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

The FemINDICAtor® qPCR Plant Gender Detection Assay uses a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of plant gender for every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting gender, resulting in the minimization of false negatives due to reaction set-up errors or failing experimental conditions.

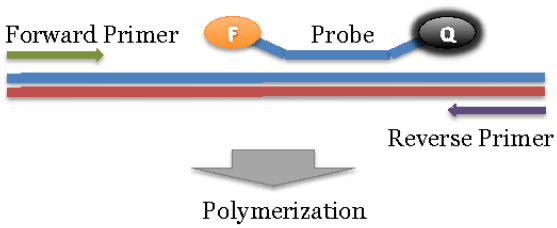
Plant Gender and Plant Pathogen Analysis Quick Table

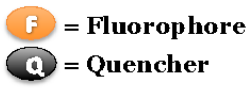
Assay	Cq Value	Fluor	Negative Control (Cq)	Cq threshold
Gender - Male	< 35	FAM	No Value	Presence/Absence
Gender - Female	> 35	FAM	No Value	Presence/Absence
Internal Control*	<35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Controls	<35	FAM		

Process Overview

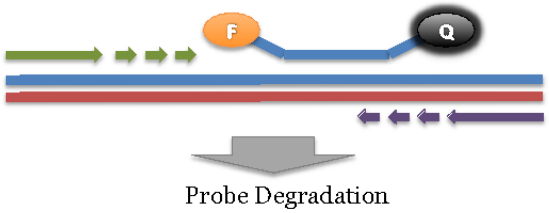
The process for determining gender uses real-time quantitative PCR (qPCR) using a multiplex system of primers to detect both cannabis DNA and the target of interest. Below is a simplified depiction of the qPCR assays.

Step 1:
Primers and probe bind to target DNA.

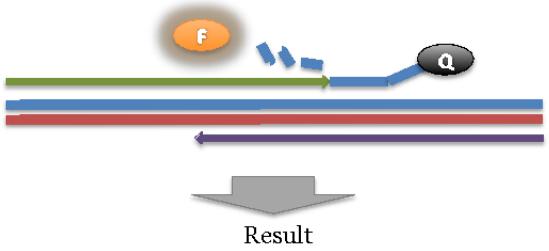




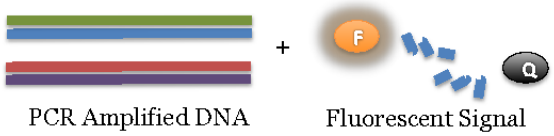
Step 2:
PCR occurs, primers are extended on forward and reverse DNA strands.



Step 3:
Probe is degraded as a result of polymerization and fluorescent signal is generated.



Step 4:
Target DNA is amplified and fluorescent signal can be measured and quantified .



Kit Specifications

The SenSATIVAx® MIP/Extract DNA Purification Kit contains 200 reactions (Medicinal Genomics #420004) worth of reagents.The qPCR Master Kit contains 200 reactions (Medicinal Genomics # 420201). Each FemINDICAtor Plant Gender Kit contains 200 reactions worth of reagents. Each FemINDICAtor® Positive Control contains 60 reactions worth of reagents.

Materials:

Supplied by Medicinal Genomics

- SenSATIVAx MIP/Extract DNA Extraction Kit (Medicinal Genomics #420004)
- qPCR Master Kit v3, store at -15 to -20ºC upon arrival (Medicinal Genomics #420201)
- Plant Gender Detection Assay store at -15ºC to -20ºC upon arrival (Medicinal Genomics #420112)
- Plant Gender Positive Control store at -15ºC to -20ºC upon arrival) (Medicinal Genomics #420312)

Materials Supplied by the User

Hardware

- Bio-Rad CFX96 Touch Real-Time System (Contact Bio-Rad)
- Bio-Rad Personal PC (Contact Bio-Rad)
- Single channel pipette P10, P20, and P200
- Compact PCR Tube Rack (USA Scientific, #2300-9602 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)
- Lab nutating mixer
- Mortar & Pestle
- Refrigerator, 4ºC
- Freezer, -20ºC and -80ºC (-80 is Optional)

Consumables

- 96 well optical qPCR plates (Bio-Rad Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR Plates, Bio-Rad # HSP-9601 or Fisher Scientific 96-Well Armadillo PCR Plate, Fisher # AB2396)
- Adhesive optical seal for qPCR plates (Bio-Rad Microseal® # MSB-1001 or USA Scientific TempPlate® RT Optical Film # 2978-2100)
- Filtered pipette tips for P10, P20, P50, and P200
- 15mL tubes
- 1.5 mL snap cap tubes
- 2.0 mL snap cap tubes
- Laboratory Gloves, (USA Scientific, # 4904-3300 or similar)
- Lab spatula/spoon

Reagents

- Proteinase K (New England Biolabs, P8107S)
- 10% bleach
- Nuclease free H_2O
- Chloroform (Fisher Scientific, C298-1)

Seed Sampling:

Note: If possible, store Mortar, pestle, and seed material at -80ºC overnight, in lieu of -80ºC store at -20ºC.

1. Grind seed material with the mortar and pestle until a consistency of a fine powder.

   NOTE: Medicinal Genomics has successfully extracted 50 or 100 seeds per extraction

   a. Clean mortar and pestle with 10% bleach between sample grinding. Make sure they are completely dry before proceeding.
2. Using a clean spatula, transfer seed powder to a 15 mL conical tube containing 3 mL MIP Solution A and vortex thoroughly
3. Place the lid on the tube and incubate on a lab rotator for 10 minutes. If you do not have a lab rotator, re-vortex tube every few minutes
4. Split the 3 mL Seed/Solution A mixture into two 2 mL tubes, 1.5 mL of seed/solution A mixture in each.
5. Add 7.5µL NEB Proteinase K (20 mg/mL) to each tube and vortex thoroughly
6. Incubate tubes at 60ºC for 30 minutes
7. Spin tubes in benchtop mini centrifuge for 2 minutes
8. Transfer 600µL of sample supernatant to a new tube.
9. Add 600 uL of chloroform to each 600 uL sample and vortex thoroughly
10. Spin tube(s) for 5 minutes at 14,000 rpm
11. Transfer 100µL of supernatant four times from each tube and add each to an individual well of an extraction plate
    a. Each tube will result in 4 wells with 100 uL each
12. Add 100 uL Solution B to each extraction plate well containing 100 uL sample supernatant.
13. Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer for at least 30 seconds.
14. Add 200µL of MGC Binding Buffer to each sample, and pipette tip mix 15 times.

    Note: Be careful to avoid adding too many bubbles by pipetting gently when tip mixing. This is extremely important as to not contaminate the wells in proximity.
15. Incubate the extraction plate on the bench for at least 5 minutes.
16. Place the extraction plate onto the 96-well plate magnet plate for at least 5 minutes.
17. After the 5 min incubation, remove supernatant and discard. Be careful not to disturb or aspirate the beads. See Figure 5.
18. Add 400µL of 70% ethanol (EtOH) to each well, with the extraction plate still on the magnet plate.
19. Wait at least 30 seconds and remove all the EtOH.

    Note: Place the pipet tip at the bottom center of the well to remove all liquid.

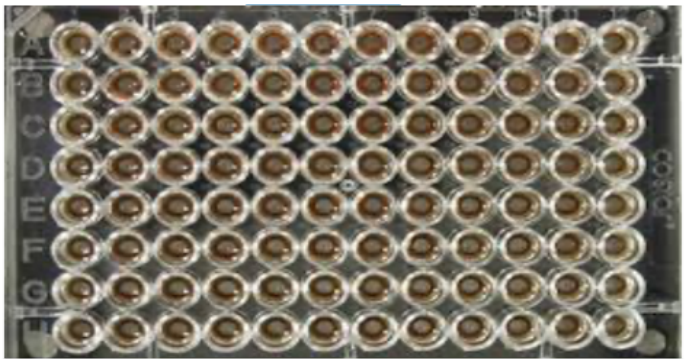


Figure 5: Extraction plate during wash step on magnetic plate.

20. Repeat 400 µL 70% EtOH wash with the extraction plate still on the magnet plate. Wait at least 30 seconds and remove all the EtOH.

    Note: If EtOH still remains in the wells, go back in with a smaller pipet tip to remove the excess. Leftover EtOH can inhibit qPCR efficiency.

21. After all the EtOH has been removed, let the beads dry at room temperature on the magnet plate for 15 minutes.

    Note: It is important to NOT allow the beads to dry for an extended period of time. Over-drying can cause a reduction in DNA yield.

22. Remove the extraction plate from the magnet plate and add 50 µL of MGC Elution Buffer.
23. Tip mix approximately 15 times or until the beads are completely re-suspended.

    Note: The re-suspensions may appear varied in their appearance, but the result will be the same. See Figure 6.

24. Incubate the plate for at least 1 minute on the bench, before returning the plate to the magnetic plate.
25. Let the plate sit on the magnet for at least 1 minute before transferring the eluent to a new clean well in the same plate or new plate

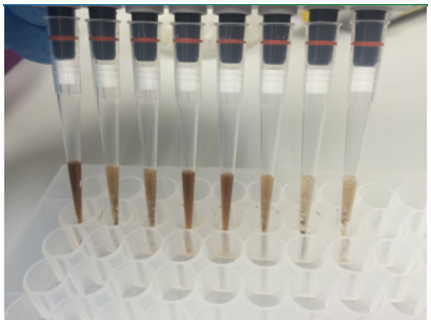


Figure 6: Multichannel pipette tips showing magnetic beads resuspended in elution buffer.

26. Dilute a portion of eluted DNA 1:10 with nuclease free water for qPCR reactions. For example transfer 5 uL of elution into 45 uL nuclease free water. This results in a 1:10 dilution. Mix well.

27. If not performing qPCR right away, 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. If not performing qPCR until another day, store the sealed plate at -20°C until ready to perform the qPCR protocol.

Real-Time Quantitative PCR (qPCR) Protocol

1. Remove qPCR Reagents, Assay Probe Mix tube, and positive control from the -20°C freezer.
   a. Place qPCR Master Mix on ice or leave at -20C until ready to use. Allow remaining tubes to defrost at room temperature. Once defrosted, place tubes on ice.

2. Before preparing the master mixes, invert or vortex and spin-down the reagents according to instructions below:
   a. Assay Probe Mix tube – Vortex quickly followed by a pulse spin-down in a micro centrifuge.
   b. Assay Positive Control tube – Vortex quickly followed by a pulse spin-down in a micro centrifuge.
   c. qPCR Master Mix – Invert the tube 5 times, followed by a pulse spin-down in a micro centrifuge.
   d. Reaction Buffer - Vortex quickly followed by a pulse spin-down in a micro centrifuge.
   e. Water - Vortex quickly followed by a pulse spin-down in a micro centrifuge.
   f. Return all reagents to the ice.

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

3. Prepare Assay master mix in a 1.5mL tube (the probe mix also contains the internal plant control, SCCG probe mix). Always prepare enough Assay 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 two extra reactions for the qPCR positive and negative control. For example, if testing 10 plants for gender, you would need to make enough master mix for 13 or 14 reactions, which would account for 1 or 2 excess.

   Note: *It is best to add the largest volume reagent first, in this case H_2O.*

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 (FemINDICAtor)	1µL	25µL	50µL
Reaction Buffer	0.8µL	20µl	40µl
Water	8.2	205µL	410µL
Total	13.75µL	343.75µL	687.5µL

   a. Once combined carefully tip mix or invert the tube 5 times to combine the master mix reagents.
   b. Pulse spin-down in micro centrifuge.

c. 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
   a. 1µL of Positive Control dilute with 9µL of water (found in the kit)
   b. For the negative control, use water (found in the kit).

   Note: *It is best to add the largest volume reagent first, in this case* H_2O***.***

5. Transfer 5µL of each 1:10 dilution prepared from each sample eluent into a separate well of the qPCR plate.
6. Add 5µL of the diluted Positive Control to its corresponding well. Then add 5µL of water to the corresponding Negative Control well

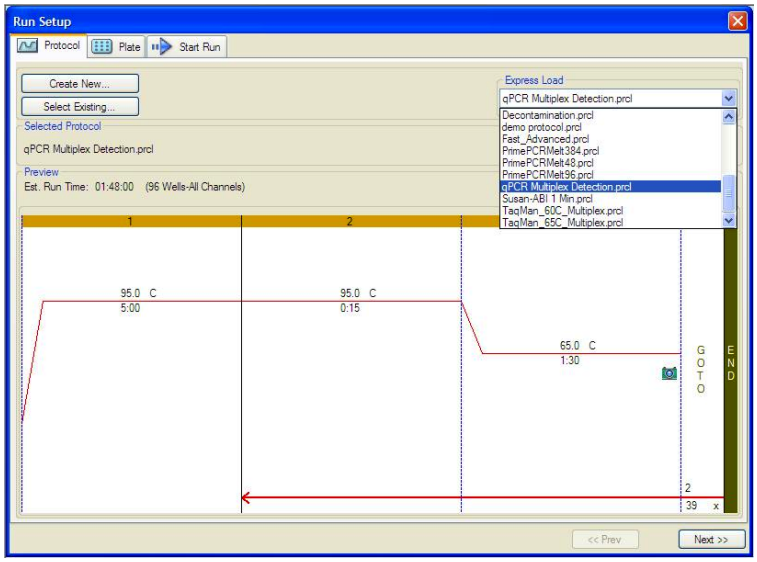
   Note*: ALWAYS use a fresh tip for every liquid transfer into the qPCR plate*

7. Add 13.75µL of Assay Probe MM to each corresponding sample well, positive control well, and negative control well in the qPCR plate. Gently tip mix a few times after each addition of qPCR master mix. Be careful to not introduce bubbles during this mix.

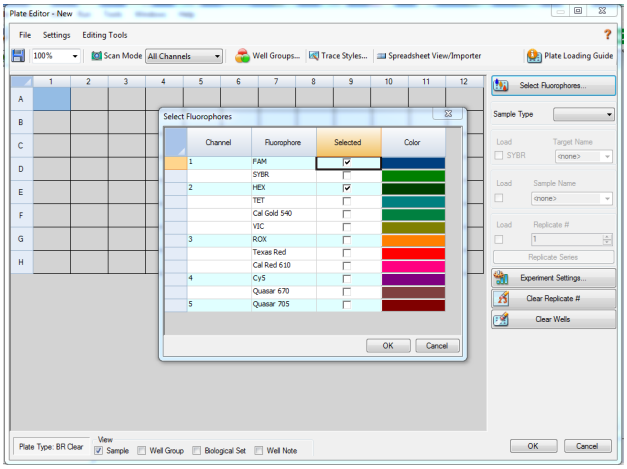
8. Seal the plate with the adhesive seal, making sure to completely seal the plate wells using a pen or flat object to slide back and forth along the seal.
   a. Spin-down for at least 1 minute in the plate micro centrifuge.

   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.*
   b. Label the plate as qPCR Plate_[date].
   c. Place the sealed plate onto the Bio-Rad qPCR instrument, positioning the A1 well in the top left corner.

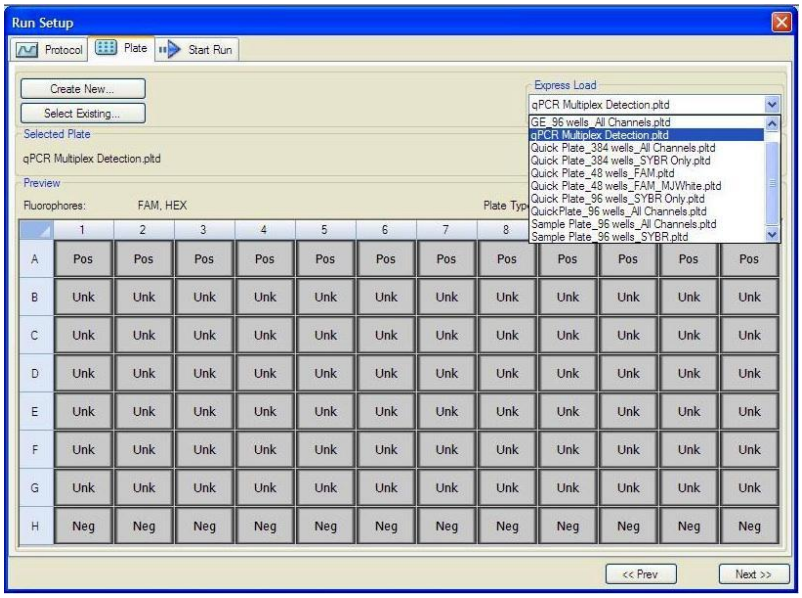
9. **Use the program labeled qPCR Multiplex Detection on the Bio-Rad qPCR instrument.**
   a. Select User-Defined in the Startup Wizard under Run Setup.
      - Use the Express Load drop down menu to pick the qPCR Multiplex Detection Program and click Next.
   - If not already pre-programmed, create 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.



b. Click on “Create New” and the plate editor window will appear. Choose FAM and HEX Fluorophores and click “OK”.



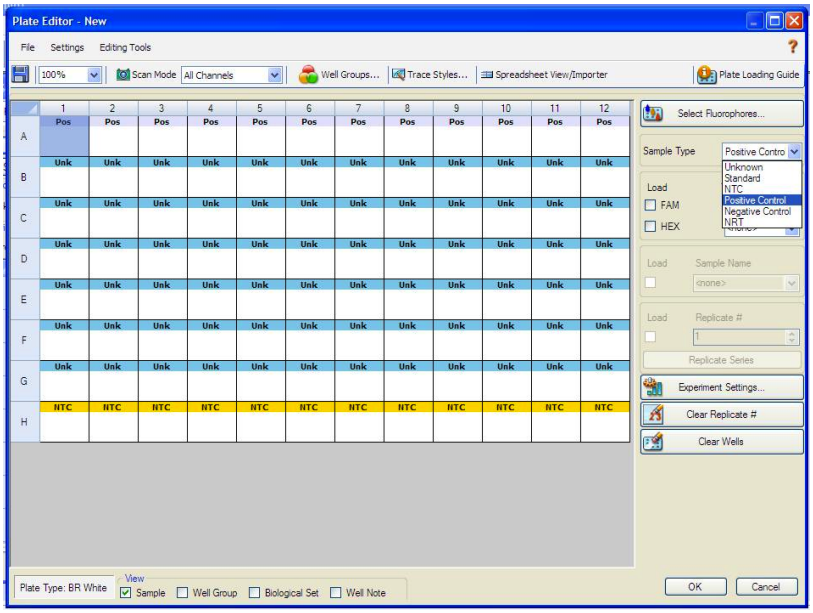
c. If plate layout was previously saved, choose it from the drop-down menu and click “Edit Selected” to move to the Plate Editor Screen.



d. 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

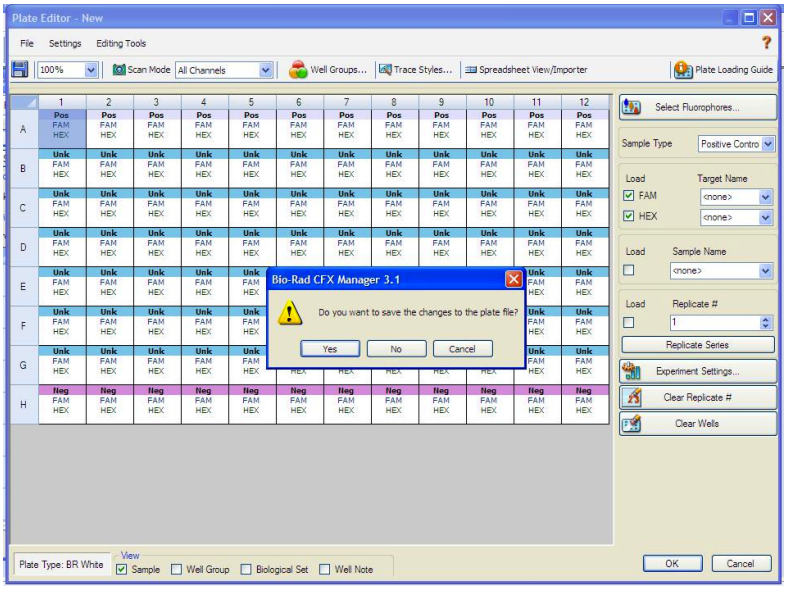
e. Make sure All Channels is selected from the dropdown menu at the top.



f. Attach the fluorophores to the wells being used.
1. Highlight all the wells being used

2. Highlight the well locations and click on FAM and HEX.

g. When the plate is designed correctly click OK.
h. 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).

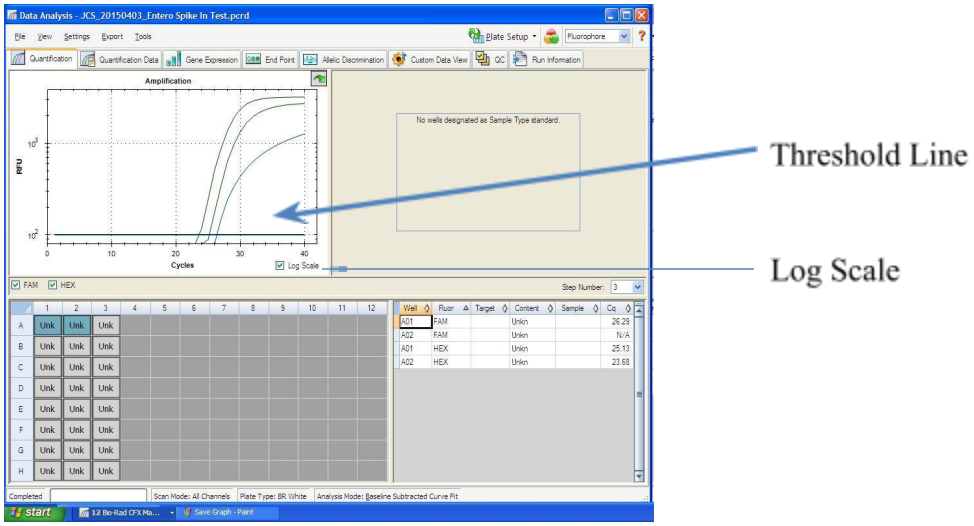
i. Close the lid and click Start Run.
j. Save the experiment with the [User] and [date]
k. When run is complete, immediately dispose of the plate after qPCR. Do not remove the plate seal after the run to avoid contamination in the lab.

Detailed Assay Data Analysis:

Presence / Absence Assay: Gender

a. The Data Analysis window will open automatically when the run is complete.
b. Highlight the well of interest.
- The graph will appear above.
- The Cq values will appear to the right.

c. To analyze the results
- Start by turning the graph to Log Scale and manually moving the thresholds to 10^2 for the FAM and HEX fluorophore.
    - To turn the graph to Log Scale, click on the box at the bottom right of the graph.
    - To move the threshold, click on the horizontal lines, and move them to 10^2 on the y-axis.



- **Controls**

1. Male specific Positive Control, on the FAM fluorophore, has a Cq value < 35.
    - Visually confirm with the curve on the graph.
2. Male specific Negative Control, on the FAM fluorophore, has a Cq value > 35 or no Cq value.
    - Visually confirm with the curve on the graph.

    NOTE: 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.

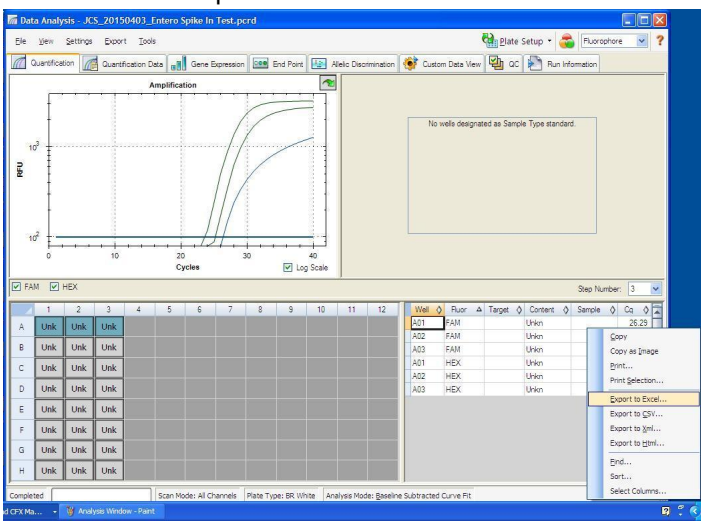
- **Unknown Samples**

1. Internal Control, on the HEX fluorophore, has a Cq value < 35.
    a. Visually confirm with the curve on the graph
2. A “presence or male” result for the unknown sample.
    a. Any Cq value for the FAM fluorophore < 35.
    b. Visually confirm with the curve on the graph.
3. An “absence or female” result for the unknown sample.

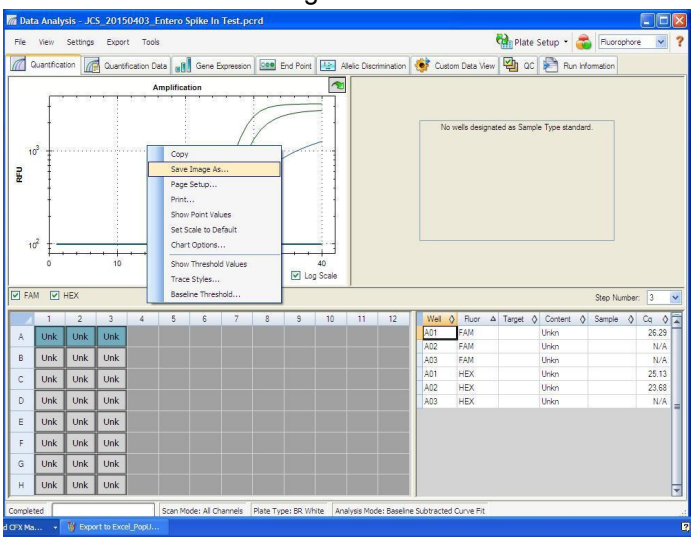
a. No Cq value for the FAM fluorophore or a Cq value >35.
b. Visually confirm no curve on the graph.

2. Export the Data

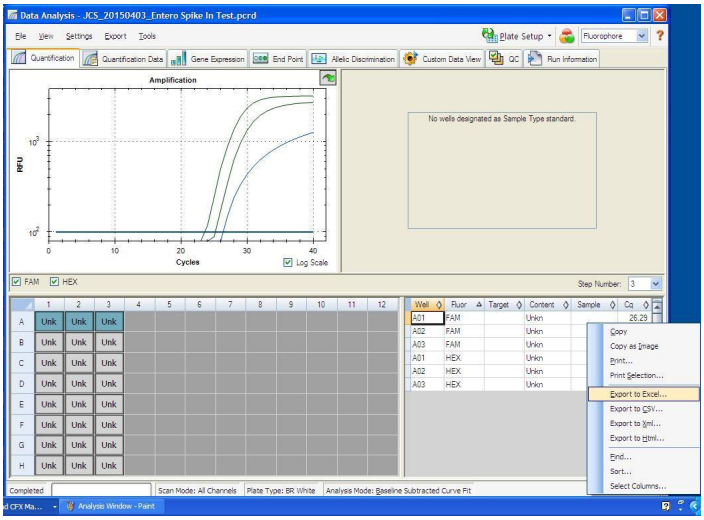
a. Exporting the Cq values into an Excel spreadsheet.
- To export the Cq values to an Excel spreadsheet, right- click on the chart on the bottom right of the screen.
- Choose Export To Excel…



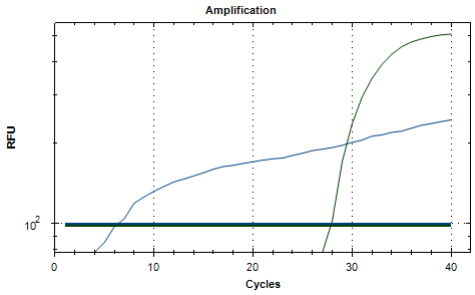
b. Saving a visual of the graph
- To save a picture of the graph, right click on the graph on the top left of the screen.
- Choose Save Image As

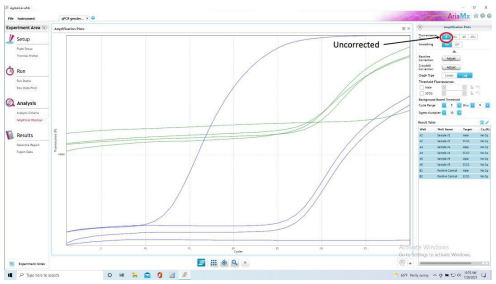


c. Exporting the Cq values into an Excel spreadsheet.
   - To export the Cq values to an Excel spreadsheet, right- click on the chart on the bottom right of the screen.
   - Choose Export To Excel…



Troubleshooting Guide

Symptom	Reason	Solution
Internal control (SCCG Primer) failure	Extraction Failure	Repeat Seed Extraction and qPCR by following the protocol.
	Mix up in Reaction Setup	Repeat qPCR by following the protocol.
	Missing Fluorophore on plate set up	Open the data file and go to the Plate Setup tab found on the left hand column. All wells should have both FAM and HEX. Analysis will 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.
Background Amplification 	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.

	Uncorrected amp plots	Looking at the Fluorescence Term 'R' view instead of 'ΔR' can provide more insight into what should be considered real amplification.

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 contaminates.

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.