FemINDICAtor® qPCR Cannabis Sex Detection Assay

User Guide v2

Real Time PCR (qPCR) Assay for the detection of plant sex in cannabis

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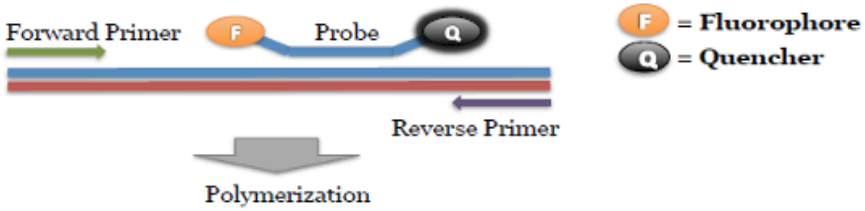
Introduction

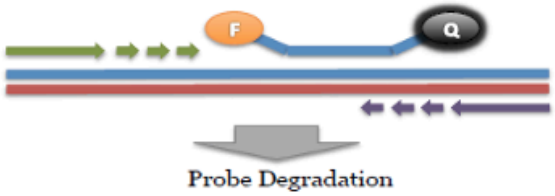
The FemINDICAtor® qPCR Cannabis Sex detection Assay uses a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of plant sex for every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting sex, resulting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

Process Overview

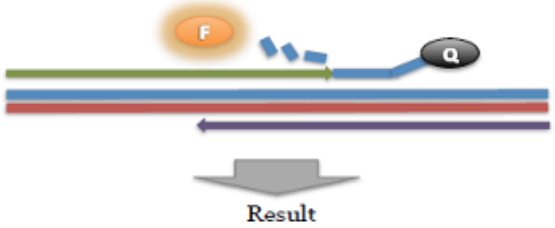
Step 1:
Primers and probe bind to target DNA.



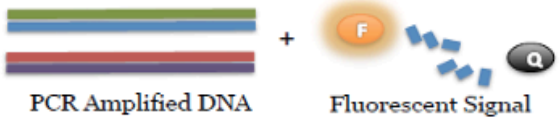
Step 2:
PCR occurs, primers are extended on forward and reverse DNA strands.



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 .



Materials

Item P/N	Component Name	Qty Provided	Storage Conditions
420240	Quick Lysis	12 x 0.2 mL PCR 8-tube strips in a 96 well carrier	RT (20–28 °C)
	Hole punches (grommets) are included with Leaf Punch Lysis	50 per Bag	RT (20–28 °C)
420201	PathoSEEK® qPCR Master Kit v3	1 Kit (200 -300 rxn)	-15 to -20 ºC
	Reaction Buffer (10x)	1 tube	-15 to -20 ºC
	Nuclease Free Water	2 tubes	RT, 4 ºC or -15 to -20 ºC
	qPCR Master Mix	1 tube	-15 to -20 ºC
420154	FemINDICAtor® qPCR Cannabis Sex Detection Assay v2	1 Tube	-15 to -20 ºC
420312	FemINDICAtor® qPCR Positive Control	1 Tube (50 uL)	-15 to -20 ºC

Shelf Life and Storage

Once received, each kit component must be stored at its designated storage condition. Reagents stored properly can be used until the expiration date indicated on each component label.

Required Equipment and Supplies

Equipment:

- Agilent AriaMx Real-Time PCR System G8830A, containing the following Optical Channels: FAM, ROX, and HEX - **Medicinal Genomics P/N 420387**
    - Software version 3.1.2306.0602
    - Agilent HP Notebook PC option 650 or lab-supplied Windows PC
    - 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.
- Bio-Rad CFX96 Touch™ Real-Time System.
    - Software version 10.0.26100
    - Bio-Rad supplied or own Windows PC
- Bio Molecular Systems Mic 4-Channel PCR Instrument - **Medicinal Genomics P/N 420241**
    - Software version 1.4.10
    - BMS supplied or lab-supplied Windows PC
    - Mic Tubes and Racked Caps - **Medicinal Genomics P/N 420244**
    - Mic Tubes and Caps (Bulk) - **Medicinal Genomics P/N 420243**
- Adjustable, variable volume pipettes (single or multichannel).—P10, P20, P200, and P1000
- Adjustable, variable volume filter pipettes tips.—For P10, P20, P200, and P1000
- 96 Well PCR Cryogenic Rack (optional)
- 1.5 mL Tube Benchtop Cryogenic Rack (if no ice available)
- Freezer—Capable of maintaining -20 °C
- Table Top Mini Plate Centrifuge
- Table Top Mini Centrifuge
- Vortex-Genie Pulse
- Eppendorf Tube Rack

Supplies:

- 96-well Optical qPCR plate — **Medicinal Genomics P/N 100164**
- Adhesive optical seals for qPCR plates — **Medicinal Genomics P/N 100177**

- Crushed ice
- 1.5 mL Eppendorf Tubes
- 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.

- 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.
- 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

The quality of results depends on the strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR:

- Never circulate lab equipment from one workstation to another.
- Always use a positive and negative control for each series of amplification reactions.
- Periodically verify the accuracy and precision of pipette, as well as correct functioning of the instruments.
- Change gloves often, especially if you suspect contamination.
- Clean workspaces periodically with 10% bleach and other decontaminating agents.
- Use powder-free gloves and avoid fingerprints and writing on tube caps. Both can interfere with data acquisition.

DNA Lysis

For Plant Sampling and DNA lysis, see the Quick Lysis for Leaf instructions in the Sample Preparation Guide, which should be followed *before* setting up the FemINDICAtor qPCR.

Real-Time Quantitative PCR (qPCR) Setup

Two options are provided for assay setup and cycling parameters. Option 1 provides optimal conditions. Option 2 can be used in situations when the sample is free of potential contaminants and amplification inhibitors.

Option 1: Legacy qPCR Master Mix volumes and cycling parameters best suited for smaller batch sizes.

Option 2: Decreased qPCR Master Mix volumes and cycling parameters intended for higher throughput.

qPCR Setup

1. Remove qPCR reagents including qPCR Master Mix, water, reaction buffer and assay probe mixes to be used from the -20 ºC 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. Prepare the 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.

Option 1 - Smaller Batch Sizes

qPCR Reagent Volumes

Reagents	1 Reaction	24 Reactions (Plus 1 excess rxn)	48 Reactions (Plus 2 excess rxn)
qPCR Master Mix v3	3.75 µL	93.75 µL	187.5 µL
Assay Probe Mix	1 µL	25 µL	50 µL
Reaction Buffer	0.8 µL	20 µL	40 µL
Water	8.2 µL	205 µL	410 µL
Total Assay Probe MM	13.75 µL	343.75 µL	687.5 µL

Option 2 - High Throughput

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

4. Once combined gently, tip mix or invert the tube 5 times to combine the master mix.
    a. Pulse spin-down tube in microcentrifuge.

b. Place qPCR Master Mix tubes on ice until used.

5. Positive Control Dilutions
    a. Standard qPCR Instruments (Bio-Rad CFX96 and Agilent AriaMX)
        i. Prepare a 1:10 dilution from stock
            1. After fully thawed, vortex and quick spin positive control stock tube prior use.
            2. Add 2 µL of stock positive control to 18 µL nuclease free water (found in the kit) and vortex to mix. This is a 1:10 dilution.
    b. BMS MIC
        i. Prepare a 1:10,000 dilution from stock
            1. After fully thawed, vortex and quick spin positive control stock tube prior to use.
            2. Add 2 µL of positive control to 198 µL nuclease free water (found in the kit) and vortex to mix. This is a 1:100 dilution.
            3. Add 2 µL of the 1:100 positive control dilution to 198 µL nuclease free water and vortex to mix. This is a 1:10,000 dilution.

    Note: It is best to add the largest volume reagent first, in this case the 198 µL water then the 2 µL of positive control, pipette mix or vortex control dilution to ensure control DNA is in solution.

6. For the negative control, use water (found in the kit).
7. Use a 96-well optical qPCR plate and label the plate "qPCR Plate_ [date]".
8. Transfer samples and master mix to the PCR plate.
    a. **Option 1:**
        i. Transfer 5 µL of each sample, 5 µL of diluted assay positive control, and 5 µL of water to separate wells of a qPCR plate.
        ii. Transfer 13.75 µL of freshly prepared qPCR Assay Master Mix to each well and slowly tip mix 5 times. Avoid adding bubbles to the mixture.
    b. **Option 2:**
        i. Transfer 9 µL of freshly prepared qPCR Assay Master Mix to each well.
        ii. Transfer 5 µL of each sample, 5 µL of diluted assay positive control, and 5 µL of water to separate wells of a qPCR plate and slowly tip mix 5 times. Avoid adding bubbles to the mixture.

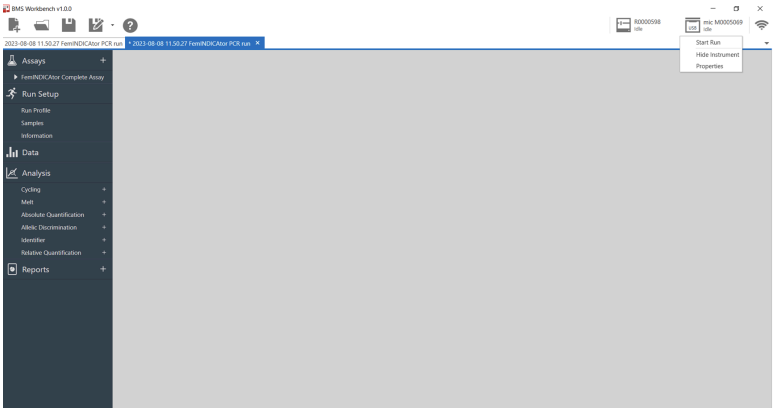
9. Seal the plate with strip caps or an adhesive seal.
10. Spin-down for at least 1 minute in plate microcentrifuge to bring well contents to the bottom of wells and help to rid of reaction bubbles.

    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.
11. 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.
12. Place the sealed plate onto the PCR instrument, positioning the A1 well in the top left corner.
13. Follow the software specific instructions to initiate the run.

BMS MIC

Setup

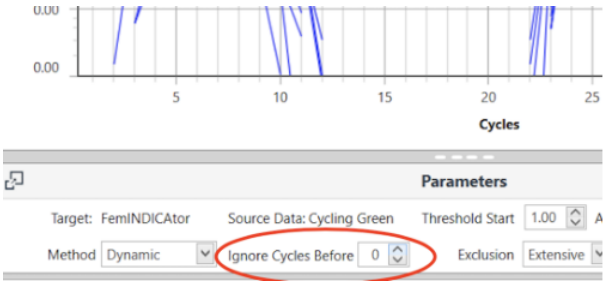
1. Open the BMS Workbench software and create a new file.
2. Select qPCR Run.
3. Select the appropriate template by clicking the "+" sign next to assays or ensure that the appropriate thermal cycling conditions are entered:
    a. Option 1: Hot Start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC for 15 seconds and 65ºC for 90 seconds.
    b. Option 2: Hot start at 95 ºC for 5 minutes, followed by 40 cycles of 95 ºC for 15 seconds and 65 ºC for 30 seconds.
4. Click on the Mic icon in the upper right-hand corner, and select "Start run" from the menu that appears.



5. A pop-up will appear asking for the reaction volume, which will be auto-filled with the correct volume based on the template chosen. Close the lid and the instrument will start.
   Note: If the cover isn't closed, the program will not start.
6. Let the Mic run to completion before analyzing the data.

Data Analysis

1. When the run has completed, click on the "+" sign next to the "Cycling" tab and select IC (Internal Control). The selection will appear under the "Cycling" tab. Next, select *FemINDICAtor*.
2. When these have been expanded, select the "+" sign next to the "Identifier" tab and select the available report.
3. If the automatic calls are blank, then one of the cycles is out of threshold. To fix this, find "Ignore Cycles Before" in the "Parameters" section, and increase its value one whole number at a time until a gray bar appears on the graph. This might have to be done with one, or all of the filters.



4. To review an automatic report, click the "+" sign next to the "Identifier" tab then select the relevant "Complete Assay". This feature will call the samples tested as detect, non-detect, or inconclusive based on the qPCR data. These results will be displayed on the right side of the screen.
5. Data may be exported by selecting the "Report" tab, then clicking the Export icon.

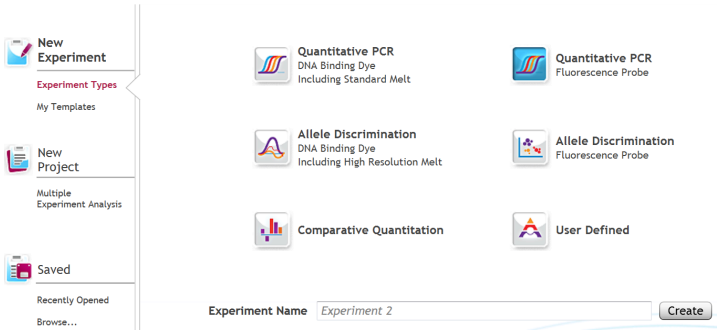
Aria MX

Setup

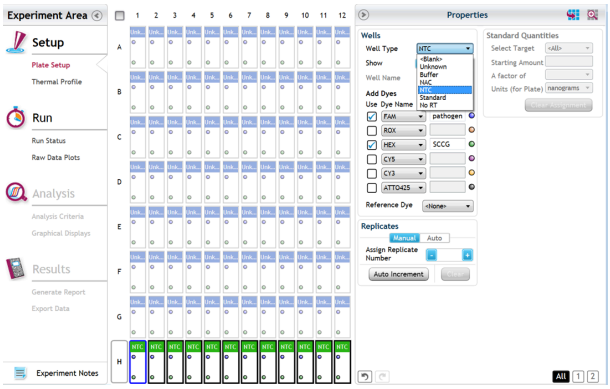
The following species will be detected on the following Fluorophores:

- Y Chromosome: FAM
- Cannabis DNA: HEX

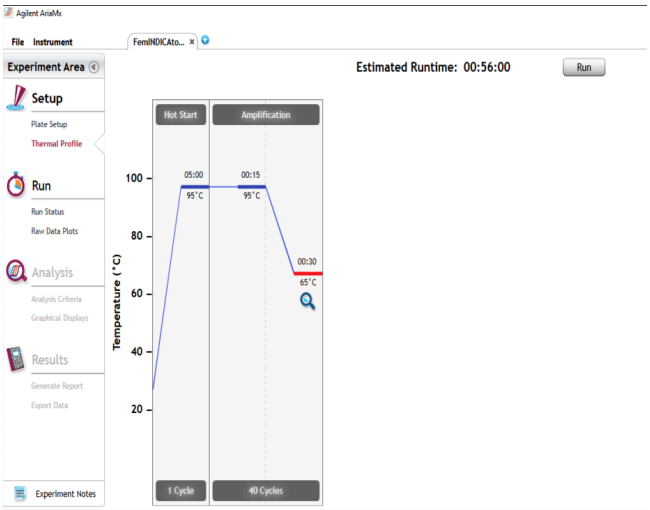
1. Create a New Experiment on the Agilent qPCR instrument.
2. Select “Quantitative PCR” from Experiment Types. Under Setup>Plate Setup, highlight wells that contain reactions and select FAM and HEX under Add Dyes.



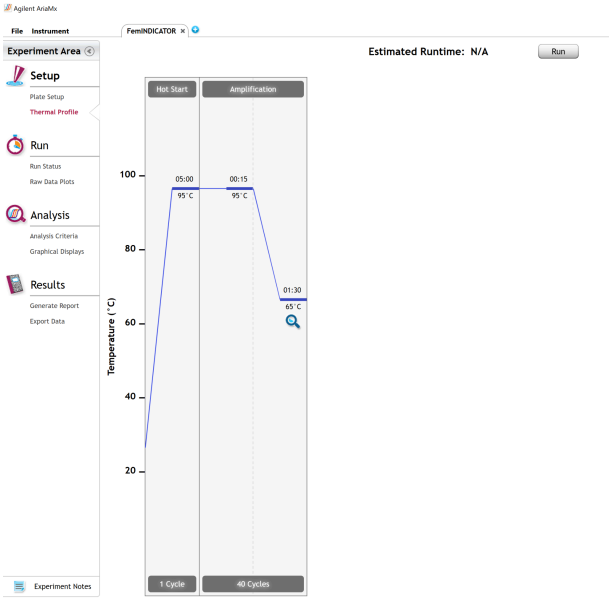
3. Change the well types to reflect your plate set up. If desired, add Target names to include “Male” for FAM and IC (Internal Control) or SCCG (single copy control gene) for HEX.



4. Under Setup>Thermal Profile, create the desired PCR thermal profile:
    a. **Option 1:** Hot Start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC for 15 seconds and 65ºC for 90 seconds.



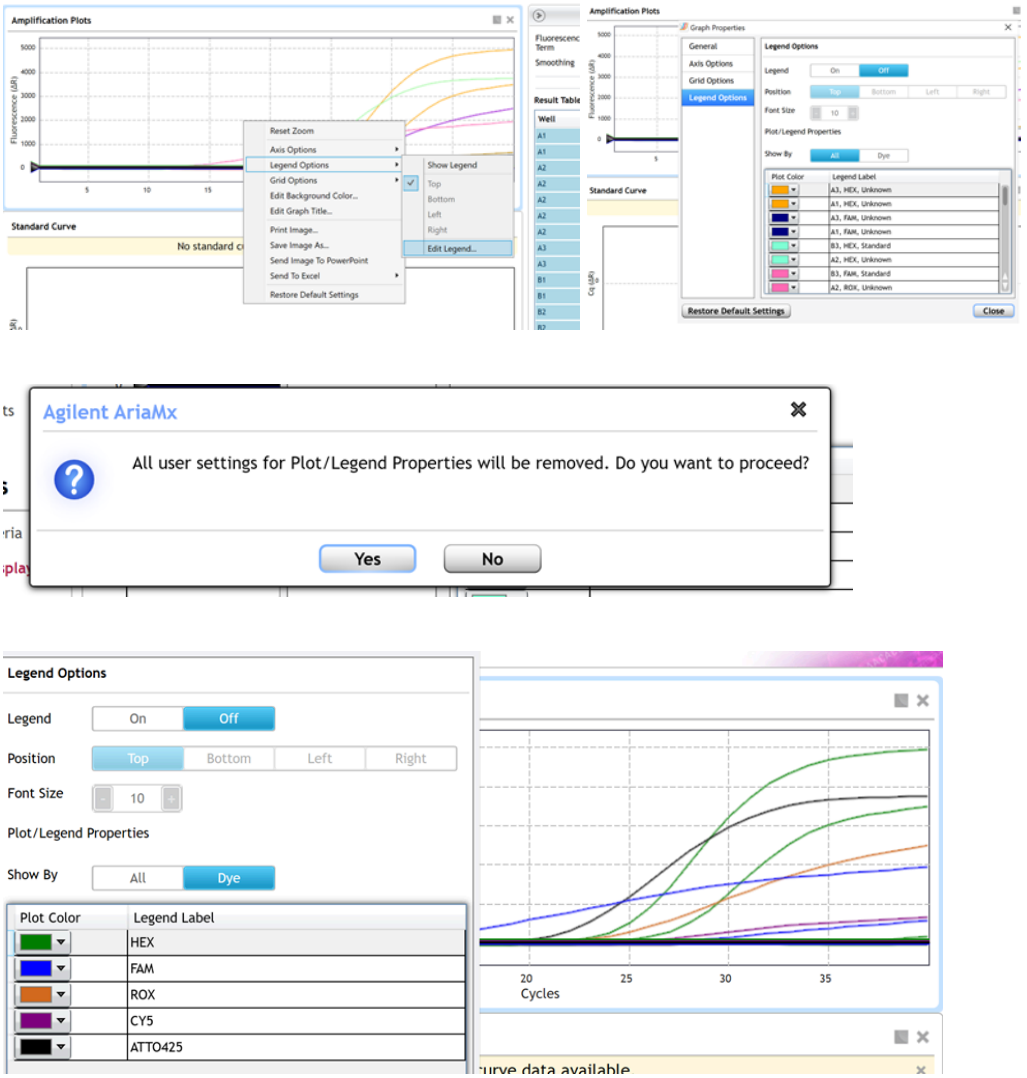
    b. **Option 2:** Hot Start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC for 15 seconds and 65ºC for 30 seconds.



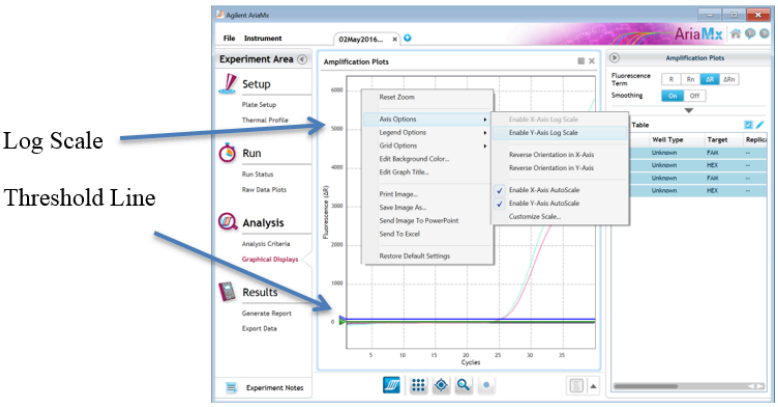
5. Close the hot top and main lids and click “Run”.
6. Save the experiment with the [User] and [date]
7. When the run is complete, dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

Data Analysis

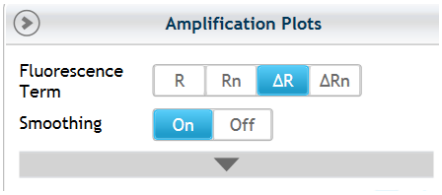
1. Open the Data Analysis window when the run is complete.
2. Highlight the wells of interest in the Analysis Criteria under Analysis, then select Graphical Display
    a. Amplification plots will be available for viewing
    b. The Cq values will appear to the right in the table
    c. Right click inside 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.

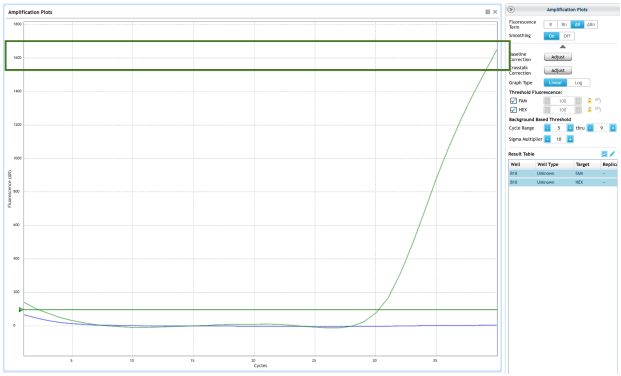


b. Expand the amplification plots settings by clicking on the triangle (shown below).

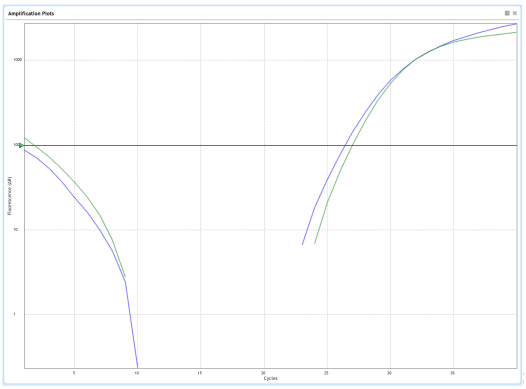


c. Manually adjust thresholds to 100 RFU for the FAM and HEX fluorophores.

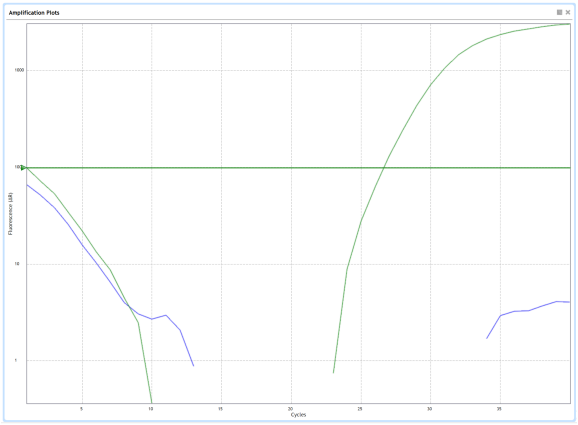
4. Controls
   a. Positive Control, on the FAM Fluorophore, has a Cq value ≤ 35.
      i. Visually confirm with the curve on the graph.
   b. Negative Control, on the FAM Fluorophore, has no Cq value.
      i. Visually confirm with the curve on the graph.
   c. Internal Control, on the HEX Fluorophore, has a Cq value ≤ 35 for all samples.
      i. Visually confirm with the curve on the graph.
      ii. Ensure that the RFU Value is greater than 500 in the ΔR, Linear view.



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).
        iii. Below is an example of a male result.



    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.
        iii. Below is an example of a female result.

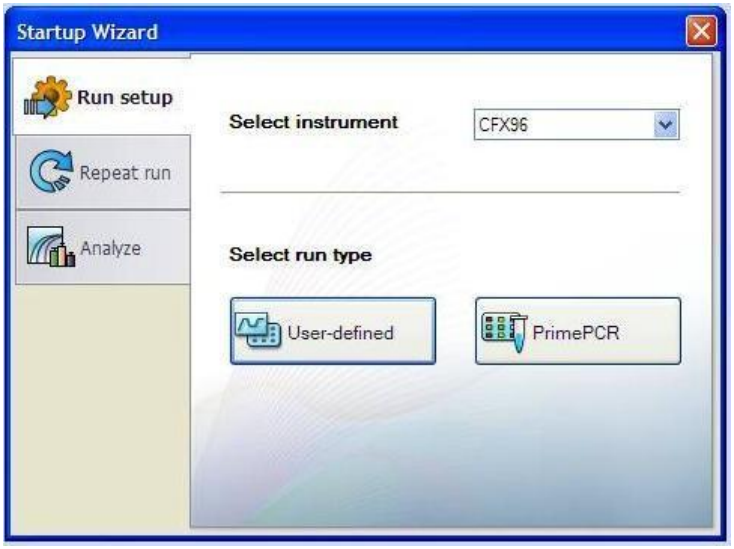


Bio-Rad CFX 96

Setup

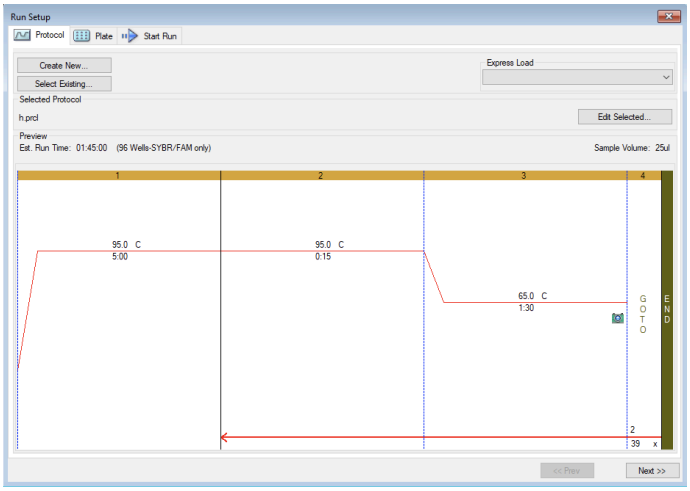
- Y Chromosome: FAM
- Cannabis DNA: HEX

1. Start the qPCR Cycling program.

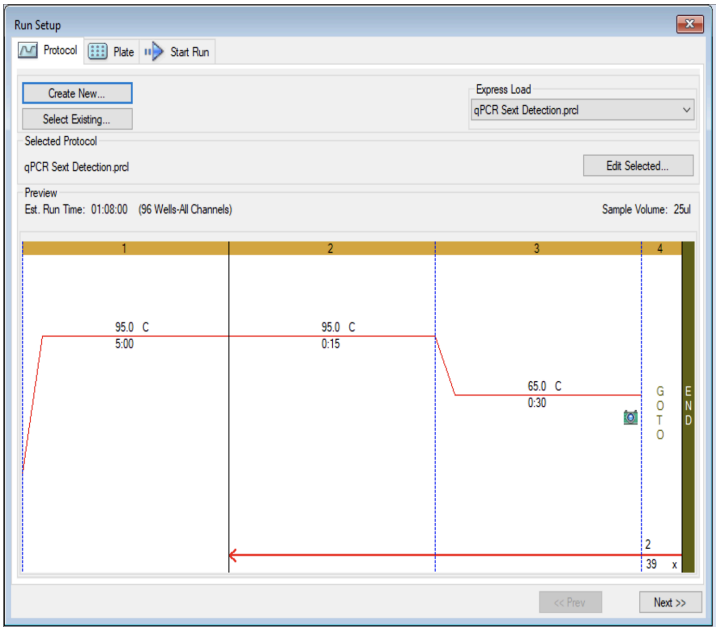


2. Select User-Defined in the Startup Wizard under Run setup.
3. Use the Express Load dropdown menu to pick the qPCR Sex Detection Program and click "Next".
4. If not already pre-programmed, create a cycling program with the following specifications and save as "qPCR Sex Detection":

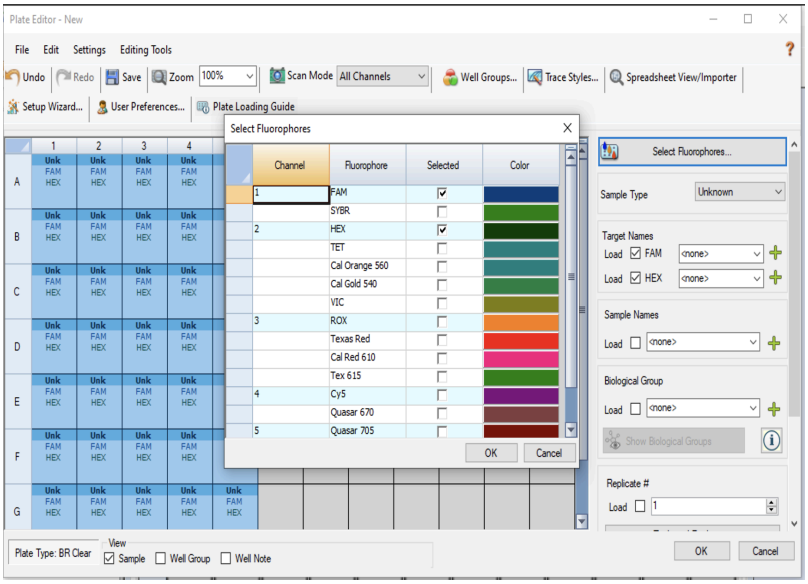
a. Option 1: Hot Start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC for 15 seconds and 65ºC for 90 seconds.

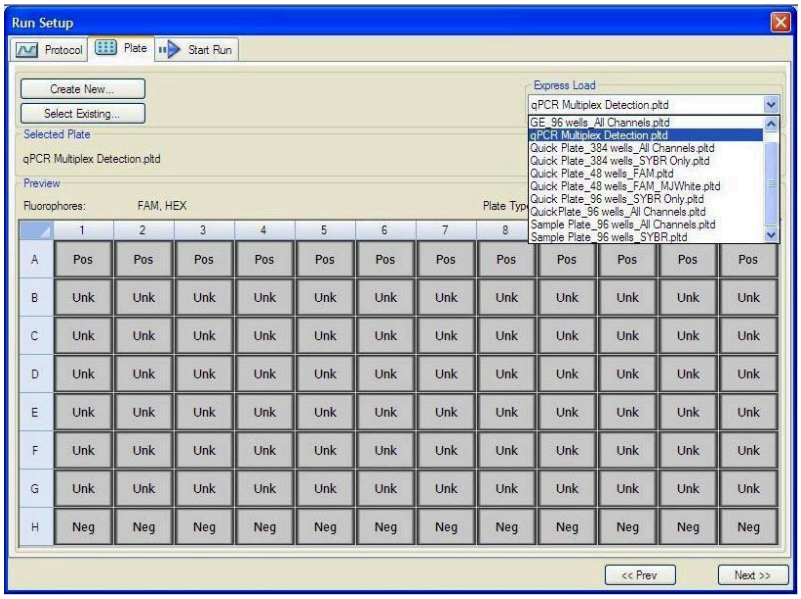


b. Option 2: Hot Start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC for 15 seconds and 65ºC for 30 seconds.



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. The 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.

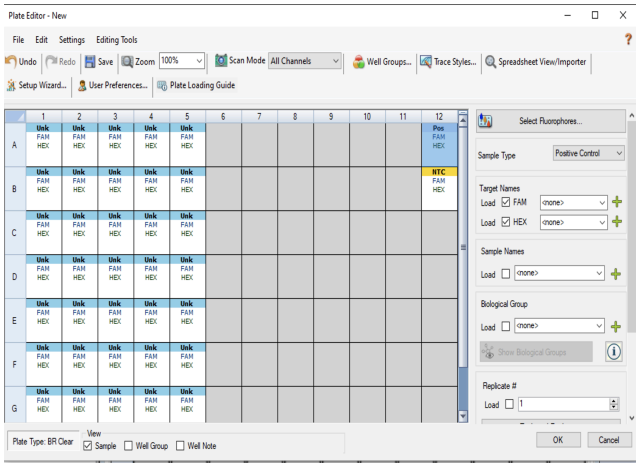
NOTE: To select the Sample Type, highlight the wells you would like to define, then choose from the dropdown menu one of three types:

Unknown

Positive Control

Negative Control

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



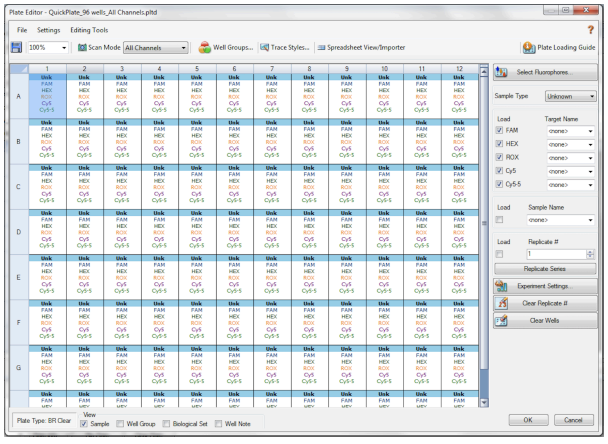
f. Attach the fluorophores to the wells being used.

   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.

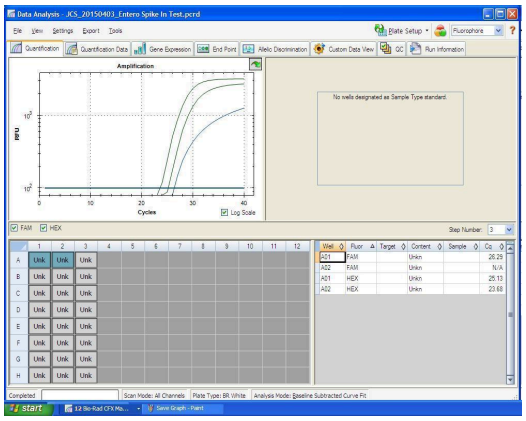
i. Close the lid and click Start Run.

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.

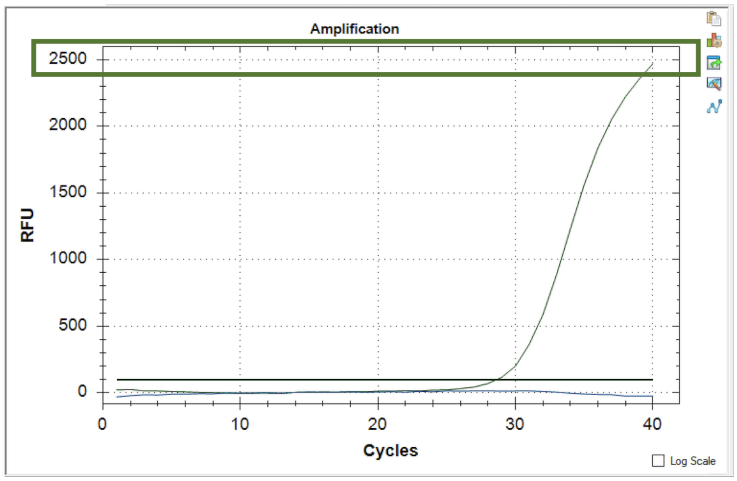
Data Analysis

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 Cq 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 10^2 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 ≤ 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 ≤ 35 for all samples.
        i. Visually confirm with the curve on the graph.

ii. Ensure that the RFU Value is greater than 500 in the Baseline Subtracted Curve Fit view.

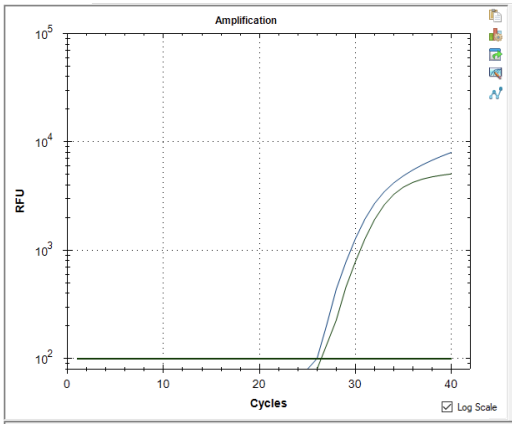


e. A Presence result for the unknown samples.

i. Male Sample Result: Check Cq Value on the FAM Fluorophore. See Table I for Cq cutoff value.

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).

iii. Below is an example of a male result.

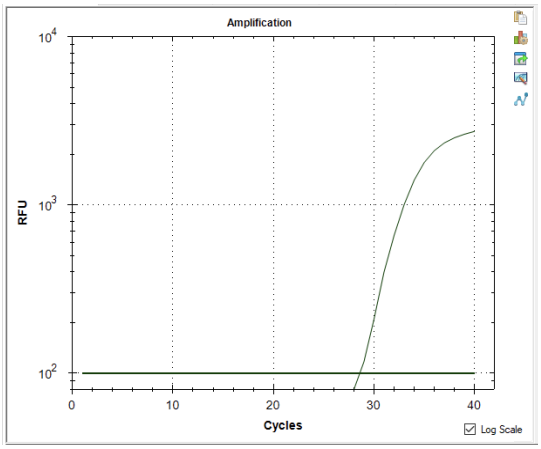


f. An Absence result for Male DNA

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.

iii. Below is an example of a female result.



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 cannabis DNA	
Assay Positive Control	≤ 35	FAM		

Troubleshooting Guide

Symptom	Reason	Solution
Internal control failure	Nucleic Acid Isolation Failure	Repeat Quick Lysis or PurePrep by following the protocol.
	If using PurePrep Residual ethanol in elution	Ethanol is an inhibitor to RT-qPCR. Return to the PurePrep protocol and repeat all steps.
	qPCR inhibition	Dilute extracted or lysed samples 1:10 with qPCR grade water and repeat the RT-qPCR.
	Mix up in Reaction Setup	Repeat the RT-qPCR by following the protocol.
	Missing Fluorophore on plate set up	Check Plate Setup to ensure the correct fluorophores were chosen on setup of run. They can be corrected post run.
Amplification of the Internal control is not expected in the assay positive or negative control wells. 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 RT-qPCR setup
Positive Negative Control	Small Cq value <15	Visually confirm that there is a true amplification curve. If not, this may be considered a background trace.
	Contamination	Repeat the RT-qPCR by following the protocol.
Positive Negative Control (Continued)	Insufficient pre-setup bleaching	Clean workspace and all equipment with 10% Bleach, repeat rt-qPCR.
Negative Positive Control	Mix-up in Reaction Setup	Repeat the RT-qPCR by following the protocol.
Total run failure	Excessive vortexing the RT-qPCR Master Mix	Repeat the RT-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 displays 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 Cq 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 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, ROX and Cy5 Fluorophores.

Amplification of the **Internal Control** or the microbial target of interest is expected in every reaction containing DNA isolated from a cannabis sample. It ensures the DNA isolation procedure was successful or the presence of microbial contamination. The internal cannabis control targets the cannabis genome, using the HEX Fluorophore.

MIP is short for Marijuana Infused Product. A MIP is cannabis plant material or concentrate mixed into a consumable.

Revision History

Version	Date	Description
v1	February 2022	New user guide format
v2	June 2025	• Improved Positive Control dilution update for MIC • Addition of MIC Data Analysis • HEX amplitude requirement in data analysis • Addition of previous master mix volumes and cycling conditions for difficult samples • Removed plant sampling and referencing plant sampling user guide

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.

This test has not been validated on remediated (irradiated, ozone treated, acid treated, hydrogen peroxide treated, etc.) samples. Samples that have undergone remediation may cause discordant results between plating methods and PathoSEEK methods. When remediated samples produce a result above the action limit on qPCR, we recommend confirming viability with an approved plating method.

Results may vary based on laboratory conditions. Altitude and humidity are among factors known to affect the growth of bacterial and fungal species.