FemINDICAtor® qPCR Cannabis Sex Detection Assay

User Guide v3

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

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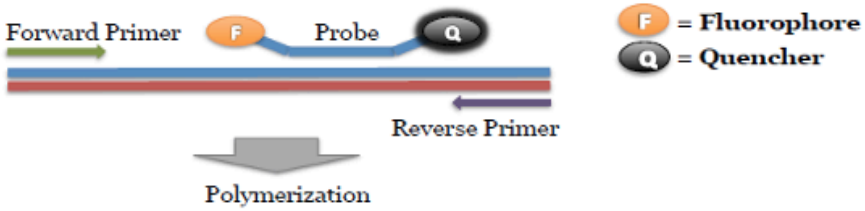
Introduction

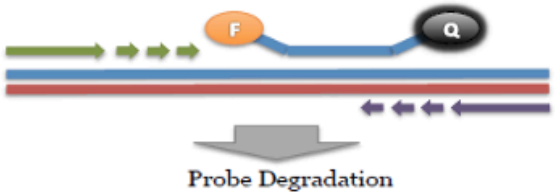
The FemINDICAtor® qPCR Cannabis Sex detection Assay uses a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of plant sex for every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting sex, resulting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

Process Overview

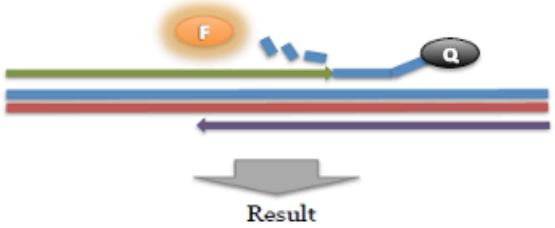
Step 1:
Primers and probe bind to target DNA.



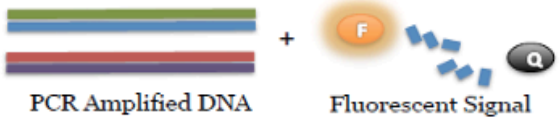
Step 2:
PCR occurs, primers are extended on forward and reverse DNA strands.



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

FemINDICAtor Cannabis Sex Detection Assay v3 Kit - P/N 420546 (**200 reactions**)

Component Name	Qty Provided	Storage Conditions
PathoSEEK® Amplification Mix *Includes 2 tubes nuclease free water for resuspension*	4 Vials (50 rxns/each)	RT / -20 ºC*
FemINDICAtor Cannabis Sex Detection Assay v3	1 Tube (200 µL)	-20 ºC

Note: Actual fill volumes include overage

*The PathoSEEK Amplification Mix can be stored lyophilized at Room Temperature for up to 2 years. Once re-hydrated it must be stored at -20 ºC for up to 3 months.

Additional Required Components Not in Kit:

Item P/N	Item 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)
420312	FemINDICAtor Positive Control	1 Tube (50 µL)	-20 ºC
100184	PCR Grade Water	1 Bottle (500 mL)	RT
100177	Optical Adhesive Seals	Case of 100	RT
100164	96-Well Optical qPCR plate	Case of 25	RT

*4mm Grommets for leaf punches are included with Quick Lysis reagent (Qty 100)

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, and HEX
    - 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:

- 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

qPCR Setup

1. Remove PathoSEEK Amplification Mix, FemINDICAtor v3 detection assay, and FemINDICAtor Positive Control from the -20 ºC freezer.
    a. If lyophilized Amplification Mix has not been previously rehydrated, rehydrate with 550 µl of Nuclease Free Water. Swirl or pipette tip mix. After resuspension, store remainder Amplification Mix at -15 to -20 °C when not in use.
    b. Allow all frozen reagents to defrost at room temperature (20-28 °C). Once defrosted, place tubes on ice.
2. Before preparing the Master Mix, invert or vortex and pulse spin down the reagents in a mini centrifuge
    a. FemINDICAtor Assay v3 tube (probe mix) – vortex tube quickly followed by a pulse spin down in a minicentrifuge.
    b. FemINDICAtor Positive Control tube – vortex tube quickly followed by a pulse spin down in a minicentrifuge.
    c. PathoSEEK Amplification Mix – Invert the bottle 5-10 times to mix or briefly vortex.
    d. Return all reagents to the ice.
3. Prepare Master Mix in a 1.5 mL tube. Label the tube as “Master Mix”. See Table 1 (PathoSEEK Amplification Master Mix).

    Note: Always prepare enough Master Mix for an additional one or two reactions to account for pipetting and dead volumes. Be sure to include 2 extra reactions for the qPCR positive and negative controls. For example, if testing 10 samples, you would need to make enough Master Mix for 13 or 14 reactions, which would account for 1 or 2 excess.

Table 1: PathoSEEK Amplification Master Mix Reagent Volumes

Reagent	Volume for 1 qPCR Reaction
PathoSEEK Amplification Mix	10 µL
FemINDICAtor Assay v3	1 µL
Nuclease Free Water	4 µL
Total	15 µL

4. Once the Master Mix is combined, cap the tube and vortex to mix.
    i. Pulse spin down tube in minicentrifuge.
    ii. Place the Master Mix tube on ice until used.
5. For the negative control, use nuclease free water that was used to rehydrate your Amplification Mix.
6. Positive Control Dilutions
    a. Standard qPCR Instruments (Bio-Rad CFX96 and Agilent AriaMX)
        i. Prepare a 1:10 dilution from the stock
            1. After the positive control stock tube is fully thawed, vortex and quickly spin prior to use.
            2. Add 2 µL of stock positive control to 18 µL nuclease free water (found in the kit) and vortex to mix. Quick spin the tube after mixing. This is a 1:10 dilution.
    b. BMS MIC
        i. Prepare a 1:100,000 dilution from stock
            1. After the positive control stock tube is fully thawed, vortex and quickly spin prior to use.
            2. Add 2 µL of positive control to 198 µL nuclease free water (found in the kit) and vortex to mix then quick spin the tube. 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 then quick spin the tube. This is a 1:10,000 dilution.
            4. Add 10 µL of 1:10,000 FemINDICAtor Positive Control to 90 µL of nuclease-free water. Vortex and pulse spin down in a mini centrifuge. This is the 1:100,000 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. This dilution is stable when it is stored at -20°C when not in use for six months.

7. For qPCR reactions use a 96-well optical qPCR plate, optically clear qPCR tubes, or Mic tubes.
8. Transfer lysed samples into qPCR plate wells or tubes
    a. Carefully remove the caps from the Quick Lysis tubes..

    Note: If lysed samples were frozen, let the DNA thaw completely and spin the tubes in centrifuge to avoid cross contamination between samples. Tip mix thawed samples wells before transferring to the qPCR plate or tubes.

    b. Pipette transfer 5 µL of each sample lysate into the corresponding qPCR tube or well on the qPCR plate.
    c. Add 5 µL of the appropriately diluted Positive Control to the corresponding positive control plate well or tube.
    d. Add 5 µL of nuclease-free water to the corresponding negative control plate well or tube.

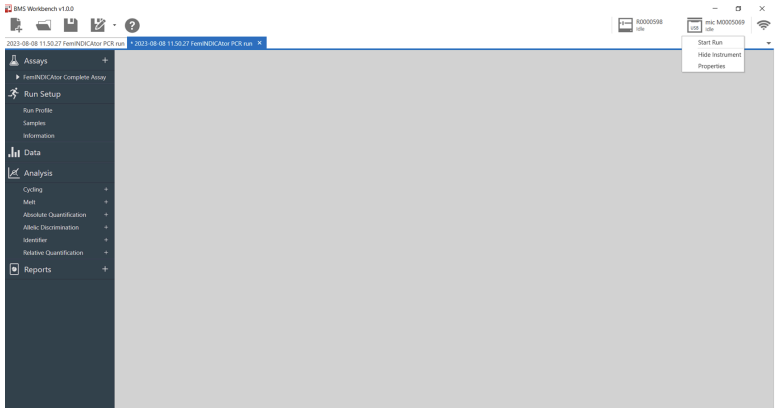
    Note: ALWAYS use a fresh tip for every liquid transfer of sample, positive, or negative control into the qPCR plate
9. Add 15 µL of Master Mix to each corresponding sample well, positive control well, and negative control well in the qPCR plate or tubes. Gently tip mix a few times after each addition of Master Mix. Be careful not to introduce bubbles during this mix. Use a fresh tip for each transfer of Mater Mix to each well.
10. Seal the plate with strip caps or an adhesive seal, or seal qPCR tubes with strip caps or Mic tube caps.
11. For the Bio-Rad CFX and Agilent AriaMX, spin down qPCR plate or tubes for at least 1 minute in plate (or tube) mini centrifuge to bring well contents to the bottom of wells (or tubes) and help to get rid of reaction bubbles.

    Note: Check for bubbles in the wells or tubes (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the wells (or tubes), spin down for another minute in mini centrifuge.
12. Place the sealed plate or tubes onto the PCR instrument.
13. Follow the software specific instructions for each qPCR platform to initiate the run.

Bio Molecular Systems Mic

Setup

1. Open the Workbench v2 software and create a new file.
2. Select qPCR Run.
3. Select the FemINDICAtor template by clicking the "+" sign next to assays or ensure that the appropriate thermal cycling conditions are entered:
    a. Hot Start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC for 15 seconds and 65ºC for 90 seconds.
4. Click on the Mic icon in the upper right-hand corner, and select "Start run" from the menu that appears.

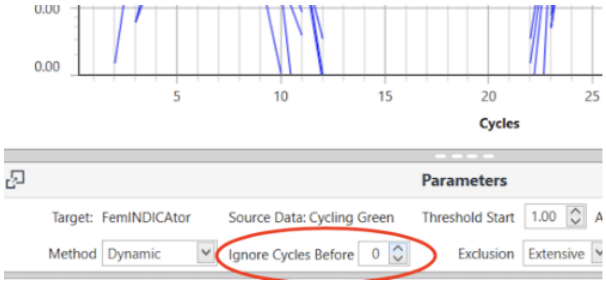


5. A pop-up will appear asking for the reaction volume, which will be auto-filled with the correct volume based on the template chosen. Close the lid and the instrument will start.
   Note: If the cover isn't closed, the program will not start.
6. Let the Mic run to completion before analyzing the data.

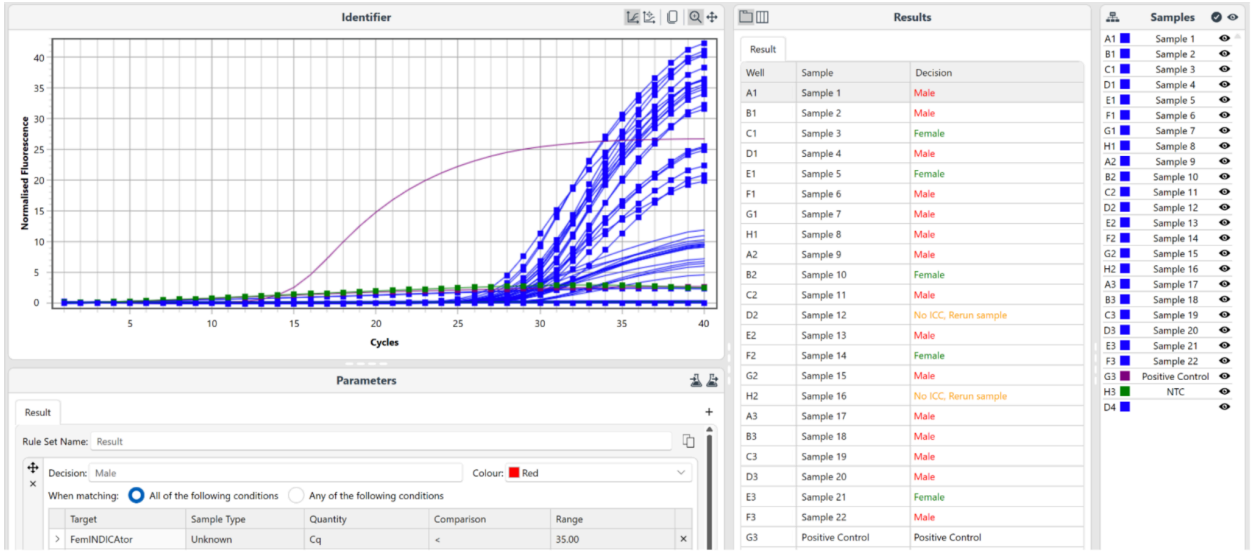
Data Analysis

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 female, male, 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.

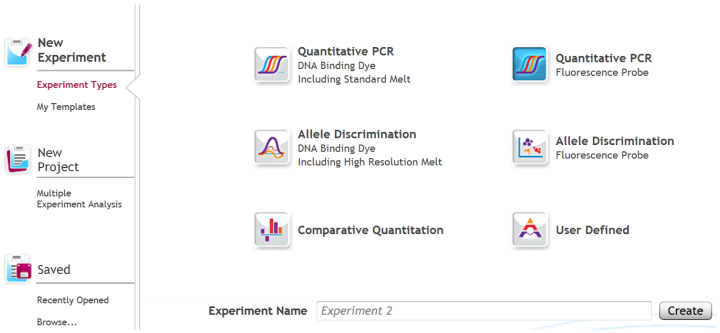
Agilent AriaMX

Setup

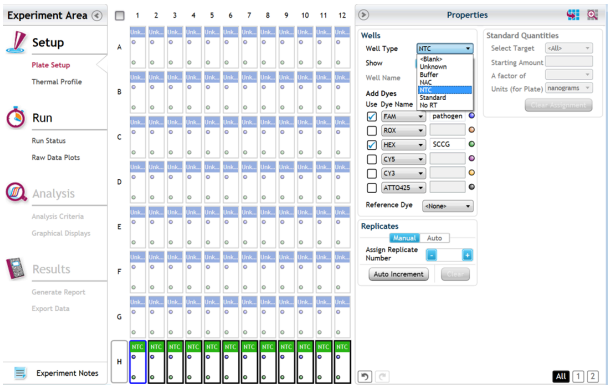
The following species will be detected on the following Fluorophores:

- Y Chromosome: FAM
- Cannabis DNA: HEX

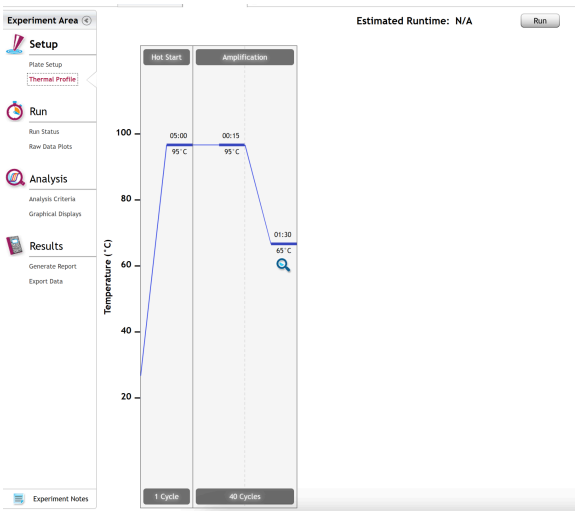
1. Create a New Experiment on the Agilent qPCR instrument.
2. Select “Quantitative PCR” from Experiment Types. Under Setup>Plate Setup, highlight wells that contain reactions and select FAM and HEX under Add Dyes.



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) for HEX.



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

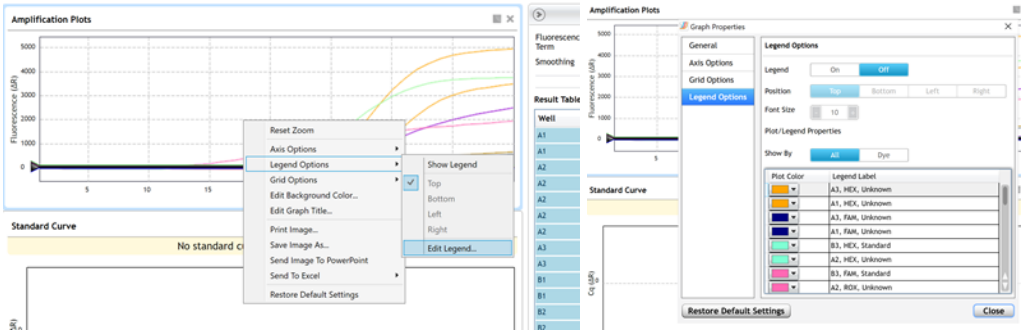


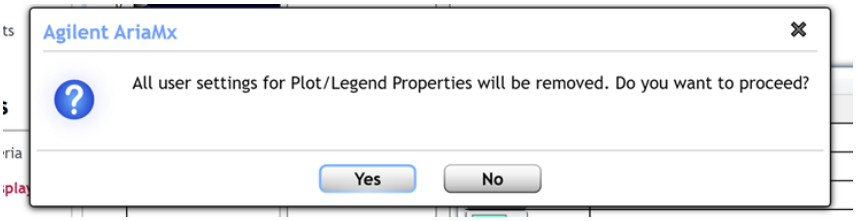
5. Close the hot top and main lids and click “Run”.
6. Save the experiment with the [User] and [date]
7. When the run is complete, dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

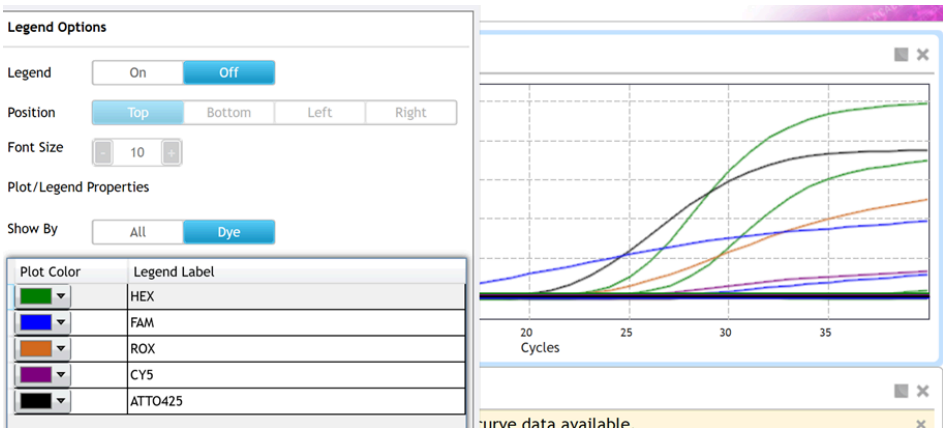
Data Analysis

1. Open the Data Analysis window when the run is complete.

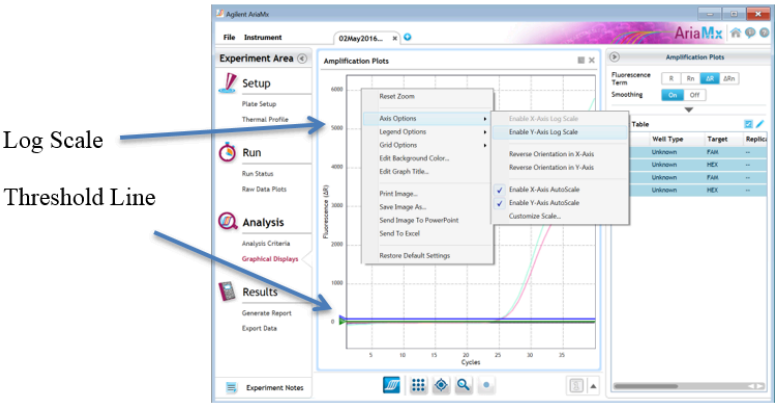
2. Highlight the wells of interest in the Analysis Criteria under Analysis, then select Graphical Display
    a. Amplification plots will be available for viewing
    b. The Cq values will appear to the right in the table
    c. Right click inside the graph, select Edit Legend under Legend Options
    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.



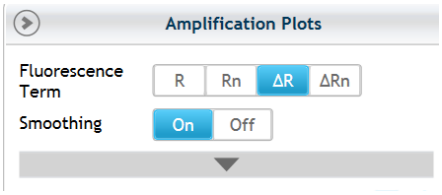




3. To analyze the results:
    a. Start by turning the graph to Log Scale by right clicking on the chart and selecting Axis options. Enable y-axis log scale.

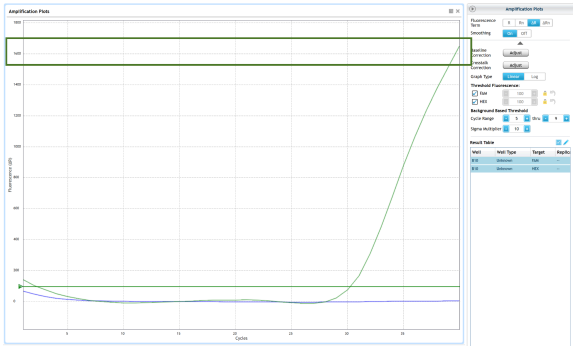


    b. Expand the amplification plots settings by clicking on the triangle (shown below).

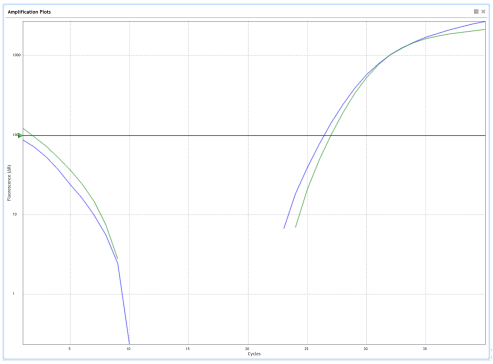


    c. Manually adjust and lock thresholds to 100 RFU for the FAM and HEX fluorophores.

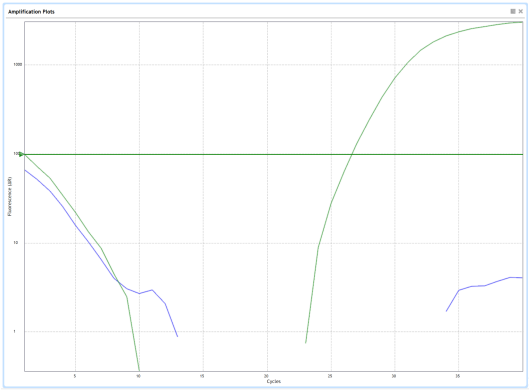
4. Controls
    a. Positive Control, on the FAM Fluorophore, has a Cq value ≤ 35.
        i. Visually confirm with the curve on the graph.
    b. Negative Control, on the FAM Fluorophore, has no Cq value.
        i. Visually confirm with the curve on the graph.
    c. Internal Control, on the HEX Fluorophore, 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.

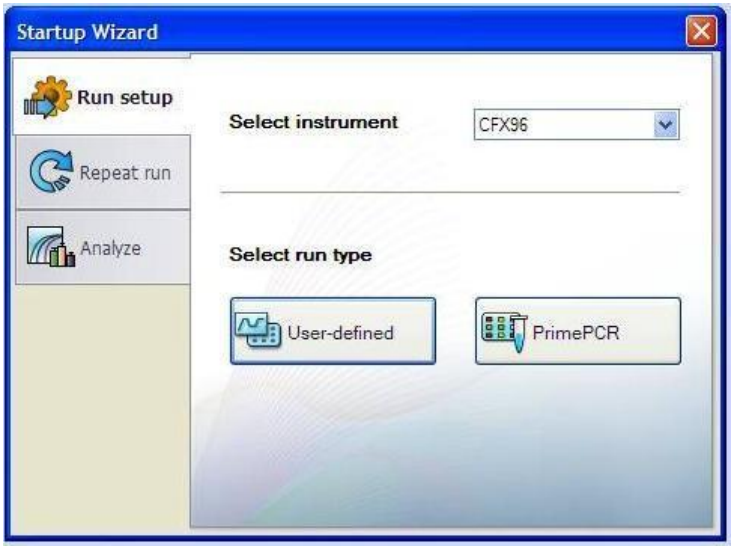


Bio-Rad CFX 96

Setup

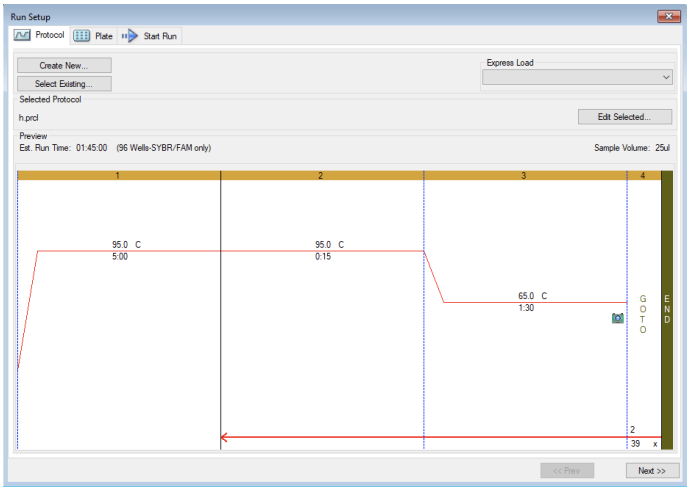
- Y Chromosome: FAM
- Cannabis DNA: HEX

1. Start the qPCR Cycling program.

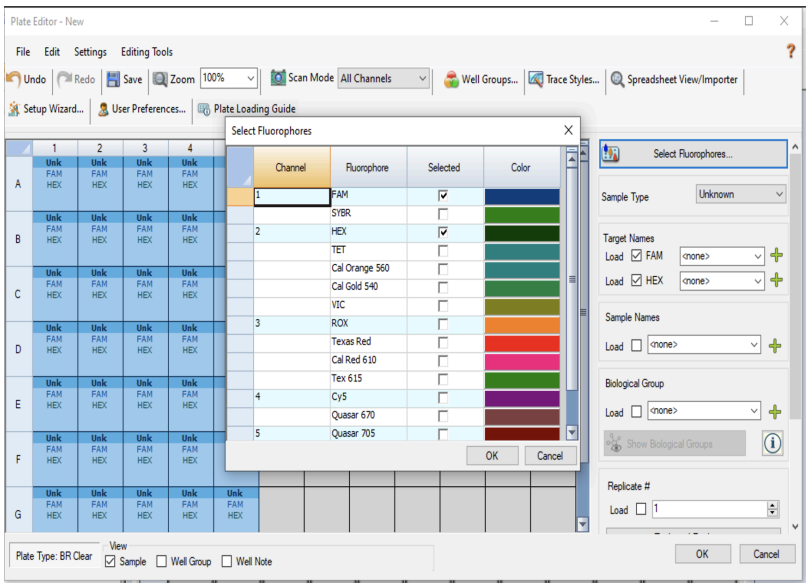


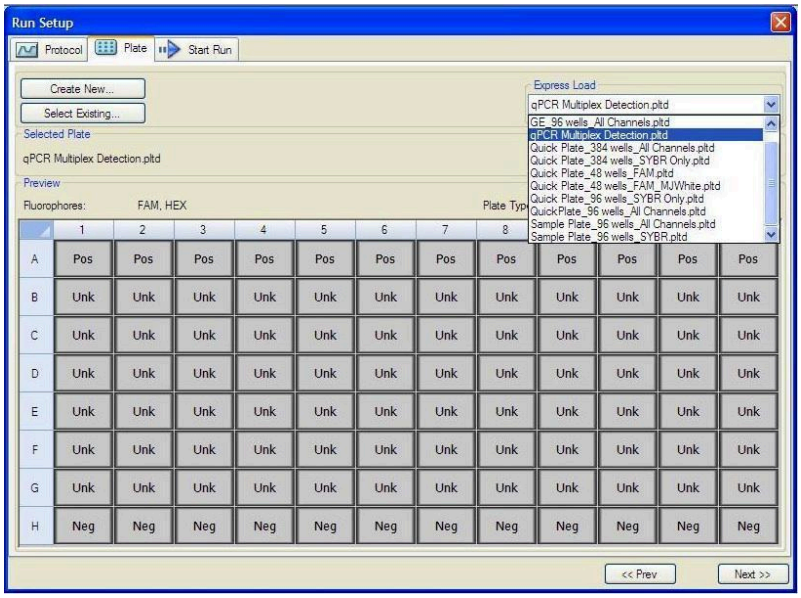
2. Select User-Defined in the Startup Wizard under Run setup.
3. Use the Express Load dropdown menu to pick the qPCR Sex Detection Program and click "Next".
4. If not already pre-programmed, create a cycling program with the following specifications and save as "qPCR Sex Detection":

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

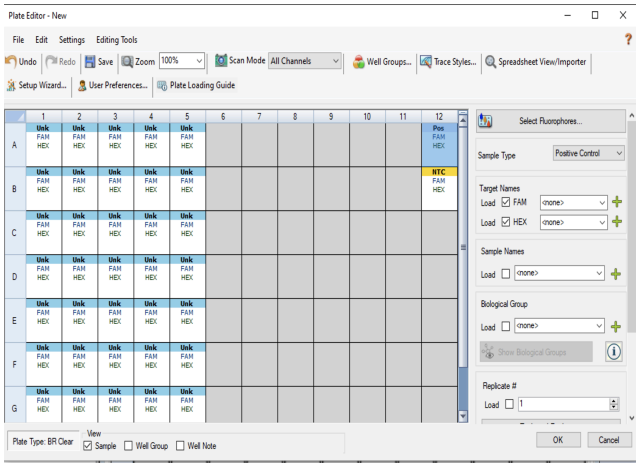
NOTE: To select the Sample Type, highlight the wells you would like to define, then choose from the dropdown menu one of three types:

Unknown

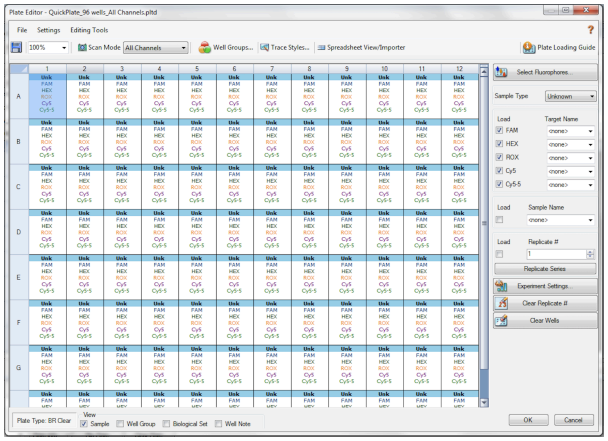
Positive Control

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.



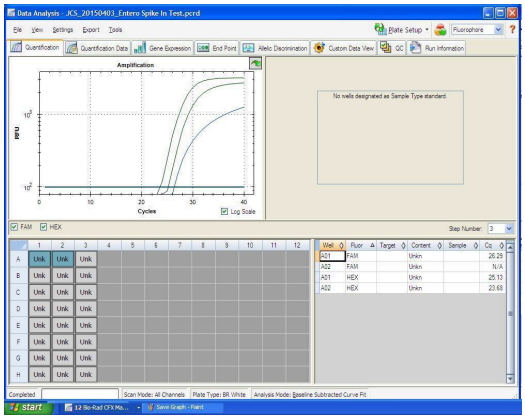
Note: Saving will override the template but will not cause any issues.

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.

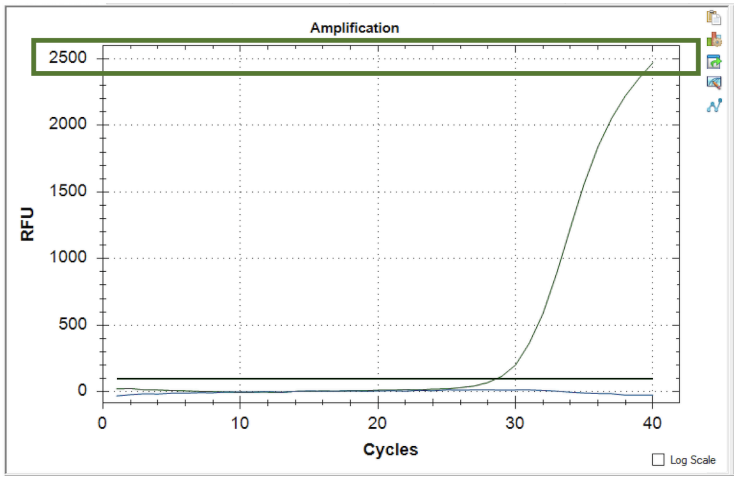
Data Analysis

1. The Data Analysis window will open automatically when the run is complete.
2. Highlight the wells 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.

iii. Alternatively, set an exact threshold value by selecting a single fluorophore at a time beneath the graph, then Settings > Baseline Threshold. In the next window select User Defined and enter 100.



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.

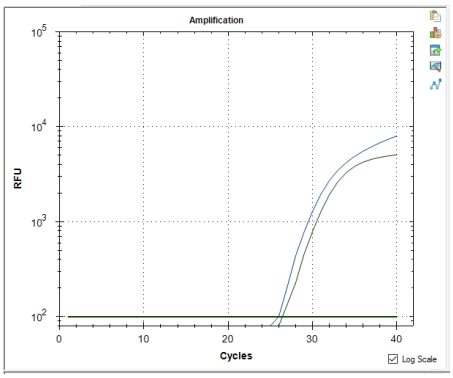


e. A Presence result for the unknown samples.

   i. Male Sample Result: Check Cq Value on the FAM Fluorophore. See the FemINDICAtor Sex Detection Assay Data Analysis Quick Reference Table 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.

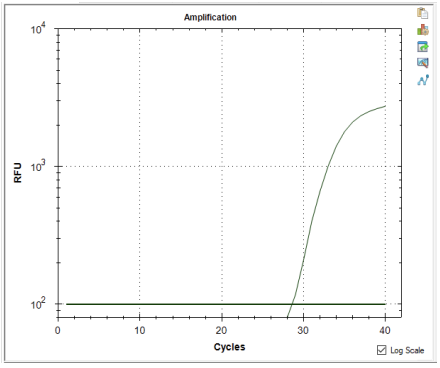


f. An Absence result for Male DNA

   i. Female Sample Result: Check Cq Value on the FAM Fluorophore. See the FemINDICAtor Sex Detection Assay Data Analysis Quick Reference Table 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** 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 control targets the cannabis genome, using the HEX Fluorophore.

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
v3	November 2025	• Updating qPCR Master Mix v3 to Amplification Mix • Update to Kit format • Removed Master Mix volume and cycling options - reverted back to original optimal parameters

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.

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