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Introduction

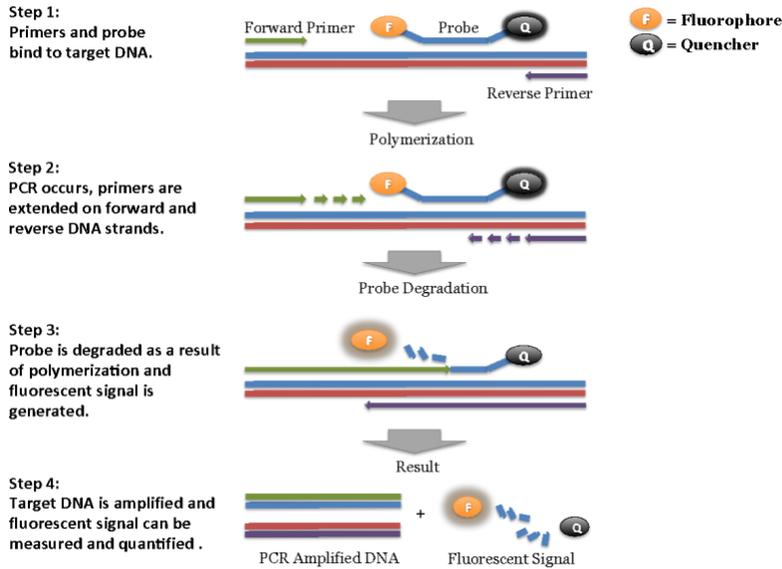
The PathoSEEK® plant pathogen detection kits use a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of plant pathogen for every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting pathogens, resulting 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 or 420201). Each PathSEEK® Plant Pathogen Assay Kit contains 200 reactions worth of reagents. Each PathSEEK® 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
 - qPCR Master Kit v2, Medicinal Genomics #420200) or
 - qPCR Master Kit v3 (Medicinal Genomics #420201)

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

- Agilent AriaMx Real-Time PCR System G8830A Option 010 FAM and HEX (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 401494 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
- Compact PCR Tube Rack (USA Scientific, #2300-9602 or similar)
- 1.5mL tubes (USA Scientific #1415-2600)
- Reservoirs (Fisher Scientific #14-222-398 or similar)
- Crushed ice or cold racks (1.5ul Tube Bench-top Cryogenic Racks, VWR #89004-558 or similar)
- Table top mini plate centrifuge (Fisher Scientific #14-100-143 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
- Toothpicks for punching out leaf punches (can use pipette tips as well)

Reagents

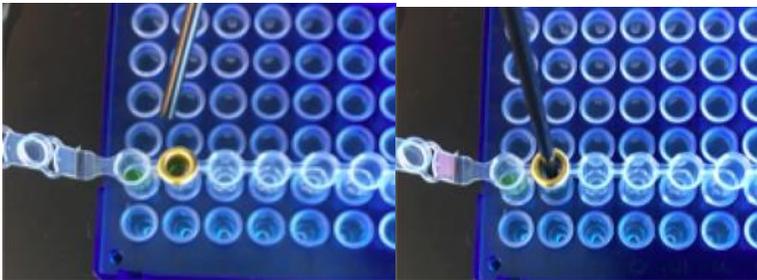
- 10% bleach

Leaf Sampling:

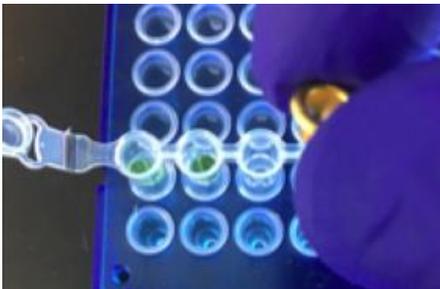
1. Put on a new pair of sterile gloves.
2. Remove necessary number of pre-aliquoted Leaf Punch Lysis Solution strip tubes to PCR tube rack and let thaw.
3. Once thawed quick spin using mini centrifuge with strip tube attachment to bring all contents of solution to the bottom of the tubes.
4. Label the 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 "Leaf_Lysis" program on Agilent AriaMX Instrument.

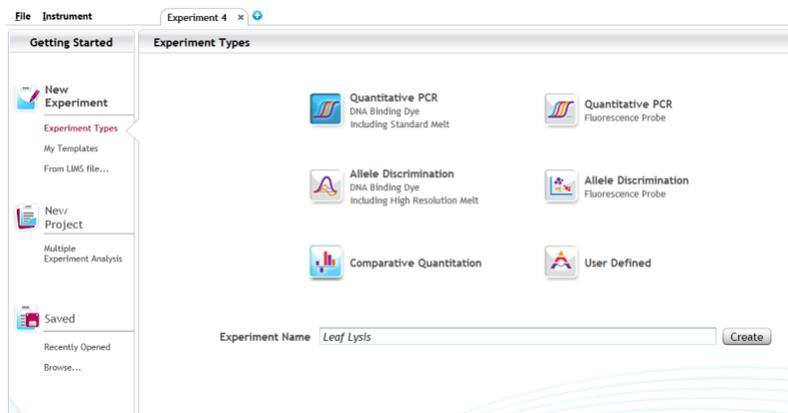
Lysis Reaction Setup:

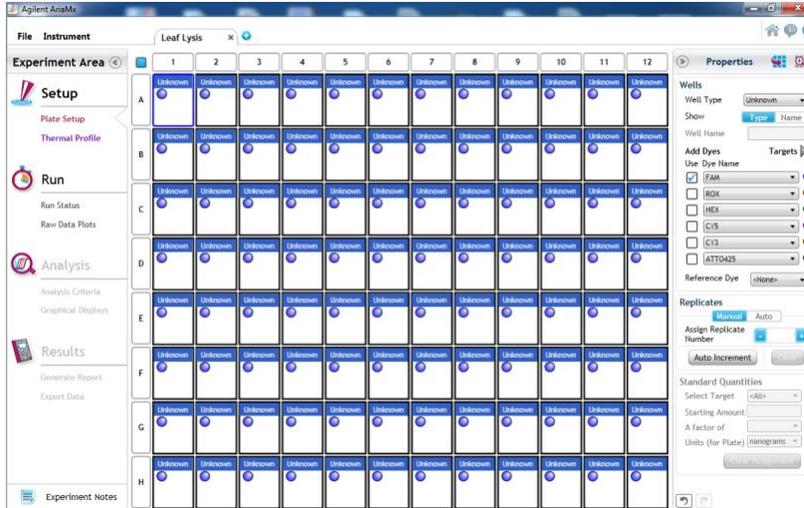
1. Make sure all of the strip tube caps are firmly snapped into place on each tube.
2. Briefly spin all strip tubes using mini-centrifuge with strip tube attachment to ensure that the Lysis Solution and leaf punch are in the bottom of the wells of the strip tubes. In some cases, the leaf punch may float 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 AriaMx.

NOTE: If only loading a few strip tubes, load on far left or right columns as seen below to avoid warping of tubes.



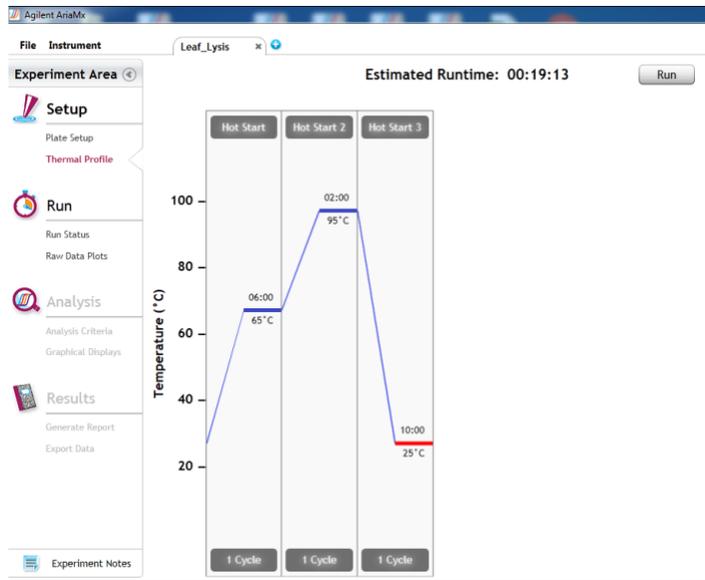
4. Create a new experiment on the Agilent qPCR Instrument called “Leaf_Lysis”
 - 4.1. Select “Quantitative PCR” from Experiment Types. Under Setup>Plate Setup, select FAM channel collection. There are no fluorescence readings in the lysis step however you have to choose one for the instrument to run the program.





4.2. Click on 'Thermal Profile' to create Leaf_Lysis method

- If not already pre-programmed, create a program with the following specifications and save as "Leaf_Lysis"
 - 6 minutes at 65°C
 - 2 minutes at 95°C
 - 10 minutes at 25°C
 - When lysis is complete, place lysed sample at 4C or on ice for 10 minutes to allow to cool before transferring to qPCR reactions.



- 4.3. Close the lid and click "Run".
- 4.4. 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.
- 4.5. 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).

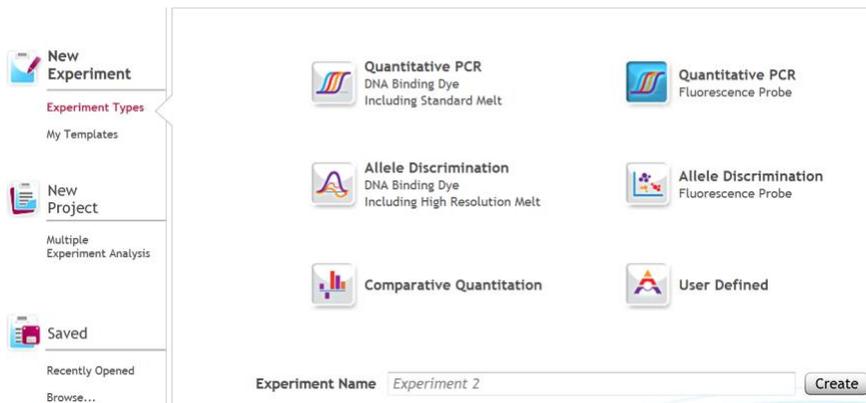
Note: It is best to add the largest volume reagent first, in this case H₂O.
5. Open cap of tube containing lysed leaf sample carefully (pay attention not to spill any liquid). Transfer 5µL of each lysed sample into a separate well of the qPCR plate.
6. Add 5µL of the diluted Positive Control to it's 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 plate. 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 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.
 - 8.1. Spin-down for at least 1 minute in the plate micro centrifuge.

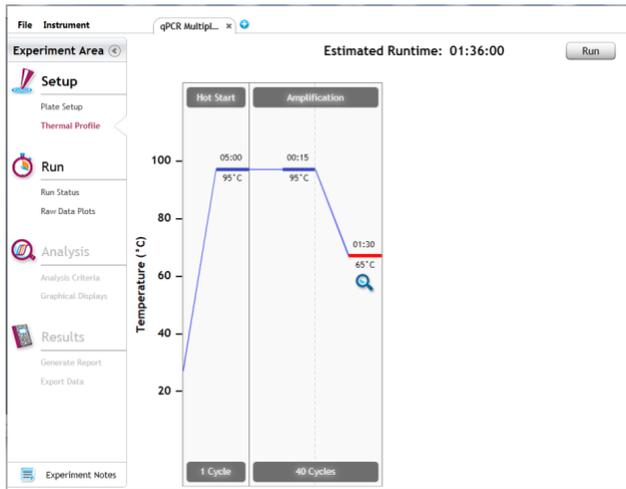
Note: Check for bubbles at the bottom of the wells (bubbles on the surface of the liquid is acceptable). If bubbles remain in the bottom of the wells, spin-down for another minute.
 - 8.2. Label the plate as qPCR Plate_[date].
 - 8.3. Place the sealed plate onto the Bio-Rad qPCR instrument, positioning the A1 well in the top left corner.

9. Create a New Experiment on the Agilent qPCR instrument.
 - 9.1. Select “Quantitative PCR” from Experiment Types. Under Setup>Plate Setup, select FAM and HEX channel collection.



10. Thermal Profiles:

- 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 Run”.
12. Save the experiment with the [User] and [date]
13. When run is complete, immediately dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

Detailed Assay Data Analysis

1. Presence / Absence Assay: Fusarium, Powdery Mildew, Botrytis, Russet Mite

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

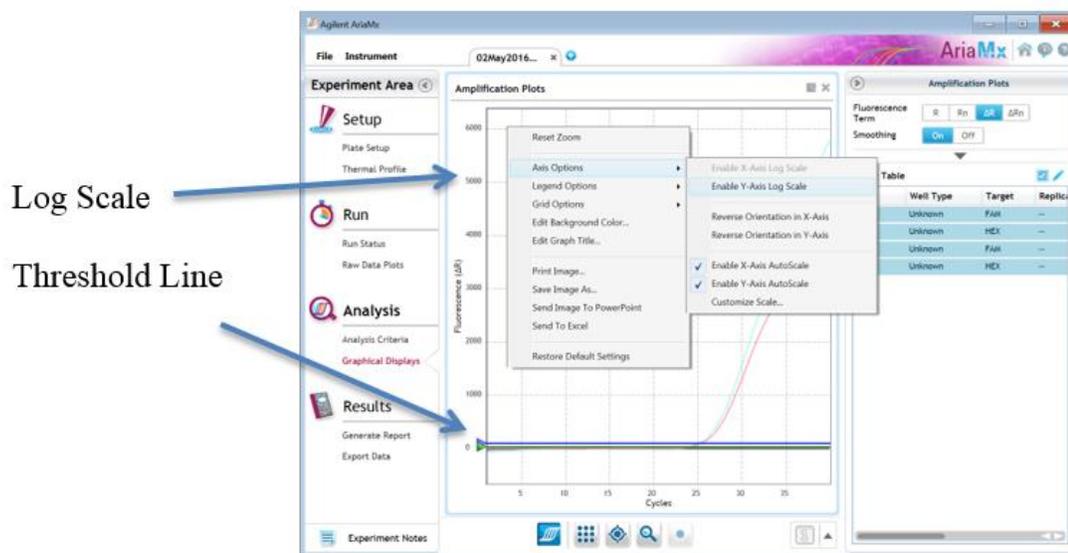
- Amplification plots will be available for viewing
- The Cq values will appear to the right in the table

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 set by typing the threshold to 100 RFU for the FAM fluorophore. Also set by typing the threshold for the HEX fluorophore to 100 RFU

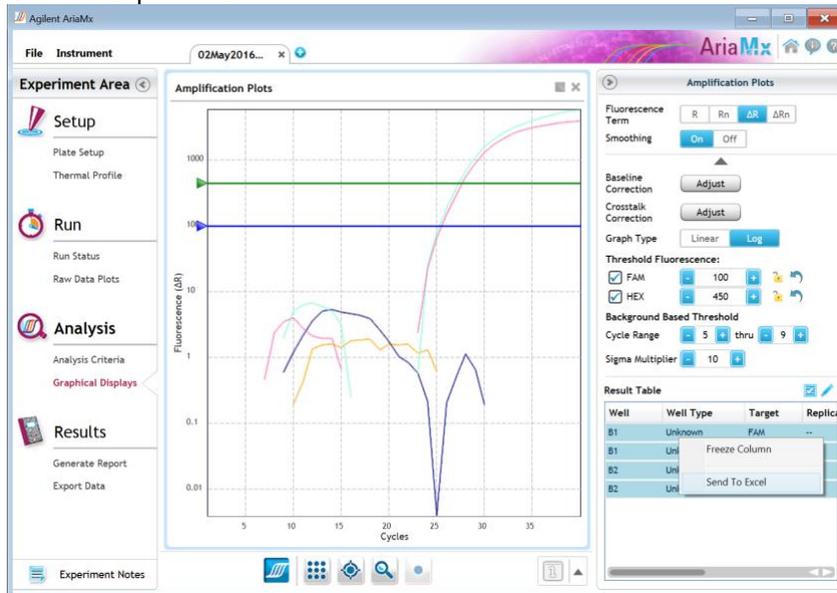


- Controls
 - Plant Pathogen specific Positive Control, on the FAM fluorophore, has a Cq value < 35.
 - Visually confirm with the curve on the graph.
 - Plant Pathogen specific Negative Control, on the FAM fluorophore, has a Cq value has no Cq value.
 - Visually confirm with the curve on the graph.
- Unknown Samples (Plant Pathogen)
 - Internal Control, on the HEX fluorophore, 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 fluorophore < 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 fluorophore
 - Visually confirm no curve on the graph.

2. Export the Data

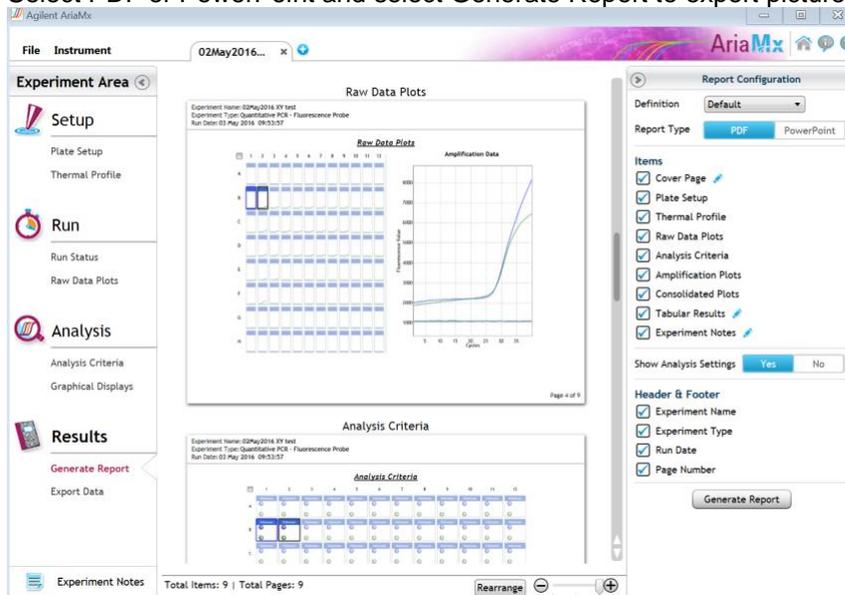
2.1. Exporting the Cq values into an Excel spreadsheet.

- To export the Cq values to an Excel spreadsheet, right- click on the chart on the bottom right of the screen.
- Choose Export to Excel...

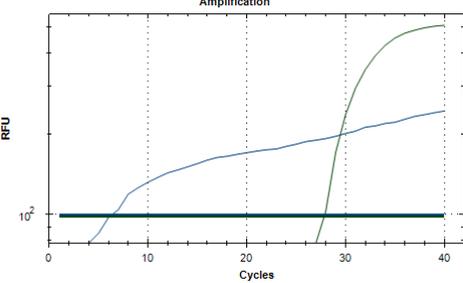


2.2. Saving a visual of the graph

- To save a picture of the graph, navigate to the Results section of the software and select Generate Report. Select PDF or PowerPoint and select Generate Report to export pictures



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 florescent 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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