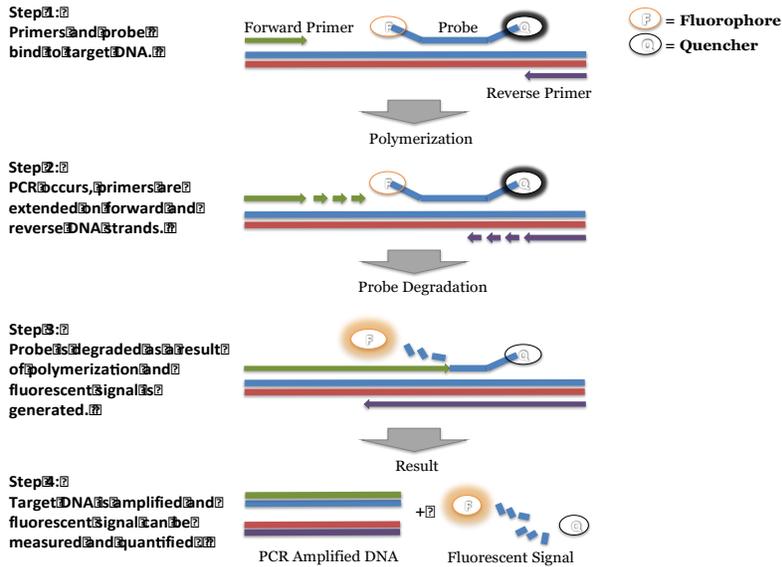
The PathoSEEK® Plant Pathogen detection kits use a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of a plant pathogen for every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting a plant pathogen, resulting in the minimization of false negatives due to reaction set-up errors or failing experimental conditions.

Plant Pathogen Analysis Quick Table

Assay	Cq Value	Fluor	Negative Control (Cq)	Cq threshold
Powdery Mildew	<40	FAM	No Value	Presence/Absence
Fusarium oxysporum	<40	FAM	No Value	Presence/Absence
Botrytis	<40	FAM	No Value	Presence/Absence
Russet Mites	<40	FAM	No Value	Presence/Absence
Internal Control*	<35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Controls	<35	FAM		

Process Overview

The process for determining the presence of a plant pathogen uses real-time quantitative PCR (qPCR) using a multiplex system of primers to detect both cannabis DNA and the target of interest. Below is a simplified depiction of the qPCR assays.



Kit Specifications

The Leaf Punch Lysis Solution contains 96 reactions (Medicinal Genomics # 420208), The qPCR Master Kit contains 200 reactions (Medicinal Genomics # 420200). Each PathoSEEK® Plant Pathogen Detection Assay Kit contains 200 reactions worth of reagents. Each PathoSEEK® Plant Pathogen Positive Control contains 60 reactions worth of reagents.

Materials:

Supplied by Medicinal Genomics

Used with all assays:

- Leaf Punch Lysis Solution, store at -15 to -20°C upon arrival (Medicinal Genomics #420208)
- Leaf hole punches (included with Leaf Punch Lysis Buffer)
- qPCR Master Kit, store at -15 to -20°C upon arrival (Medicinal Genomics #420200)

For Plant Pathogen testing (store at -15°C to -20°C upon arrival):

- Powdery Mildew Detection Assay (Medicinal Genomics #420115)
- Powdery Mildew Positive Control (Medicinal Genomics #420216)
- Fusarium oxysporum Detection Assay (Medicinal Genomics #420117)
- Fusarium oxysporum Positive Control (Medicinal Genomics #420220)
- Botrytis Detection Assay (Medicinal Genomics #420116)
- Botrytis Positive Control (Medicinal Genomics #420222)
- Russet Mite Detection Assay (Medicinal Genomics #420146)
- Russet Mite Positive Control (Medicinal Genomics #420224)

Materials Supplied by the User

Consumables & Hardware

- Chai Bio Open PCR Dual Channel Catalog Number E013201
- 0.1mL PCR 8Tube Strips, Optical Caps (USA Scientific, #1402-2300 or similar)
- Multi-channel pipette P50 or P20 (optional)
- Single channel pipette P10, P20 and P200
- Filtered pipette tips for P10, P20, P50, and P200
- 1.5mL tubes (USA Scientific #1415-2600)
- Reservoirs (Fisher Scientific #14-222-398 or similar) (optional)
- Crushed ice or cold racks (1.5ul Tube Bench-top Cryogenic Racks, VWR #89004-558 or similar)
- Dual rotor personal microcentrifuge, (USA Scientific #2641-0016)
- Table top Vortex Genie (Scientific Industries #SI-0236 or Similar)
- Refrigerator, 4°C
- Freezer, -20°C
- Laboratory Gloves, (USA Scientific, # 4904-3300 or similar)
- Permanent Marker (Sharpie)
- Plant Stakes (Optional for labeling)
- Toothpicks for punching out leaf punches (can use pipette tips as well)

Reagents

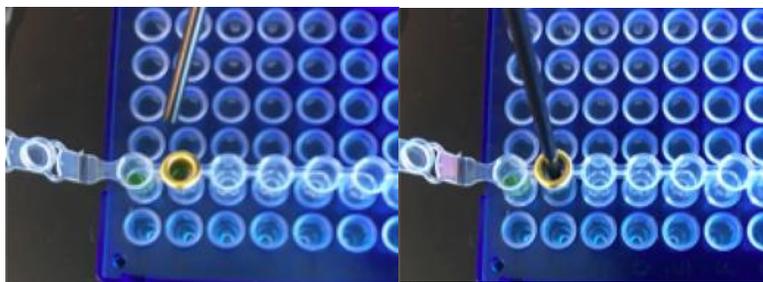
- 10% bleach

Leaf Sampling:

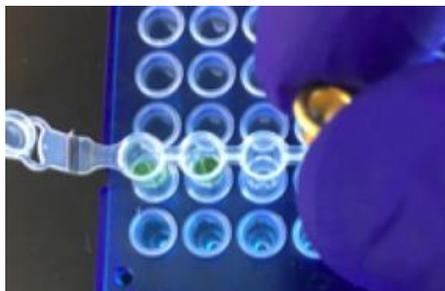
1. Put on a new pair of sterile gloves.
2. Remove necessary number of pre-aliquoted Leaf Punch Lysis Solution strip tubes to PCR tube rack and let thaw.
3. Once thawed quick spin using mini centrifuge with strip tube attachment to bring all contents of solution to the bottom of the tubes.
4. Label the wells of the Leaf Punch Lysis Solution strip tubes being used.
5. Obtain a disposable leaf punch. Press leaf punch down into leaf on sterile surface and rotate to remove a small section.



6. Remove cap from one strip tube and place leaf punch into the top of the strip tube. Discharge leaf punch with toothpick or pipette tip. Use a fresh toothpick or pipette tip for every leaf punch.



7. Close cap of strip tube and discard leaf punch.



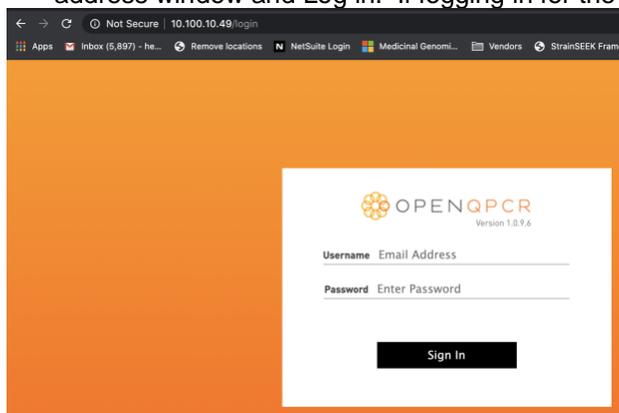
8. Repeat steps 5-7 for each sample being tested.
9. Change gloves in between each leaf sampling.
10. Once finished with leaf sampling, run Lysis program on ChaiBio Instrument.

Lysis Reaction Setup:

1. Make sure all of the strip tube caps are firmly snapped into place on each tube.
2. Briefly spin all strip tubes using mini-centrifuge with strip tube attachment to ensure that the Lysis Solution and leaf punch are in the bottom of the wells of the strip tubes. In some cases, the leaf punch may float onto top of the Lysis Solution. This is OK as long as it is in contact with the liquid.
3. Place all of the sealed strip tubes onto the Open PCR.



4. Sign on to the instrument's unique website by typing the IP address on the instrument screen into your browser address window and Log in. If logging in for the first time, create username and password.

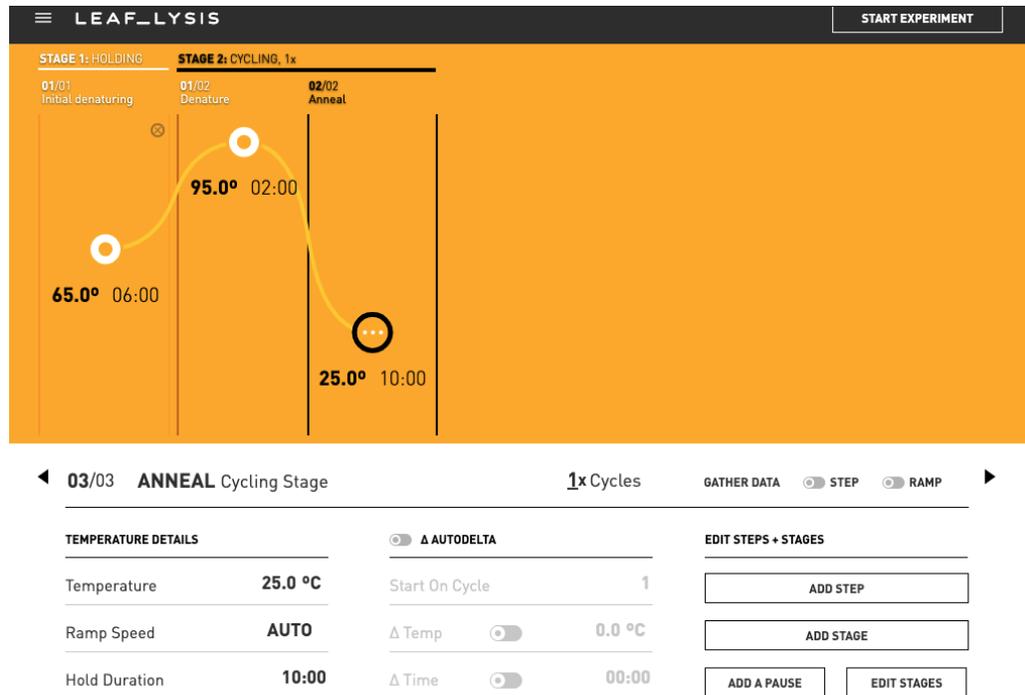




5. Create a new experiment on the Open qPCR Instrument called “Leaf_Lysis”

5.1. Program the proper Thermal Profile for the Leaf_Lysis method

- The Temperature Parameters are as follows:
 - 6 minutes at 65°C
 - 2 minutes at 95°C
 - 10 minutes at 25°C
- Program the “Initial Denaturing” step to be 6 minutes at 65°C.
- Program the “Denature” Cycling Stage to be 2 minutes at 95°C. Change the Number of Cycles to be 1 cycle.
- Program the “Anneal” Cycling Stage to be 10 minutes at 25°C. Turn off the “Gather Data” option at this step, as there are no fluorophores included in the lysis step.



- 5.2. Close the lid and click “Start Experiment”.
- 5.3. After approximately 8 minutes when the lysis program is complete, remove the strip tubes from the instrument and briefly spin in mini-centrifuge with strip tube attachment to bring contents of each well to bottom.
- 5.4. If not already, allow the samples to come to room temperature for at least 5 minutes before continuing to qPCR detection assay setup.

Real-Time Quantitative PCR (qPCR) Protocol

1. Remove qPCR Reagents, Assay Probe Mix tube and positive control from the -20°C freezer.
 - 1.1. qPCR Master Mix and Assay Probe Mix tube – Place qPCR Master Mix on ice. Allow remaining tubes to defrost at room temperature. Once defrosted, immediately place tubes on ice.
2. Before preparing the master mixes, invert or vortex and spin-down the reagents.
 - 2.1. Assay Probe Mix tube – Vortex quickly followed by a pulse spin-down in a micro centrifuge.
 - 2.2. Assay Positive Control tube – Vortex quickly followed by a pulse spin-down in a micro centrifuge.
 - 2.3. qPCR Master Mix – Invert the tube 5 times, followed by a pulse spin-down in a micro centrifuge.
 - 2.4. Reaction Buffer - Vortex quickly followed by a pulse spin-down in a micro centrifuge.
 - 2.5. Water - Vortex quickly followed by a pulse spin-down in a micro centrifuge.
 - 2.6. Return all reagents to the ice.

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

3. Make master mix in a 1.5mL tube (the probe mix also contains the internal plant control, SCCG probe mix). 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. Be sure to include three extra reactions for the qPCR positive and negative controls and the leaf lysis negative control. For example, if testing 10 plants, you would need to make enough master mix for 14 or 15 reactions, which would account for 1 or 2 excess.

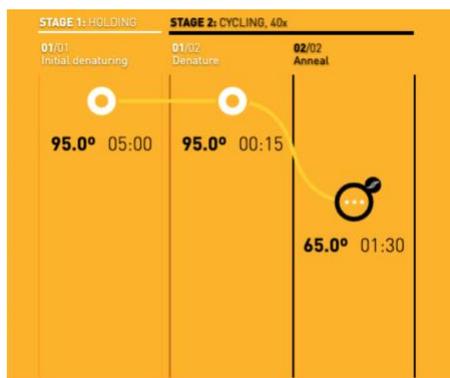
Note: It is best to add the largest volume reagent first, in this case H₂O.

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	8.2	205µL	410µL
Total	13.75µL	343.75µL	687.5µL

- 3.1. Once combined carefully tip mix or invert the tube 5 times to combine the Assay MM tubes
- 3.2. Pulse spin-down in micro centrifuge.
- 3.3. Place MM tube on ice until used in step 5.
4. For the positive control(s), make a 1:10 dilution of assay positive control being run
 - 4.1. 1µL of Positive Control dilute with 9µL of water (found in the kit)
 - 4.2. For the negative control, use water (found in the kit).

Note: It is best to add the largest volume reagent first, in this case H₂O.
5. Open cap of tube containing lysed leaf sample carefully (pay attention not to spill any liquid). Transfer 5µL of each lysed sample into a separate well of the qPCR tubes.

6. Add 5µL of the diluted Positive Control to its corresponding well. Then add 5µL of water to the corresponding Negative Control well
 Note: ALWAYS use a fresh tip for every liquid transfer into the qPCR plate
7. Add 13.75µL of Assay Probe MM to each corresponding sample well, positive control well, and negative control well in the qPCR tubes. Gently tip mix a few times after each addition of qPCR master mix. Be careful to not introduce bubbles during this mix.
8. Seal the tubes with the attached caps, making sure to completely seal the wells.
 - 8.1. Spin-down for at least 1 minute in the plate micro centrifuge.
Note: Check for bubbles at the bottom of the wells (bubbles on the surface of the liquid is acceptable). If bubbles remain in the bottom of the wells, spin-down for another minute.
 - 8.2. Place the sealed tubes onto the Chai Bio Open PCR instrument, positioning the A1 well in the top left corner.
9. Create a New Experiment on the Chai Bio website corresponding with the instrument.
10. Thermal Profile:
 - 10.1. 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



11. Close the lid and click “Start Experiment”.
12. Save the experiment with the [User] and [date]
13. When run is complete, immediately dispose of the tubes. Do not open the tube caps after the run to avoid contamination in the lab.

Detailed Assay Data Analysis

1. Presence / Absence Assay: Plant Pathogens

1.1. Select the Experiment once the Run is Complete

1.2. When the Data Analysis window opens, change the “Color By” option to “Channel” and the “Plot Type” to “Log” at the top of the page. Ensure “Baseline Subtraction” and both channels are selected.

Amplification Curve ▼

COLOR BY		PLOT TYPE	
WELL	CHANNEL	LOG	LINEAR

BASELINE SUBTRACTION

AMPLIFICATION SETTINGS

CHOOSE CHANNEL:

1 2

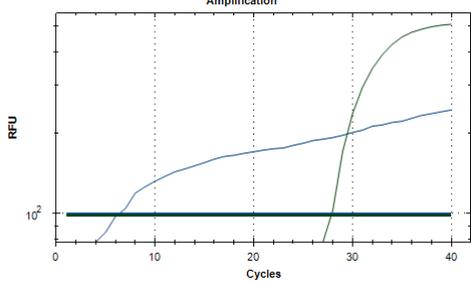
1.3. Highlight the wells of interest in the Well Map under the Graphical Display

- Amplification plots will be available for viewing
- The Cq values will appear to the right in the table

- Controls
 - Plant Pathogen specific Positive Control, on the FAM flourophore, has a Cq value < 35.
 - Visually confirm with the curve on the graph.
 - Plant Pathogen specific Negative Control, on the FAM flourophore, has a Cq value has no Cq value.
 - Visually confirm with the curve on the graph.

- Unknown Samples (Plant Pathogens)
 - Internal Control, on the HEX flourophore, has a Cq value < 35.
 - Visually confirm with the curve on the graph
 - A “presence” result for the unknown sample.
 - Any Cq value for the FAM flourophore < 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, especially when Cq reading is less than 15. See troubleshooting guide below.)
 - An “absence” result for the unknown sample.
 - No Cq value for the FAM flourophore
 - Visually confirm no curve on the graph.

Troubleshooting Guide

Symptom	Reason	Solution
Internal control (SCCG Primer) failure	Lysis Failure	Repeat Leaf_Lysis and qPCR by following the protocol.
	Mix up in Reaction Setup	Repeat 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.
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.
	Carry over	Repeat 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	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 fluorescent signal. This is usually a negative result, but should be repeated if unsure.

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 samples where no Cq is expected. It helps to ensure that all Assay specific reactions are clean of contaminants.

The **Positive Control** is the sample where a Cq is expected. It helps ensure that the qPCR Detection assay and instrument are working correctly.

The **Internal Control** is added to every sample 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.

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