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## **FemINDICator<sup>®</sup> qPCR Cannabis Sex Detection Assay**

### **User Guide**

### **Real Time PCR (qPCR) Assay for the detection of Plant Sex**

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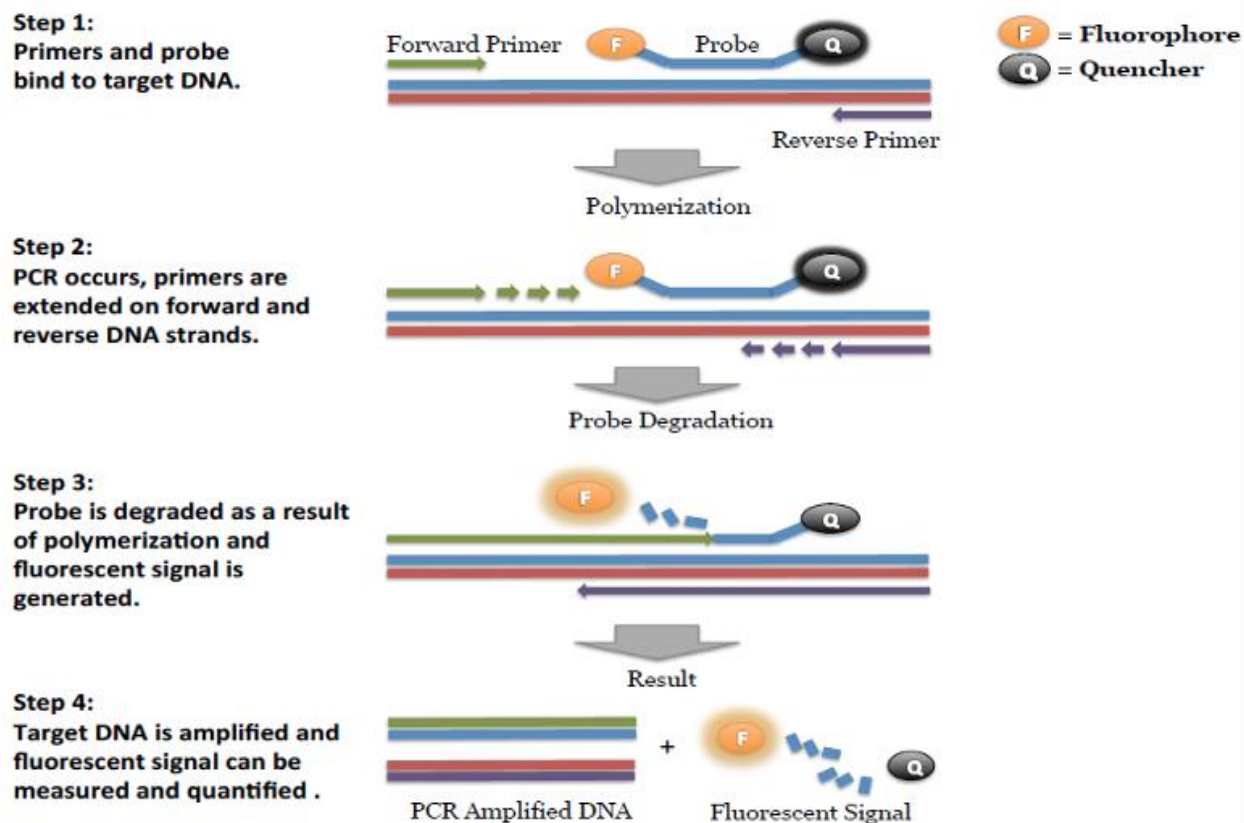
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## Introduction

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

## Process Overview



## Materials and Methods

### Test Kit Information

1. Leaf Punch Lysis Solution - P/N 420208
2. Leaf hole punches (included with Leaf Punch Lysis Solution)
3. FemINDICAtor® qPCR Cannabis Sex Detection Assay v2 - P/N 420154
4. FemINDICAtor® qPCR Cannabis Sex Positive Control - P/N 420312
5. Medicinal Genomics qPCR Master Kit v3 - P/N 420201

### Test Kit Components

1. Leaf Punch Lysis Solution - P/N 420208
  - a. 12 x 0.2 mL PCR 8-tube strips in a 96 well carrier
  - b. Leaf hole punches (included with Leaf Punch Lysis Solution)
2. qPCR Master Kit v3 - P/N 420201.
  - a. Kit (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture
    - i. Reaction Buffer (10x)— 1 tube
    - ii. Nuclease Free Water—2 tubes
    - iii. qPCR Master Mix — 1 tube
3. FemINDICAtor® qPCR Cannabis Sex Detection Assay- P/N 420154 (300 reactions).
  - a. Assay — 1 tube (Store kit at -15 to -20°C). Expires 2 Years from Date of Manufacture.
4. FemINDICAtor® qPCR Cannabis Sex Positive Control - P/N 420312 (50 reactions)
  - a. Control — 1 tube (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture.

### Supplies, Reagents, and Equipment

1. Agilent AriaMx Real-Time PCR System G8830A—Containing the following Optical Channels: FAM and HEX.
  - a. Agilent HP Notebook PC option 650
  - b. 96 Well Optical qPCR plate— Agilent #401490 or Fisher Scientific #AB2396
  - c. Adhesive optical seal for qPCR plates— Agilent #401492; USA Scientific TempPlate RT Optical Film #2978-2100

- d. Optical Strip Caps—Agilent #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.**

- 2. Bio-Rad CFX96 Touch™ Real-Time System. Bio-Rad Personal PC
  - a. 96 Well Optical qPCR plate— Bio-Rad #HSP-96601 or Fisher Scientific #AB2396
  - b. Adhesive optical seal for qPCR plates— Bio-Rad #MSB-1001; USA Scientific TempPlate RT Optical Film #2978-2100
- 3. Adjustable, variable volume pipettes (single or multichannel) —P10, P20, P50, P200 or P300 and P1000
- 4. Adjustable, variable volume filter pipettes tips—For P10, P20, P50, P200, P300 and P1000
- 5. Crushed ice
- 6. 96 Well PCR Cryogenic Rack (optional)
- 7. 1.5 mL Tube Benchtop Cryogenic Rack (if no ice)
- 8. Freezer—Capable of maintaining -20°C
- 9. Table Top Mini Plate Centrifuge
- 10. Table Top Mini Centrifuge
- 11. Vortex-Genie Pulse
- 12. 1.5 mL Eppendorf Tubes
- 13. Eppendorf Tube Rack
- 14. Refrigerator—Capable of maintaining 2–8°C
- 15. 10% bleach

## **Safety Precautions and Recommendations for Best Results**

### **Safety Precautions**

The FemINDICator Sex Detection Assay is a qPCR detection assay for the rapid detection of male plant DNA in cannabis matrices.

1. Assay users should observe standard lab practices and safety precautions when performing this assay. Wear protective gloves, lab coats, eye/face protection as indicated by your quality system.
2. It is the responsibility of each laboratory to handle waste and effluents processed according to their nature and degree of hazardousness. Waste and effluents processed must be treated and disposed of in accordance with all applicable local, state, and federal regulations.

### **Environment**

1. The quality of results depends on the strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR.
  - a. Never circulate lab equipment from one workstation to another.
  - b. Always use a positive and negative control for each series of amplification reactions.
  - c. Periodically verify the accuracy and precision of pipette, as well as correct functioning of the instruments.
  - d. Change gloves often, especially if you suspect contamination.
  - e. Clean workspaces periodically with 10% bleach and other decontaminating agents.
  - f. Use powder-free gloves.
  - g. Avoid fingerprints and writing on qPCR reaction strip tube caps because both can interfere with data acquisition.

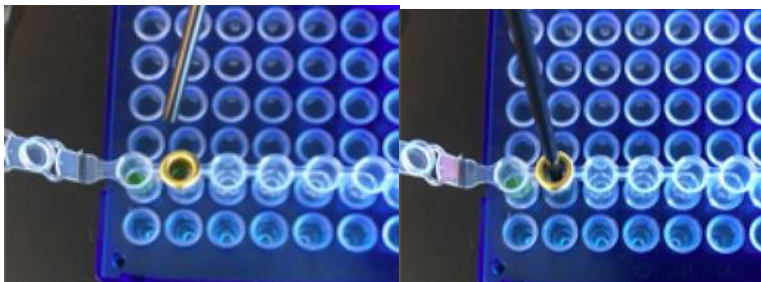
### Leaf Sampling and Lysis

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 a 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.

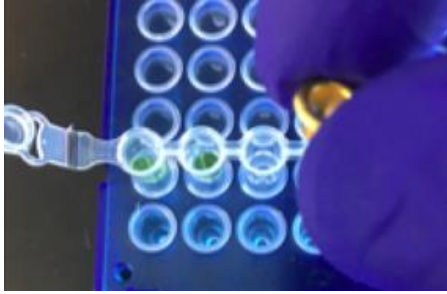
*NOTE: Only a single punch per plant is necessary for sex testing. Multiple punches may result in overloading of the qPCR assay.*



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 disposable 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 the qPCR Instrument.

### **Lysis Reaction Setup**

AriaMX:

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.

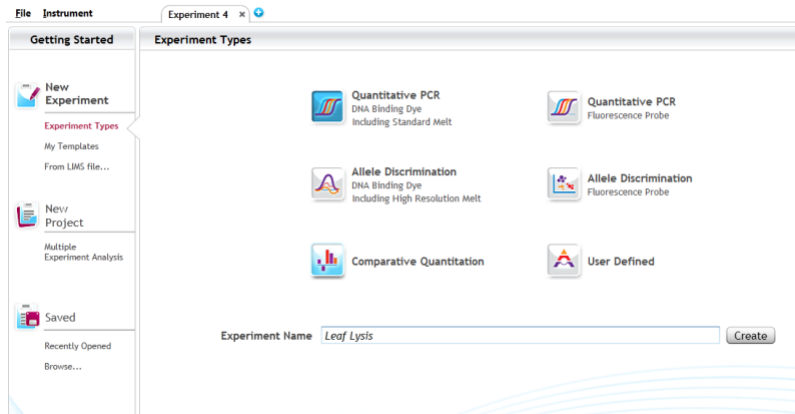
NOTE: If only loading a few strip tubes, load on far left or right columns as seen below to avoid warping of tubes.



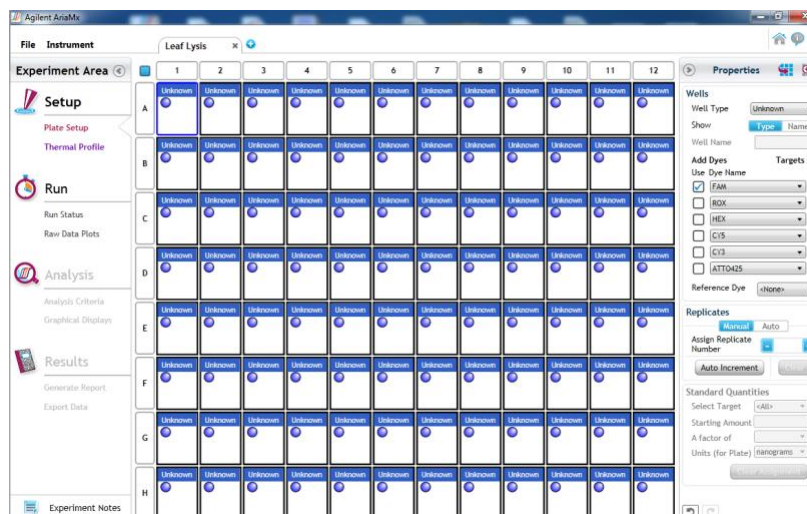
4. Create a new experiment on the Agilent qPCR Instrument called “Leaf\_Lysis”



- a. Select “Quantitative PCR” from Experiment Types.



- b. 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.



- c. 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.

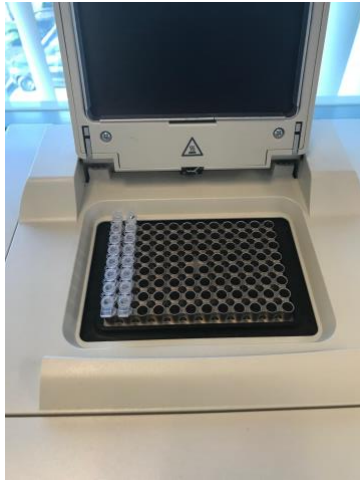


- d. Close the lid and click “Run”.
- e. After approximately 18 minutes when the lysis program is complete, remove the strip tubes from the instrument and briefly spin in mini-centrifuge with strip tube attachment to bring contents of each well to the bottom.
- f. If not already, allow the samples to come to room temperature for at least 5 minutes before continuing to qPCR detection assay setup.

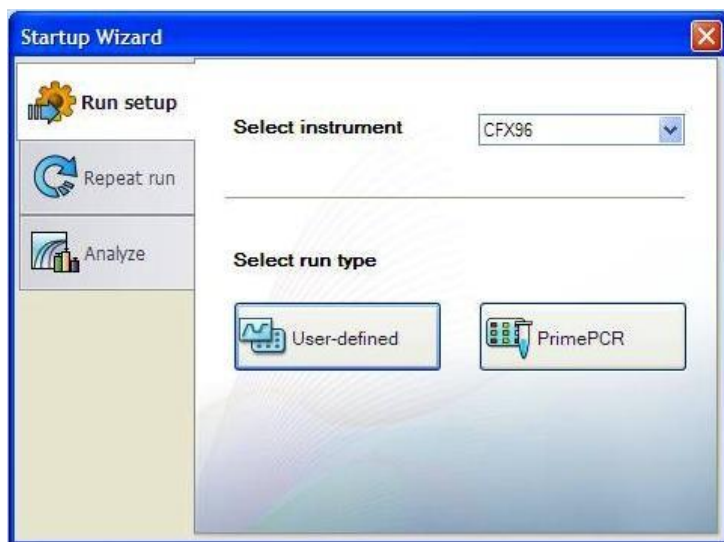
## Bio-Rad CFX96

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 Leaf Punch Lysis Solution and leaf punch are in contact with each other to ensure proper lysis. 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 Bio-Rad CFX 96.

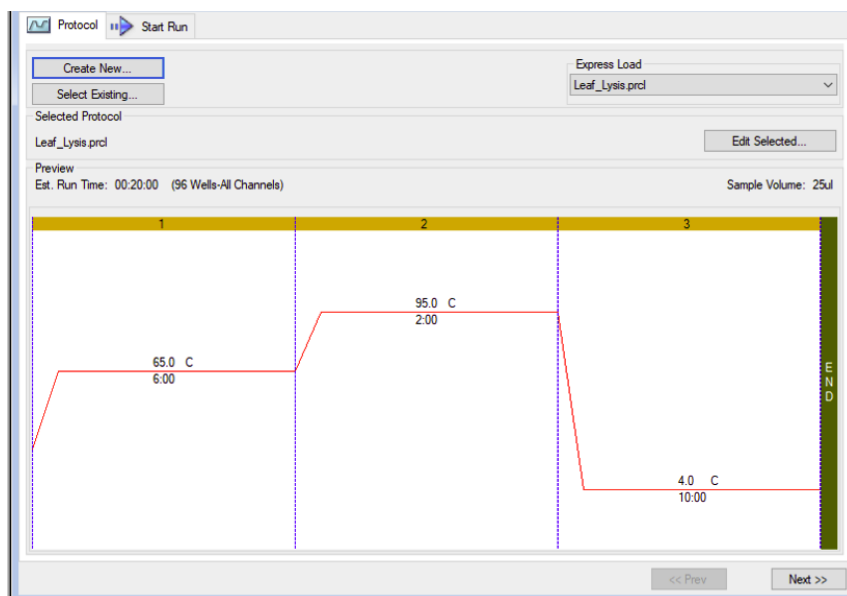
NOTE: If only loading a few strip tubes, load on far left or right columns as seen below to avoid warping of tubes.



4. Start the Leaf Lysis program
  - a. Select User-Defined in the Startup Wizard under Run setup

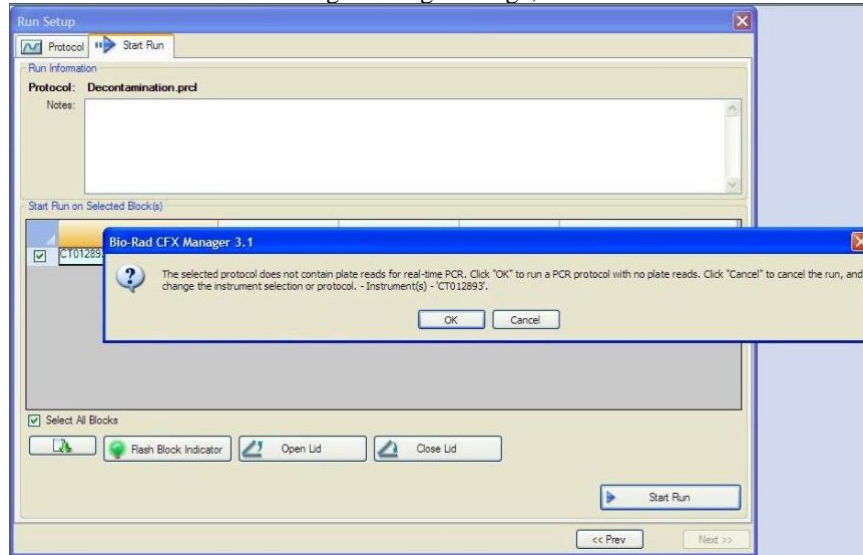


- b. Use the Express Load dropdown menu to pick the Leaf\_Lysis program and click “Next”.
- i. If not already pre-programmed, create a program with the following specifications and save as “Leaf\_Lysis”
- 6 minutes at 65°C
  - 2 minutes at 95°C
  - 10 minutes at 4°C
- \*Set Heated lid to 100°C



- c. Click Next, Close the lid and click “Start Run.”

- You will see the following warning message, Click OK.



- d. After approximately 18 minutes when the lysis program is complete, remove the strip tubes from the instrument and briefly spin in a mini centrifuge with strip tube attachment to bring contents of each well to the bottom.
- e. 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) Setup**

1. 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 or leave at -20°C until ready to use. Allow remaining tubes to thaw at room temperature. Once thawed, immediately place tubes on ice.
2. Before preparing the reaction, invert or vortex and spin-down the reagents.
  - a. Assay probe mix tubes, reaction buffer, positive controls and water – Vortex quickly followed by a pulse spin-down in a microcentrifuge.
  - b. qPCR Master Mix – Invert the tube 5 times (do not vortex), followed by a pulse spin-down in a microcentrifuge.

- c. 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 Assay Primer/Probe mix contains the internal plant control, SCCG probe mix, and the probe targeting male DNA. Label tube as 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. Add qPCR Master mix last.*

4. qPCR Reagent Volumes

Reagents	1 Reaction	24 Reactions (Plus 1 excess rxn)	48 Reactions (Plus 2 excess rxn)
qPCR Master Mix	2.5 µL	62.5 µL	125 µL
Assay Probe Mix	0.65 µL	16.25 µL	32.5 µL
Reaction Buffer	0.52 µL	13 µL	26 µL
Water	5.33 µL	133.25 µL	266.5 µL
Total Assay Probe MM	9 µL	225 µL	450µL

5. Once combined gently, tip mix or invert the tube 5 times to combine the master mix.
- Pulse spin-down tube in microcentrifuge.
  - Place qPCR Master Mix tubes on ice until used.
6. For the positive control, make a 1:10 dilution of stock
- Vortex Positive Control stock tube prior use.
  - Add 1 µL of Positive Control to 9 µL nuclease free water (found in the kit) and vortex to mix.
  - 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 or vortex control dilution to ensure control DNA is in solution.*

7. Use a 96-well optical qPCR plate and label the plate “qPCR Plate\_ [date]”.
8. Transfer 5  $\mu$ L of each sample into the corresponding well on the qPCR plate, keeping the extraction plate on the magnet when aspirating the 5  $\mu$ L.
9. Add 5  $\mu$ L of the diluted Positive Control to the corresponding well.
10. Add 5  $\mu$ L of water to the corresponding negative control well.
11. Add 9  $\mu$ L of FemINDICATOR 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 not to introduce bubbles during this mix.
12. Seal the plate with strip caps or an adhesive seal.
13. Spin-down for at least 1 minute in plate microcentrifuge to bring well contents to the bottom of wells and help to rid of reaction 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.*
14. For the Agilent Aria: 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 holes in the plate.
15. Place the sealed plate onto the PCR instrument, positioning the A1 well in the top left corner.
16. Follow the software specific instructions to initiate the run.

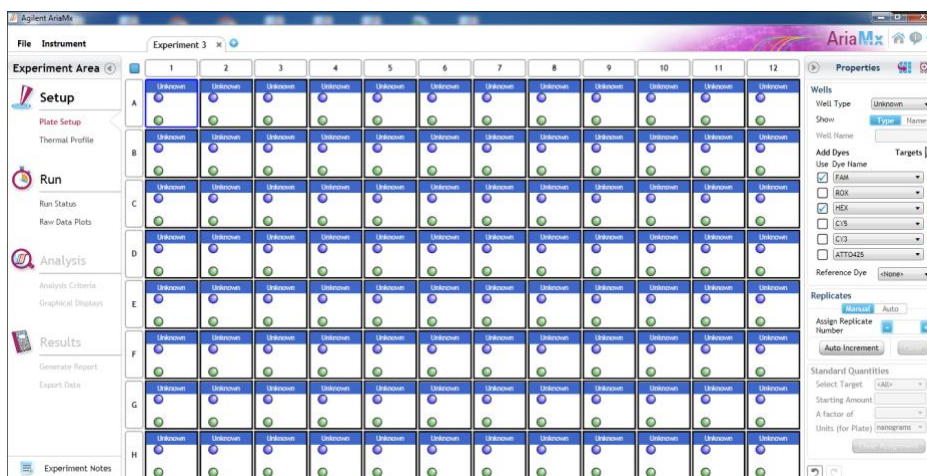
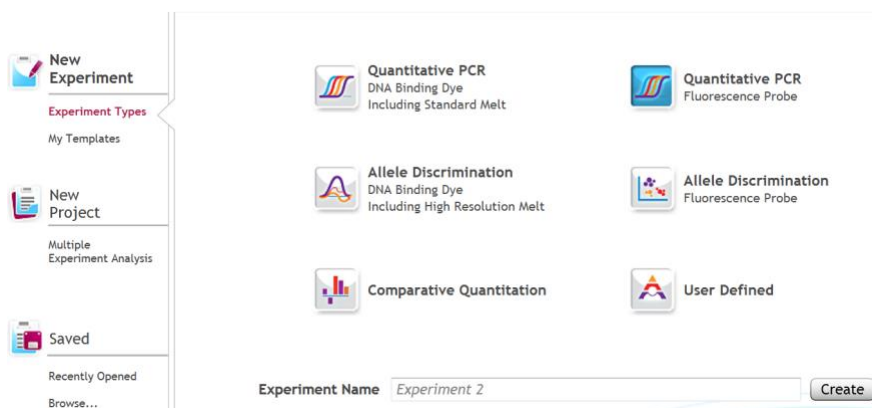
## Aria MX

### Setting up the run:

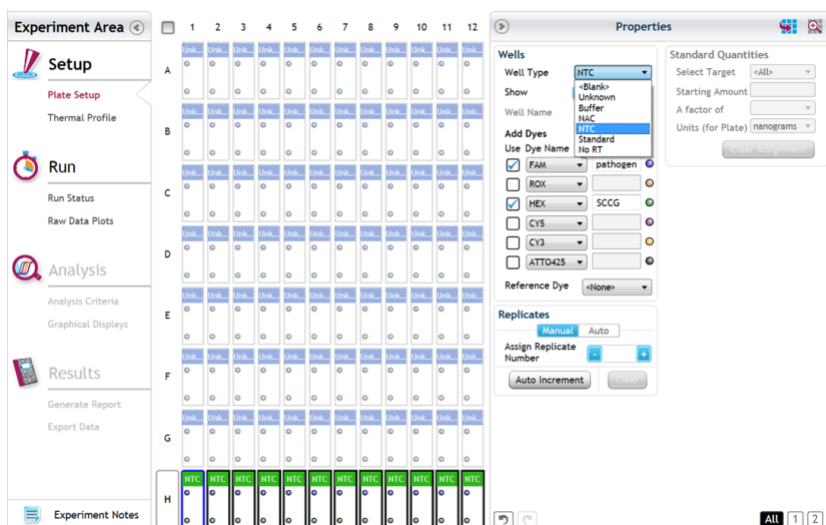
The following species will be detected on the following Fluorophores:

- Y Chromosome: FAM
- Cannabis DNA: HEX

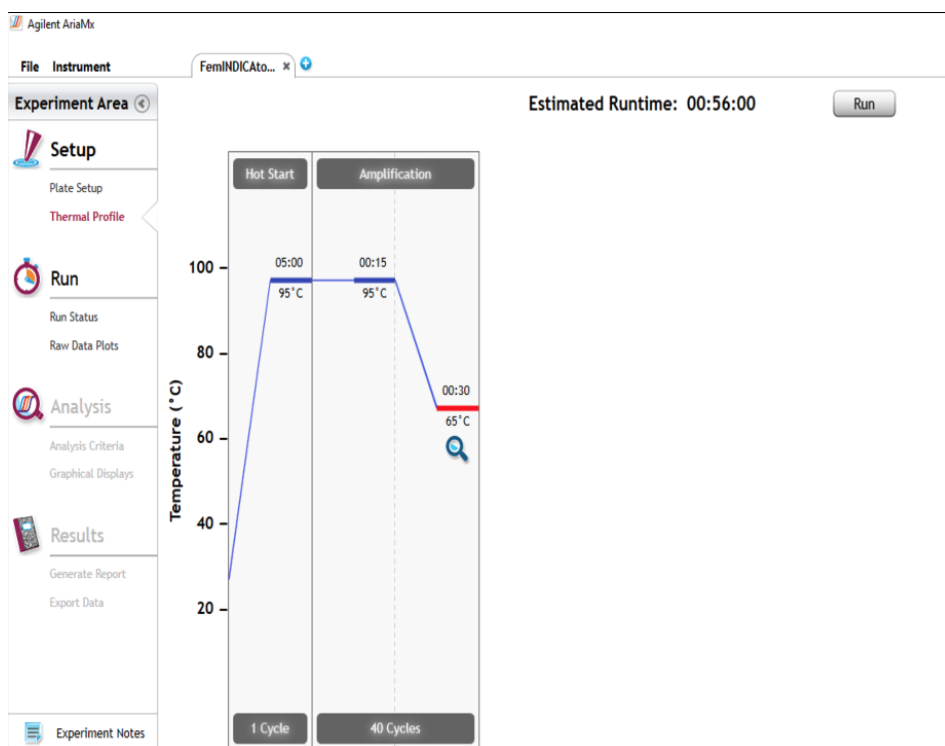
1. Create a New Experiment on the Agilent qPCR instrument.
2. Select “Quantitative PCR” from Experiment Types. Under Setup>Plate Setup, highlight wells that contain reactions and select FAM and HEX under Add Dyes.







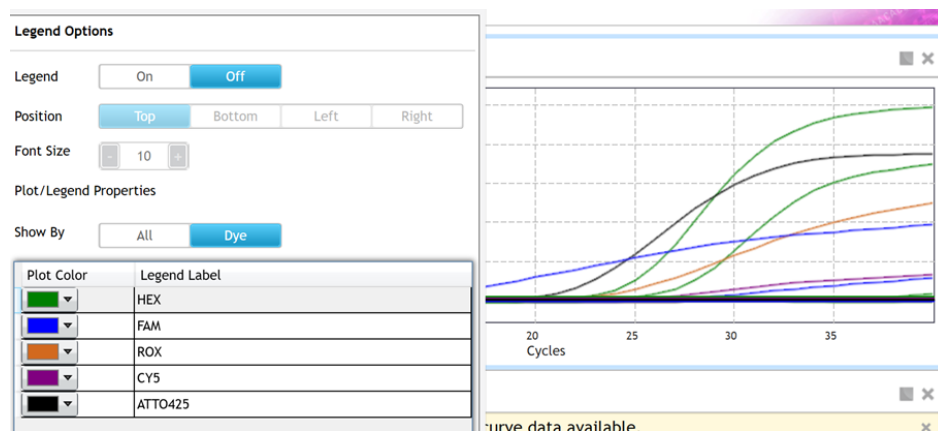
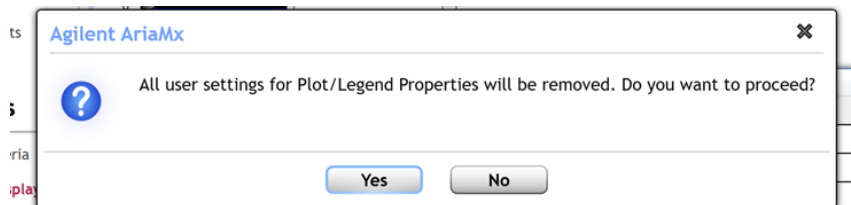
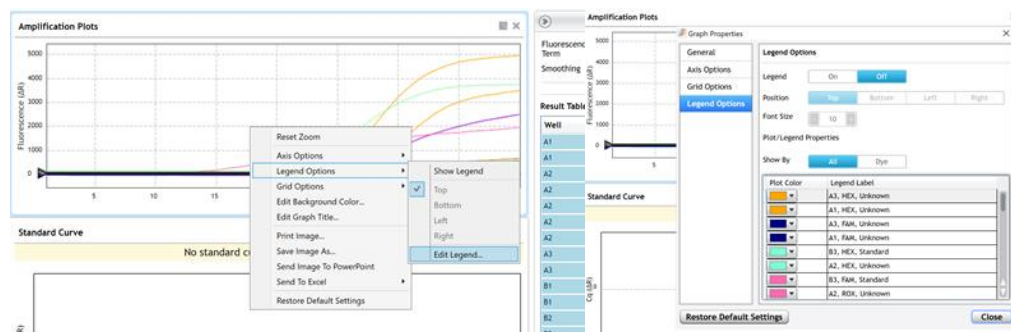
3. Change the well types to reflect your plate set up. If desired, add Target names to include “Male” for FAM and IC (Internal Control) or SCCG (single copy control gene) for HEX.
4. Under Setup>Thermal Profile, create the following PCR thermal profile:
  - a. Hot Start at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 65°C for 30 seconds.



5. Close the hot top and main lids and click “Run”.
6. Save the experiment with the [User] and [date]
7. When the run is complete, dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

### **Data Analysis:**

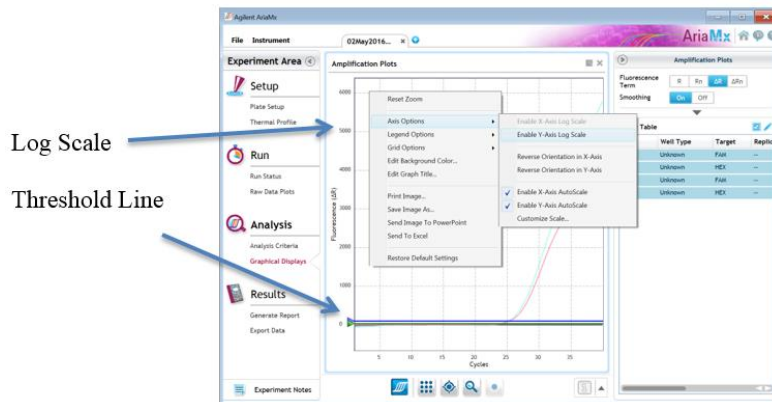
1. Open the Data Analysis window when the run is complete.
2. Highlight the wells of interest in the Analysis Criteria under Analysis, then select Graphical Display
  - a. Amplification plots will be available for viewing
  - b. The Cq values will appear to the right in the table
  - c. Right click inside the graph, select Edit Legend under Legend Options
  - d. Change “All” to “Dye”
  - e. All user settings for Plot/Legend Properties will be removed. Do you want to proceed? Select “Yes”.
  - f. This will assign a single color to each fluorophore.



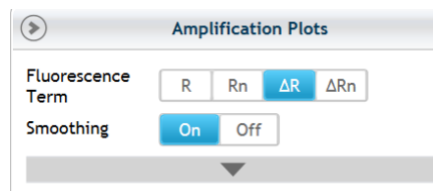
3. To analyze the results:

a. Start by turning the graph to Log Scale by right clicking on the chart and selecting Axis options.

Enable y-axis log scale.



- b. Expand the amplification plots settings by clicking on the triangle (shown below).



- c. Manually adjust thresholds to 100 RFU for the FAM and HEX fluorophores.
4. Controls
    - a. Positive Control, on the FAM Fluorophore, has a Cq value  $\leq 35$ .
      - i. Visually confirm with the curve on the graph.
    - b. Negative Control, on the FAM Fluorophore, has no Cq value.
      - i. Visually confirm with the curve on the graph.
    - c. Internal Control, on the HEX Fluorophore, has a Cq value  $\leq 35$ 
      - i. Visually confirm with the curve on the graph.
  5. Unknown Sample Targets
    - a. A Male result for the unknown Sample.
      - i. Male Sample Result: Check Cq Value on the FAM Fluorophore.

- ii. Visually confirm with the curve on the graph. It is very important to confirm with the amplification curve when a male result occurs. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15 (See troubleshooting guide below for more details).
- b. A female result for the unknown sample.
  - i. Female Sample Result: Check Cq Value on the FAM Fluorophore
  - ii. Visually confirm with the curve on the graph.

## Bio-Rad CFX 96

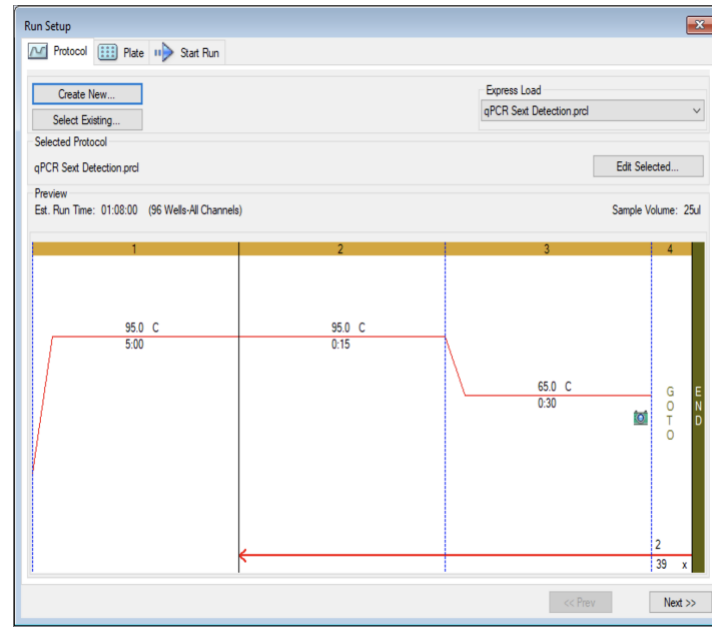
### Setting Up the Run

- Y Chromosome: FAM
- Cannabis DNA: HEX

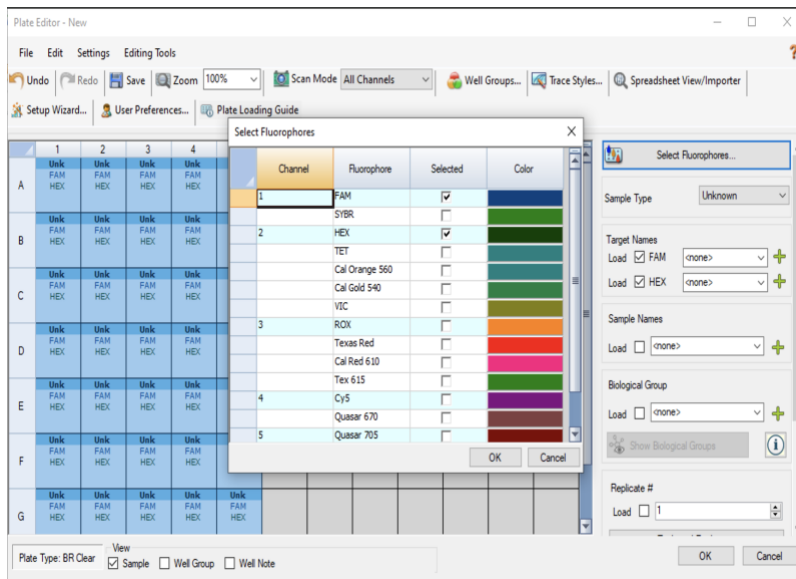
1. Start the qPCR Cycling program.

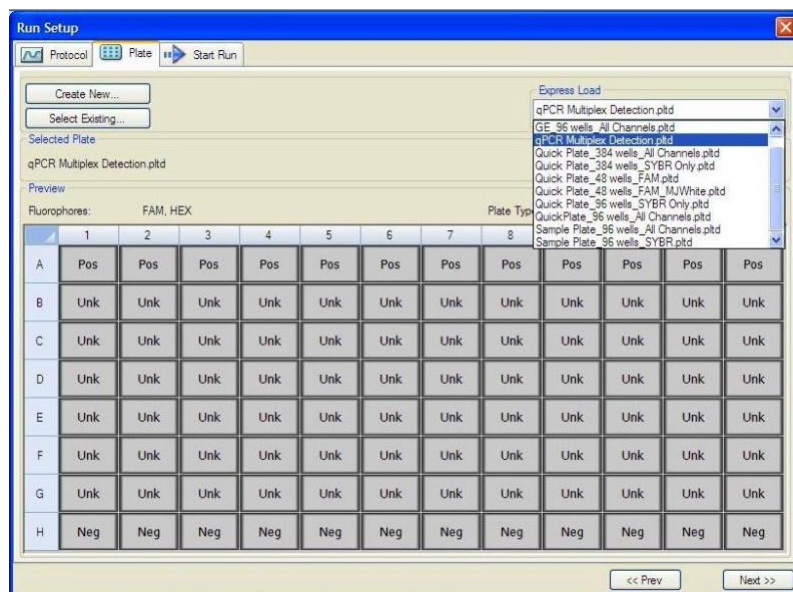


2. Select User-Defined in the Startup Wizard under Run setup.
3. Use the Express Load dropdown menu to pick the qPCR Sex Detection Program and click “Next”.
4. If not already pre-programmed, create a cycling program with the following specifications and save as “qPCR Sex Detection”:
  - a. Hot Start at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 65°C for 30 seconds.



5. Design your plate under the plate tab in the Run Setup.
  - a. Select the qPCR Sex Detection from the dropdown menu. If not already present, click “Create New”
  - b. Plate editor window will appear. Choose FAM and HEX fluorophores and click “OK”.





- c. If plate layout previously saved, 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:*

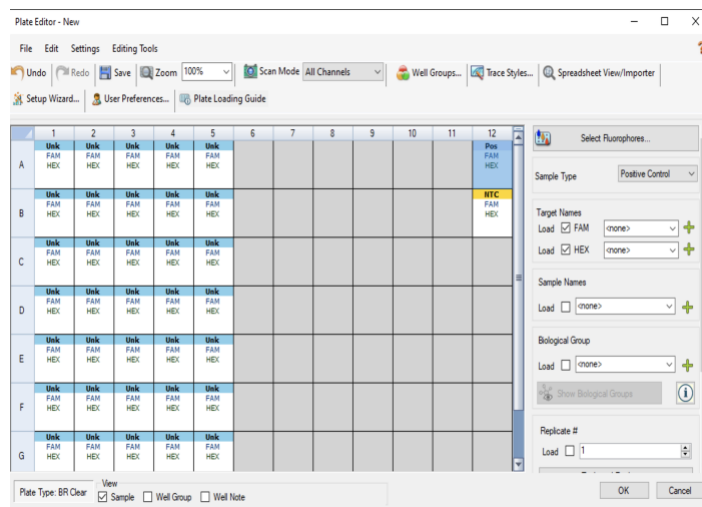
*Unknown*

*Positive Control*

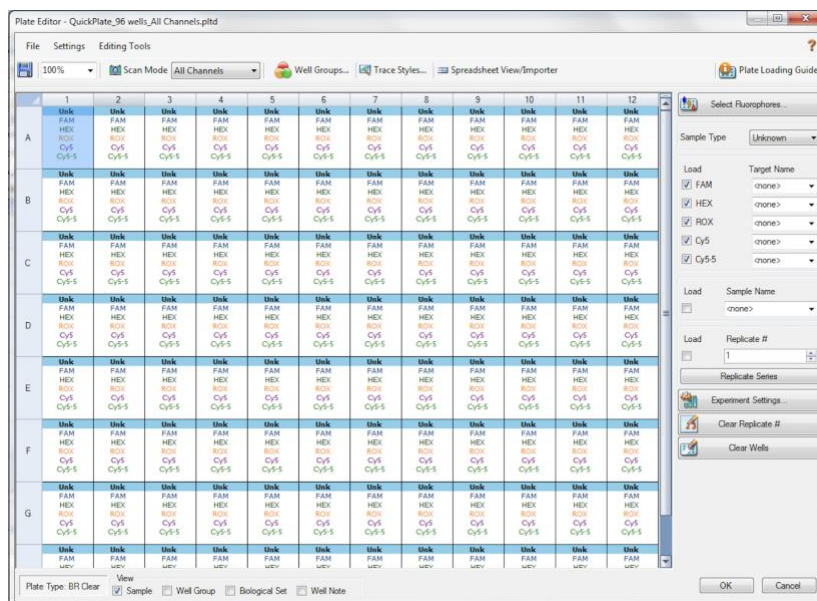
*Negative Control*

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





- f. Attach the fluorophores to the wells being used.
  - i. Highlight all the wells being used.
  - ii. For the Sex Detection Assay 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 Sex Detection”. If you do not save the plate, it will return to the default plate.

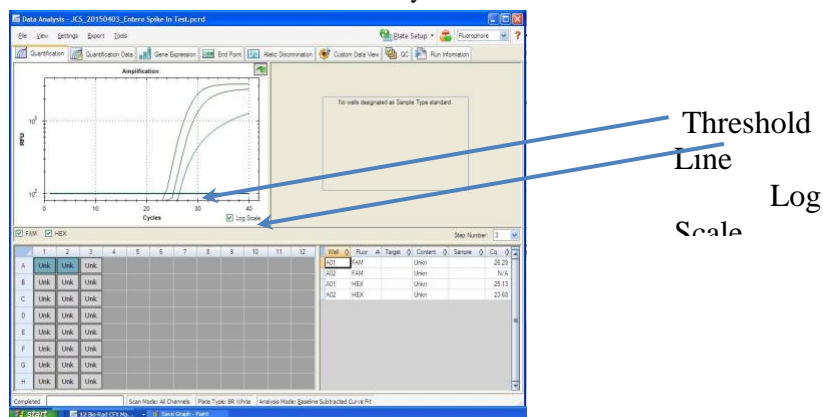


*Note: Saving will override the template but will not cause any issues.*

- i. Close the lid and click Start Run.
- j. Save the experiment with the [User] and [date].
- k. When the 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.

### **Data Analysis**

1. The Data Analysis window will open automatically when the run is complete.
2. Highlight the well(s) of interest.
3. The graph will appear above.
4. The C<sub>q</sub> values will appear to the right.
5. To analyze the results:
  - a. Start by turning the graph to Log Scale and manually moving the threshold to 10<sup>2</sup> for all fluorophores.
    - i. To turn the graph to Log Scale, click on the box at the bottom right of the graph.
    - ii. To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis.



6. Controls

- a. **Assay-specific Positive Control**, on the FAM fluorophore, has a Cq value  $\leq 35$ .
- b. Visually confirm with the curve on the graph.
- c. **Assay-specific Negative Control**, on the FAM fluorophore, has no Cq value.
  - i. Visually confirm with the curve on the graph.
- d. **Internal Control**, on the HEX Fluorophore, has a Cq value  $\leq 35$ 
  - i. Visually confirm with the curve on the graph.
- e. A **Presence** result for the unknown samples.
  - i. **Male Sample Result:** Check Cq Value on the FAM Fluorophore. See Table I for Cq cutoff value. Visually confirm with the curve on the graph. It is very important to confirm with the amplification curve when a male result occurs. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15 (See troubleshooting guide below for more details).
- f. An **Absence** result for Male DNA
  - i. **Female Sample Result:** Check Cq Value on the FAM Fluorophore. See Table I for Cq cutoff value.
  - ii. Visually confirm with the curve on the graph.

FemINDICATOR® Sex Detection Assay Data Analysis Quick Reference Table:

Assay	Cq Value	Fluor	Negative Control (Cq)	Cq threshold
Sex - Male	< 35	FAM	No Value	Presence/Absence
Sex - 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		

### **Troubleshooting Guide**

Symptom	Reason	Solution
Internal control (SCCG Primer) failure	Leaf Lysis Failure	Repeat Leaf Lysis by following the protocol.
	Mix up in Reaction Setup	Repeat the 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 the window is closed the analysis should automatically update.
Internal Control (SCCG) Positive result on positive or negative control samples or samples that do not contain plant DNA No Cq or a Cq of more than 35 is acceptable. Any Cq lower than 35 constitutes a rerun	Plant DNA contamination in a reagent	Troubleshoot which reagent was contaminated; use new reagents, thoroughly clean all pipettes and bench areas with 10% bleach solution.
	qPCR bench too close to extraction area	Designate separate benches, pipettes etc. for extractions and qPCR setup
Positive Negative Control	Small Cq value <15	Visually confirm that there is an amplification curve. If not, this is a low level background and is to be expected.

	Contamination	Repeat the 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 C <sub>q</sub> reading (<15), the curve is usually missing the exponential growth phase, but rather a gradual increase of fluorescence signal. This is usually a negative result, but should be repeated.

### **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 C<sub>q</sub> 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 C<sub>q</sub> 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 C<sub>q</sub> is expected. It ensures the effectiveness and efficiency of each reaction. The internal control targets plant DNA, or more specifically, a Single Copy Control Gene (SCCG), using the HEX Fluorophore.

## DISCLAIMER

This test was developed, and its performance characteristics determined by Medicinal Genomics Company, for laboratory use. Any deviations from this protocol are not supported by MGC.

The results may vary based on laboratory conditions. Altitude and humidity are among factors known to affect the growth of bacterial and fungal species. All thresholds were determined based on the results using the BIO-RAD CFX96 Touch® Real-Time PCR Detection System. It is recommended that thresholds be calibrated for each specific laboratory setting.

## LIMITED USE LABEL LICENSE

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