PathoSEEK® Total Aerobic Bacteria Count Assay v2 with SenSATIVAx® DNA Purification

User Guide v3

Real Time PCR (qPCR) Assay for the detection of Total Aerobic Bacteria in cannabis flower and MIP matrices

Table of Contents

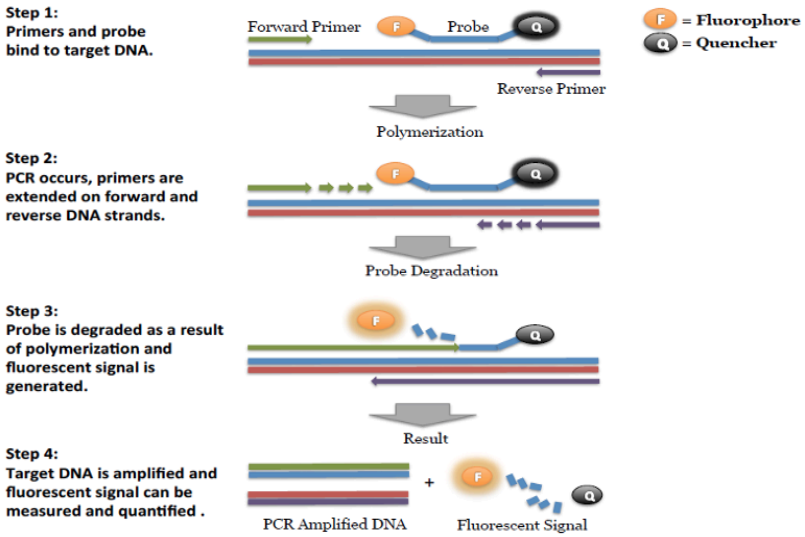
Introduction

Total Aerobic Bacteria encompasses many species. Bacteria can cause deterioration and decomposition of cannabis, and certain species of aerobic bacteria, such as Shiga Toxin producing E. coli, can cause infections in humans. Current regulations allow cannabis flower and cannabis products to contain a limit of total aerobic bacteria. The PathoSEEK® Total Aerobic Count Assay v2 with SenSATIVAx® DNA Purification Protocol is designed to detect all aerobic bacteria in a single qPCR (Quantitative Polymerase Chain Reaction) in cannabis flower, hemp flower, or non flower matrices.

Process Overview

The PathoSEEK® Total Aerobic Bacteria Count, or Total Aerobic Count (TAC) Microbial Detection Assay uses a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of aerobic bacteria as well as cannabis DNA in every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting pathogens, resulting in the minimization of false negative results due to reaction set-up errors or failing experimental conditions. Below is a simplified depiction of the qPCR assays.

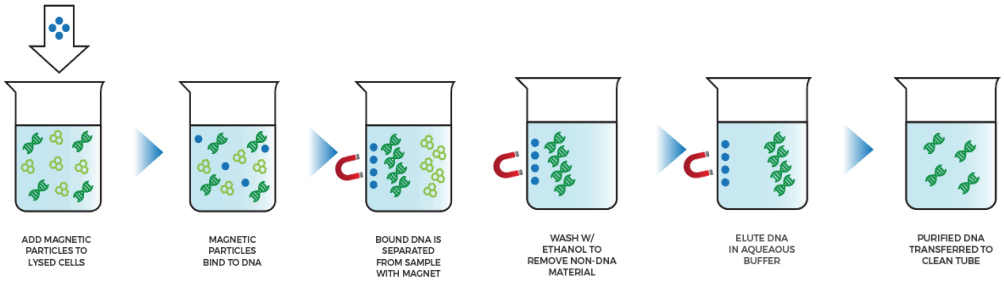
Figure 1: Overview of qPCR



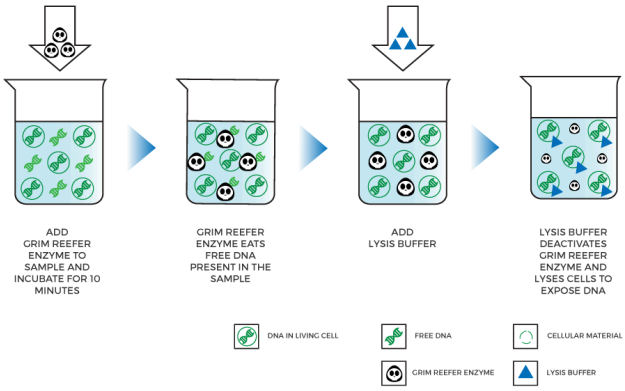
SenSATIVAx is a proprietary DNA purification process that uses magnetic particles to isolate and purify both plant and microbial DNA from a raw, homogenized plant or MIP sample. The use of magnetic particles affords 8 or 96 tip automation, enabling high throughput applications. DNA can be isolated from a single sample or a large batch in under 1 hour. Hands-on time is less than 45 minutes. The optional Grim Reefer® Free DNA Removal Kit eliminates free DNA from samples prior to qPCR analysis. Use Grim Reefer to ensure DNA from dead organisms does not inflate quantitative results from PathoSEEK® qPCR Detection Assays.

Figure 2: SenSATIVAx Extraction Protocol

NO Grim Reefer



+ Grim Reefer



Materials

SenSATIVAx® Flower & Leaf DNA Purification Kit Components - **P/N 420001**

Component Name	Qty Provided	Storage Conditions
MGC Cell Lysis Buffer	1 Bottle (12 mL)	RT (20–28 °C)
MGC Binding Buffer	1 Bottle (48 mL)	Refrigerate (2-8 ºC)
MGC Elution Buffer	1 Bottle (12 mL)	RT (20–28 °C)

SenSATIVAx® Infused Product DNA Purification Kit Components - **P/N 420004**

Component Name	Qty Provided	Storage Conditions
SenSATIVAx® Solution A	1 Bottle (350 mL)	RT (20–28 °C)
SenSATIVAx® Solution B	1 Bottle (25 mL)	RT (20–28 °C)
MGC Binding Buffer	1 Bottle (48 mL)	Refrigerate (2-8 ºC)
MGC Elution Buffer	1 Bottle (12 mL)	RT (20–28 °C)

PathoSEEK® Internal Control - **P/N 420337**

Component Name	Qty Provided	Storage Conditions
Internal Control	1 Tube (50 µL)	-15 to -20 ºC

PathoSEEK® Total Aerobic Bacteria Count (TAC) Detection Assay v3 Kit - **P/N 420541**

Component Name	Qty Provided	Storage Conditions
PathoSEEK® Total Aerobic Count Detection Assay v2	1 Tube (200 µL)	-15 to -20 ºC
qPCR Master Mix Kit v3 -Reaction Buffer	1 Tube (160 µL)	-15 to -20 ºC
qPCR Master Mix Kit v3 - Nuclease Free Water	2 Tubes (1 mL)	-15 to -20 ºC
qPCR Master Mix Kit v3 - Master Mix	1 Tube (750 µL)	-15 to -20 ºC

Optional: Grim Reef Free DNA Removal Kit - **P/N 420145**

Component Name	Qty Provided	Storage Conditions

GR Enzyme	1 Bottle (2.5 mL)	-15 to -20 ºC
GR Buffer	1 Bottle (12.5 mL)	-15 to -20 ºC

Note: Actual fill volumes include overage

Required Equipment and Supplies

Equipment:

- Bio Molecular Systems Mic 4-Channel PCR Instrument - **Medicinal Genomics P/N 420241**
    - 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**
- Agilent AriaMx Real-Time PCR System
    - 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.
    - Bio-Rad supplied or own Windows PC
- Adjustable, variable volume pipettes (single or multichannel)—P10, P20, P200 or P300, and P1000
- Adjustable, variable volume filter pipettes tips—For P10, P20, P200 or P300, and P1000
- Crushed ice
- 96 Well PCR Cryogenic Rack (optional) —VWR #89004-570
- 1.5 mL Tube Benchtop Cryogenic Rack (if no ice)— VWR #89004-558 or equivalent
- Freezer—Capable of maintaining -20 °C
- Table Top Mini Plate Centrifuge—Fisher Scientific #14-100-143 or equivalent
- Table Top Mini Centrifuge - VWR #10067-588, #2631-0006 or equivalent
- Vortex-Genie Pulse - Scientific Industries, SKU: SI-0236 or equivalent
- High Speed centrifuge - to accommodate 1.5 mL tubes such as Eppendorf model 5414R or similar with ability to spin up to speeds of at least 14,000 RCF

- Incubator - capable of maintaining 37ºC (VWR® Personal Size Incubator # 97025-630, or similar) or Heat Block capable of maintaining 37 °C (VWR Advanced Mini Dry Block Heater #10153-318) (For Grim Reefer treatment)
- Beaker or Solo Cup (optional)
- 96 Well Plate Magnet - **Medicinal Genomics P/N 420202**
- Eppendorf Tube Rack
- Scientific Scale - Capable of measuring to milligrams
- Refrigerator - Capable of maintaining 2–8°C

Supplies:

- 96-well Optical qPCR plate - **Medicinal Genomics P/N 100164**
- Adhesive optical seal for qPCR plates - **Medicinal Genomics P/N 100177**
- 96 Well Extraction Plate - **Medicinal Genomics P/N 100298**
- Filter Bags - **Medicinal Genomics P/N 100008** (Whirl-Pak #B01385WA)
- Crushed ice
- Beaker or Solo Cup. (optional)
- 1.5 mL Eppendorf Tubes
- 15 mL or 50 mL conical tubes (for MIP)
- Pipette - Aid Portable Pipetting device for serological pipettes, VWR 89166-464 or equivalent
- 25mL Serological Pipette—VWR 89130-890 or 89130-900 or equivalent
- Tryptic Soy Broth - Medicinal Genomics #420205 (Store at 2-8°C)
- 10% bleach
- 70% Ethanol **— Medicinal Genomics P/N 420030**
- Chloroform (Ethanol as preservative/ Certified ACS) - Fisher Scientific C298-1

Safety Precautions and Recommendations for Best Results

Safety Precautions

The PathoSEEK® Total Aerobic Count (TAC) is a qPCR detection assay for the rapid detection and enumeration of bacteria in cannabis matrices.

1. Assay users should observe standard microbiological 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.
3. Hazard Statement: Chloroform
    a. Harmful if inhaled or swallowed.
    b. Do not breathe vapor or mist. Do not ingest. Avoid contact with eyes, skin and clothing. Use only with adequate ventilation, which may require a chemical fume hood.
    c. Keep the container tightly closed and sealed until ready for use. Wash thoroughly after handling.
    d. Please refer to the Manufacturer Safety Data Sheet (SDS) for more information and proper disposal.

4. Hazard Statement: 70% Ethanol
    a. Highly flammable liquid and vapor may cause respiratory irritation.
    b. May cause drowsiness or dizziness. Causes damage to organs.
    c. May cause damage to organs through prolonged or repeated exposure.
    d. Please refer to the Safety Data Sheet (SDS) for more information and proper disposal.

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.

Intended User

All the components required to perform The PathoSEEK® Total Aerobic Count Assay v2 with SenSATIVAx® DNA Purification Protocol are intended to be used by trained personnel familiar with laboratory techniques associated with pathogenic organism detection.

Sample Preparation

1. Aliquot Tryptic Soy Broth (TSB).

   Note: TSB is a very good growth medium for microbes. It is best to pour the approximate amount of TSB into another sterile tube or container to avoid contaminating the whole bottle. Inspect stock of TSB for flocculants or signs of growth prior to aliquoting. Return it to the 2-8 °C refrigerator immediately after use.

2. Wipe down the workspace with a 10% bleach solution, including the benchtop and all equipment being used.
3. Remove the MGC Binding Buffer and TSB from the 2-8 °C refrigerator (it should come to room temperature, 20-28 °C, before use).
4. Prepare consumables. Label all the filter bags with "[sample name] [date]".
5. Prepare consumables. Label all the 1.5 mL centrifuge tubes needed with "[sample name]".
6. Label extraction plate with date, and if transferring eluted DNA to new plate label the destination plate also.
7. Weigh Flower or MIP samples into Whirl-pak bag or conical tubes:
   a. *Cannabis flower, **n** grams* —Weigh flower sample material into one side of the mesh liner inside the Whirl-Pak bag. Add 19 x *n* mL of TSB to each test portion. This is a 1:20 initial dilution of the sample. Close the Filter bag by folding the top over three times. Mix for 1 minute by hand.

Homogenized cannabis flower and TSB.

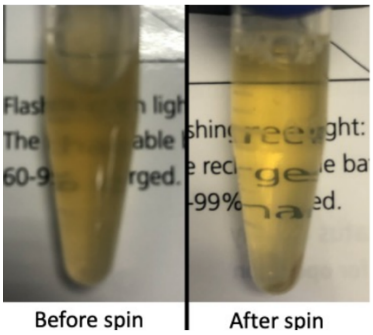
b. *MIP and concentrates, **n** grams.* — Weigh the MIP matrix into a 15 mL conical tube, 50 mL conical tube, or Whirl-Pak bag depending on MIP volume. Add 2.4 x *n* mL of TSB to each test portion. Vortex to homogenize the sample with TSB and enrich for 16-24 hours at 37°C.

8. If processing multiple samples, be sure to change gloves between each sample to ensure that there is no cross contamination between samples during the weighing process.

DNA Purification

SenSATIVAx® for Flower/Leaf DNA Extraction

1. Aspirate **1 mL** from the side of the filter bag free of plant debris and dispense into the 1.5 mL tube.
2. Spin tubes at a minimum of 14,000 RCF for 5 minutes.



Note: The supernatant should be translucent at this point. If the sample is still opaque (cloudy) spin for longer.

3. Remove and discard 950 µL of the supernatant without disturbing the pellet.
4. Resuspend the pellet by adding 200 µL of nuclease free water.
5. Pipette mix and vortex well to resuspend the pellet.

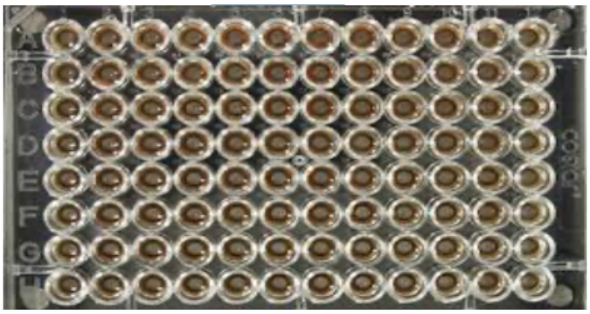
6. **(Optional) If performing the Grim Reefer extracellular DNA removal step, perform the following steps. Otherwise proceed directly to Step 7.**
    a. Add 28 µL of 10X GR Buffer and mix well by vortexing
    b. After vortexing add 5 µL of GR Enzyme and mix well by vortexing.
        i. After addition of the GR Enzyme, immediately incubate at 37 °C for 10 minutes then proceed to step 7.
7. Add 12.5 µL of MGC Cell Lysis buffer and vortex for 30 seconds then let incubate on the bench for 5 minutes.
8. Spin for at least 1-3 minutes in a bench top mini centrifuge or high speed centrifuge to pellet cellular debris.

9. Remove the 200 µL of supernatant from the 1.5 mL tube containing the centrifuged sample, being careful not to disturb the pellet at the bottom of the tube and dispense the 200 µL into the desired well of the previously labeled 96-well extraction plate.
   Note: Pellet size will vary depending on trichome density.
10. Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer.
11. Add 200 µL of MGC Binding Buffer to each sample, and pipette tip mix 15 times.
    Note: Be careful to avoid adding too many bubbles by pipetting up and down gently when tip mixing to avoid contamination of other wells within the extraction plate.
12. Incubate the plate on the bench for at least 5 minutes.
13. Place the extraction plate onto the 96-well plate magnet plate for at least 5 minutes.
14. After the 5-min incubation, remove as much of the 400 µL of the supernatant as possible. Be careful not to disturb or aspirate the beads.
15. Add 400 µL of 70% ethanol (EtOH) with the extraction plate still on the magnet plate.

16. 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.

17. 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.
18. After all the EtOH has been removed, let the beads dry at room temperature on the magnet plate for up to 15 minutes.

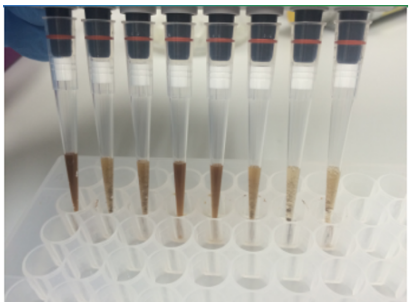


Extraction plate during wash step on magnetic plate.

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. 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.

19. Remove the extraction plate from the magnet plate and add 50 µL of MGC Elution Buffer.
    a. 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. Multichannel pipette tips showing magnetic beads resuspended in elution buffer.



Multichannel pipette tips showing magnetic beads resuspended in elution buffer

b. Incubate the plate for at least 1 minute on the bench, then return the extraction plate to the magnetic plate.
c. Let the plate sit on the magnet for at least 1 minute then transfer the eluent to a new extraction plate labeled with "Final Extract [date]".
d. 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. Leave the sealed plate on the bench for use in qPCR reactions. If not setting up qPCR right away, store at -20 °C until ready to perform the qPCR protocol.

SenSATIVAx® for MIP/Extracts DNA Purification

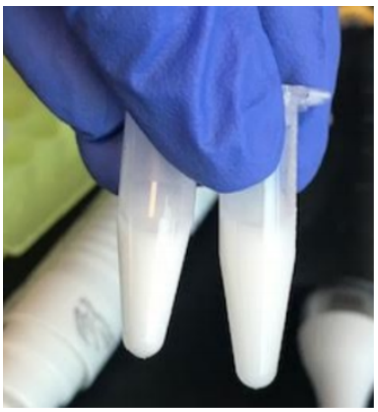
NOTE: Non flower samples should be enriched and run as 'presence/absence'. If this test results in a Cq value, the sample should be plated on culture plates of your choice to determine CFU/g.

1. Prepare an Internal Control (IC) dilution of 1:5,000
    a. Remove the IC tube from the -20 freezer and allow it to thaw completely. Once thawed, vortex to mix and centrifuge the tube to bring all contents to the bottom of the tube.
    b. Make a 1:50 dilution of the IC in a 1.5 mL snapcap tube: Transfer 2 µL of IC to 98 µL of water. Vortex thoroughly and spin down the tube.
    c. Make a 1:5,000 dilution from the 1:50 dilution prepared above: Add 2 µL 1:50 dilution to 198 µL of water. Vortex thoroughly and spin down the tube.
    Note: It's easy to mis-pipette when trying to pipette small volumes. Visually check your pipette tip after aspirating 2 µL to ensure it is in the tip before adding it to the tube for each dilution.
    d. Place on ice until use.
2. Add initial sample weight x 4.6 mL SenSATIVAx Solution A to conical tube with enriched sample/TSB. Vortex the sample vigorously until homogenized.
3. Transfer 1 mL of the homogenized sample and Solution A into a 1.5 mL tube.
4. Add 10 µL of the diluted internal control (1:5,000) to the 1.5 mL tube and vortex to mix well.
5. Centrifuge for 10 minutes at a minimum of 14,000 RCF using a high-speed bench top centrifuge.
    a. If no bench top centrifuge is available, centrifuge for 15 minutes using a mini centrifuge.
    Note: Some matrices will require the use of a high-speed centrifuge due to the presence of certain substances such as gelatin that hinder phase separation.

6. Transfer 600 µL of the solution to a new tube. Push pipette tip through the top solid layer (if one exists), without disturbing the pellet on the bottom to aspirate the sample.
7. Add 600 µL chloroform and vortex vigorously for at least 30 seconds until the solution turns a milky white color throughout.

   Note: This may require longer vortexing for some matrices

   Caution: ALWAYS WEAR GLOVES WHEN HANDLING CHLOROFORM



Example of a milky sample with chloroform in it.

8. Centrifuge for 5 minutes at a minimum of 14,000 RCF using a benchtop centrifuge.
9. If no benchtop centrifuge is available, centrifuge for 15 minutes using a mini centrifuge.
   a. **Note: If there is still any color in your aqueous layer (top layer) after centrifugation another chloroform wash may help give you a strong internal control signal (HEX) for every assay. Transfer 300 µL of the top layer to a new 1.5 mL tube and add 300 µL chloroform, vortex and centrifuge again.**
10. Transfer 100 µL of aqueous layer (TOP LAYER) to a well of the labeled 96-well extraction plate. Be careful not to disturb the lower chloroform layer.
11. Add 100 µL of SenSATIVAx Solution B to the 100 µL sample in the 96-well extraction plate and mix by pipetting up and down 5 times.
12. Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer..
13. Add 200 µL 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.
14. Incubate the plate on the bench for at least 5 minutes.
15. Place the extraction plate onto the 96-well plate magnet plate for at least 5 minutes.

16. After the 5 min incubation, remove as much of the 400 µL of the supernatant as possible. Be careful not to disturb or aspirate the beads.
    a. Add 400 µL of 70% ethanol (EtOH) with the extraction plate still on the magnet plate.
    b. Wait at least 30 seconds and remove all the EtOH.

    Note: Place the pipette tip at the bottom center of the well to remove all liquid.
17. 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 pipette tip to remove the excess. Leftover EtOH can inhibit qPCR efficiency.
18. After all the EtOH has been removed, let the beads dry at room temperature. Allow the beads to dry for up to 15 minutes. Necessary drying time will vary based on complete removal of the second ethanol wash, as well as lab environment. Visually inspect beads for residual ethanol before the elution step.

    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.
19. Remove the extraction plate from the magnet plate and add 50 µL of MGC Elution Buffer.
    a. 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.
    b. Incubate the plate for at least 1 minute on the bench, before returning the plate to the magnetic plate.
    c. Let the plate sit on the magnet for at least 1 minute before transferring the eluent to a new extraction plate labeled with "Final Extract [date]".
20. 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. Store at -20 °C until ready to perform the qPCR protocol.

Real-Time Quantitative PCR (qPCR) Setup Protocol

1. Remove qPCR reagents including qPCR Master Mix v3, water, reaction buffer, positive controls and detection assays 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 master mix, invert or vortex and spin-down the reagents.
    a. Detection Assay tubes, reaction buffer, positive controls and water – Vortex quickly followed by a pulse spin-down in a microcentrifuge.
    b. qPCR Master Mix v3 – 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 a separate master mix in a 1.5 mL tube for each assay type being run. All detection assays contain the internal plant control, IC probe mix, and the probe for the microbial targets. Label each tube with [Assay Name] MM. Always prepare enough master mix for 1 or 2 additional reactions over the total number of tests to account for pipetting and dead volumes.

    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 v3	3.75 µL	93.75 µL	187.5 µL
Detection Assay	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 MM	13.75 µL	343.75 µL	687.5 µL

5. Once combined gently, tip mix or invert the tube 5 times to combine the assay master mix.
    a. Pulse spin-down tube in microcentrifuge.
    b. Place qPCR Master Mix tubes on ice until used.
6. For the positive control, make a 1:10 dilution of stock
    a. Allow positive control to thaw. Vortex and spin before use.
    b. Add 1 µL of Positive Control to 9 µL nuclease free water (found in the kit) and vortex to mix.
    c. 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. Place the Extraction Plate on the magnet. This is to ensure that no magnetic beads are transferred into the qPCR reactions if there are some left over from the extraction elution process.
8. Use a 96-well optical qPCR plate and label the plate "qPCR Plate_ [date]".
9. Carefully remove the seal from the Extraction Plate. If frozen, let the DNA thaw completely and spin the plate before removing the seal to avoid cross contamination between samples. After thawing, tip mix samples to ensure the DNA is in solution.

   Note: ALWAYS use a fresh tip for every liquid transfer into the qPCR plate.
10. Transfer 5 µL of each sample into the corresponding well on the qPCR plate, keeping the extraction plate on the magnet when aspirating the 5 µL.
11. Add 5 µL of the diluted Positive Control to the corresponding well.
12. Add 5 µL of water to the corresponding negative control well.
13. Add 13.75 µL of Total Aerobic Count 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 air bubbles during this mix.
14. Seal the plate with strip caps or an optically clear adhesive seal.
15. 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.

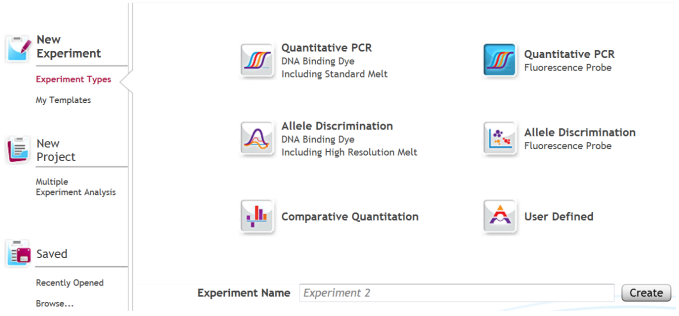
    Note: Check for bubbles at the bottom of the wells (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the bottom of the wells, spin-down for another minute.
16. For the Agilent Aria: If using an adhesive seal; place the reusable compression pad (gray side down) on the plate directly lining up the holes in the pad with the holes in the plate.
17. Place the sealed plate onto the PCR instrument, positioning the A1 well in the top left corner.
18. Follow the software specific instructions to initiate the run.

Running the Agilent AriaMX

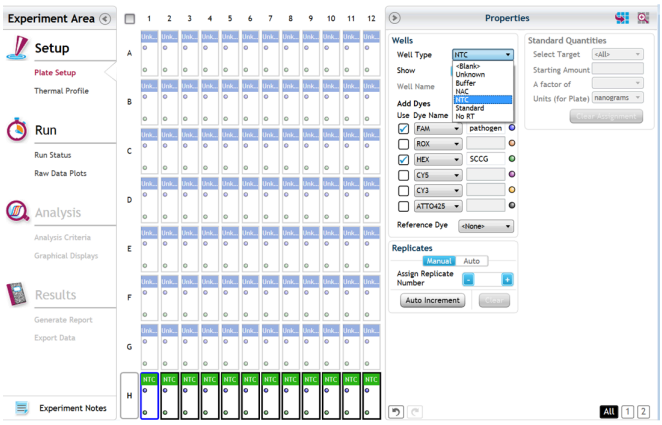
Note: The following species will be detected on the following Fluorophores:

- Total Aerobic Bacteria: FAM
- Cannabis DNA: HEX

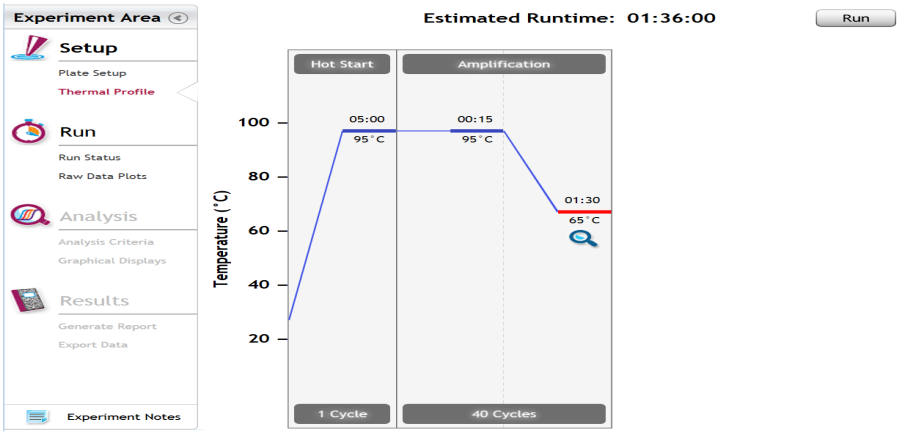
1. Create a New Experiment on the Agilent qPCR instrument.
2. Select “Quantitative PCR Fluorescent Probe” from Experiment Types. Under Setup>Plate Setup, highlight wells that contain reactions and select FAM and HEX under Add Dyes.



3. Change the well types to reflect your plate set up. If desired, add target names to include “pathogen name” for FAM and IC for HEX.



4. Under Setup>Thermal Profile, create the following 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 lid and click “Start 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 on the Agilent AriaMX

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

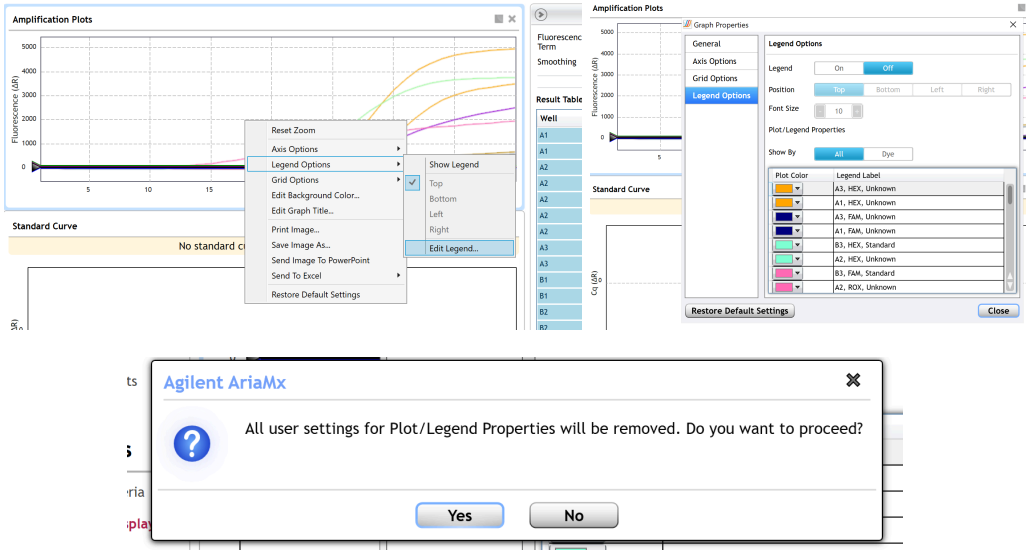
b. The Cq values will appear to the right in the table

c. Right click inside the graph, select Edit Legend under Legend Options

d. Change “All” to “Dye”

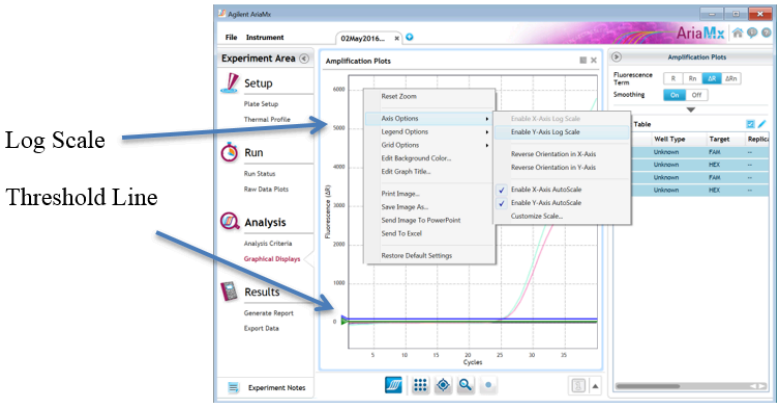
e. All user settings for Plot/Legend Properties will be removed. Do you want to proceed? Select “Yes”.

f. This will assign a single color to each fluorophore.

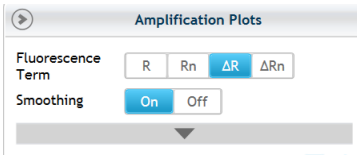


3. To analyze the results:

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

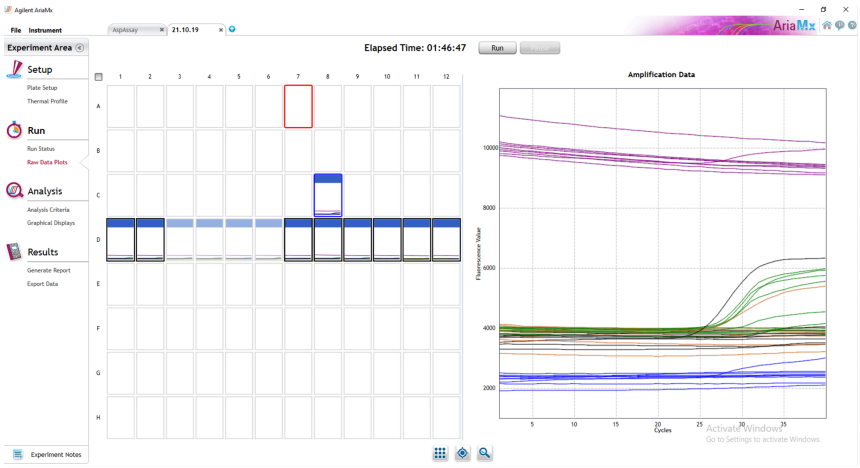


b. Expand the amplification plots settings by clicking on the triangle (shown below).

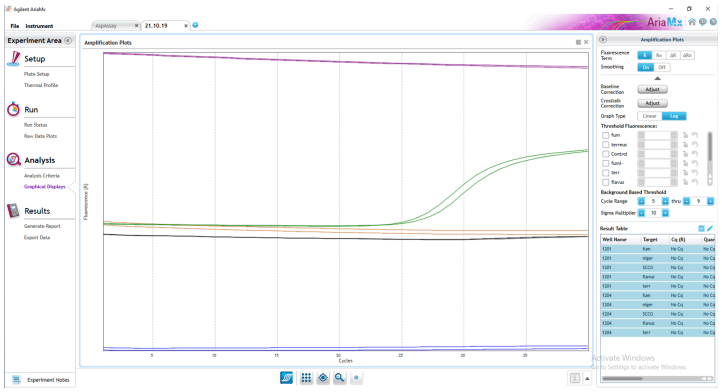


c. Manually adjust and lock thresholds to 100 RFU for the FAM and HEX fluorophores.

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 a Cq value of > 30 or 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 flower samples, < 40 for all other matrices.
      i. Visually confirm with the curve on the graph.
5. Unknown TAC Targets
   a. Check for a Cq Value on the FAM Fluorophore.
   b. If there is a Cq value, visually confirm Cq value with the curve on the graph to confirm amplification. Sometimes background amplification will give a false positive reading, especially when Cq reading is less than 15 (See troubleshooting guide below for more details). It is very important to check the raw data view (R) to confirm with the amplification curve when a presence result occurs.
      i. To review the raw data for each sample, select the wells of interest. Use the plate view of the Raw Data Plots to scroll across the plate to confirm amplification in sample wells and positive control wells. This review also ensures that thermal cycling is uniform across the plate.



ii. Look at the raw data in the "Graphical Displays" view. For every dye that gives a Cq make sure the baseline is flat and the fluorescence signal grows rapidly over 5-7 cycles as seen below. (To view the raw data, select "R" next to "Fluorescence Term" at the top right-hand side of the screen.)



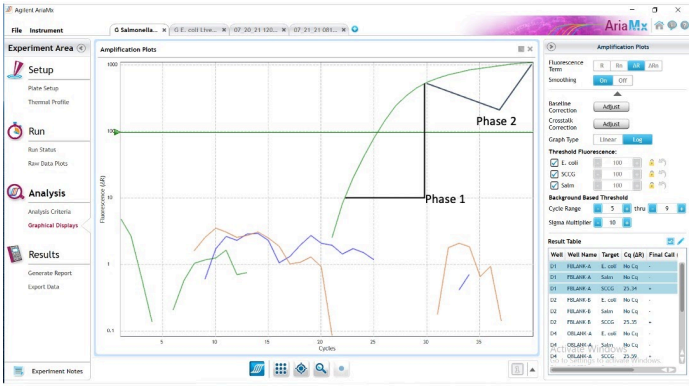
Example of a good baseline with sigmoidal shaped curve increase in the internal control fluorescence.

iii. Check the amplification plots for a semi-logarithmic curve with two distinct phases that crosses the threshold.

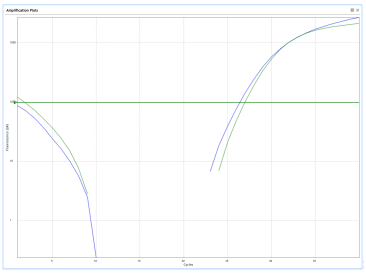
1. Select the ΔR Fluorescence Term and look at the shape of the amplification plot. The graph should have the classic semi-logarithmic shape with two visually distinct phases.
2. Phase 1 shows exponential growth in the fluorescence, in a span of around 5 cycles.

3. Phase 2 is a plateau where the amplification signal growth ends but remains level.

Example of a semi-logarithmic shape with two visually distinct phases:



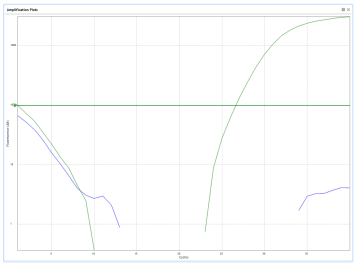
Example of a presence result:



iv. Flower: Determine resulting CFU/g by plugging the FAM Cq into the equation in the Cq to CFU conversion equation table. Assess the resulting CFU/g value against your local regulations allowable limits to determine if the sample has passed or failed.

v. Non-Flower: If this test results in a Cq value indicating the presence of the target organisms, an unenriched sample should be plated on culture plates of your choice to determine enumeration.

Example of an absence result:



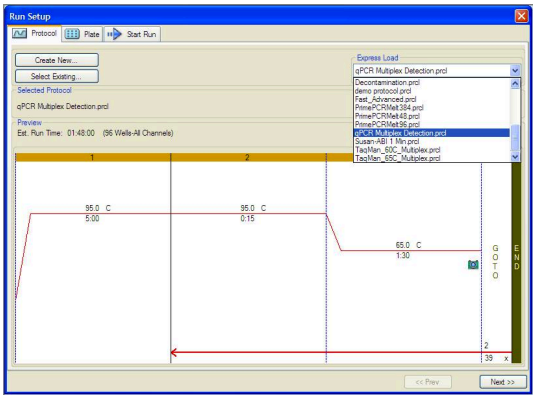
Running the BioRad CFX96

The following targets will be detected on the following fluorophores:

- TAC: FAM
- Cannabis DNA: HEX

1. Start the qPCR Cycling program.
2. Select User-Defined in the Startup Wizard under Run setup.

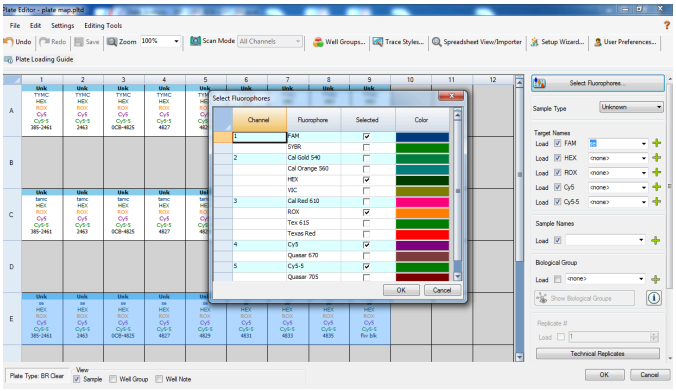
3. Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click "Next".
4. If not already pre-programmed, create a cycling program with the following specifications and save as "qPCR Multiplex Detection":
    a. Hot Start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC for 15 seconds and 65ºC for 1 minute, 30 seconds.

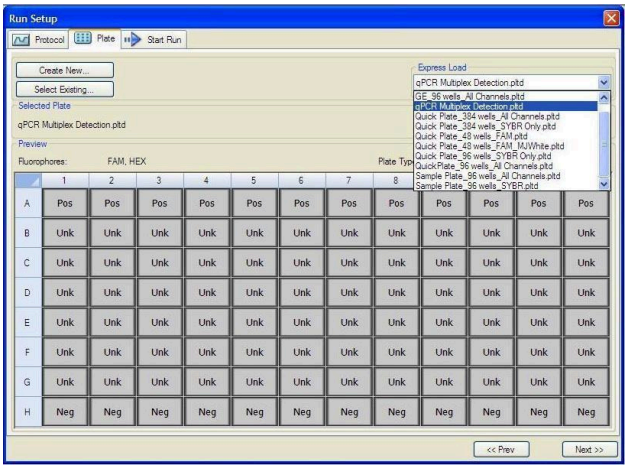


5. Design your plate under the plate tab in the Run Setup.

a. Select the qPCR Multiplex 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”.





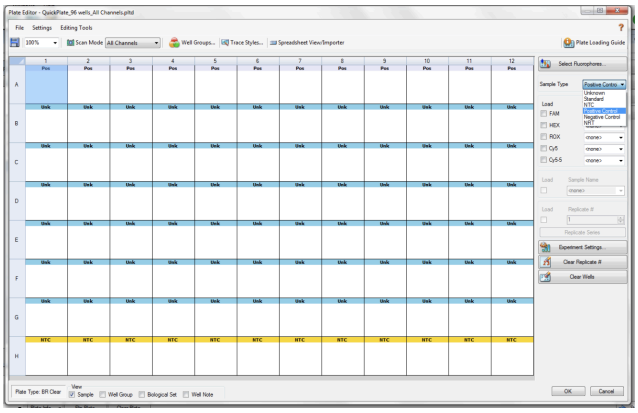
c. If plate layout previously saved, click “Edit Selected” to move to the Plate Editor Screen.

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:

1. **Unknown**
2. **Positive Control**
3. **Negative Control**

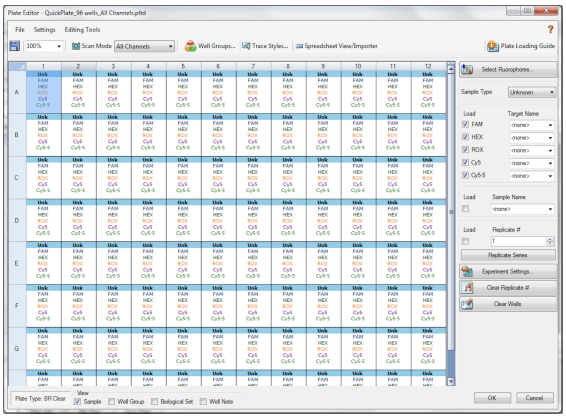
e. Make sure “All Channels” is selected from the dropdown menu at the top.



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



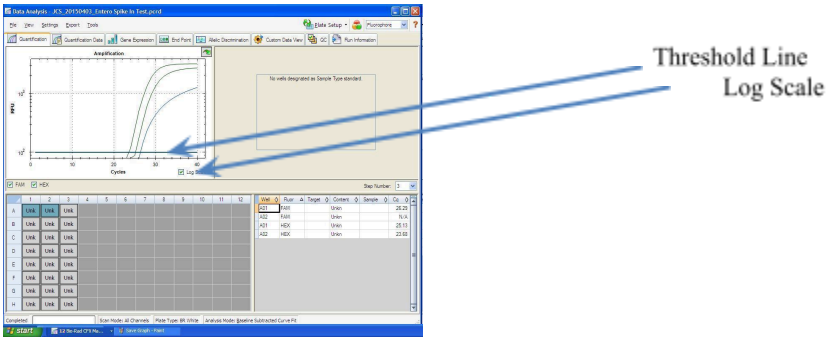
Note: Saving will override the template but will not cause any issues.

6. Close the lid and click Start Run.
7. Save the experiment with the [User] and [date].
8. 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.

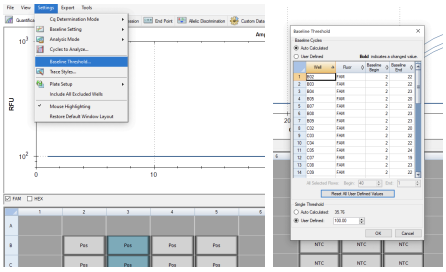
Data Analysis on the BioRad CFX96

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

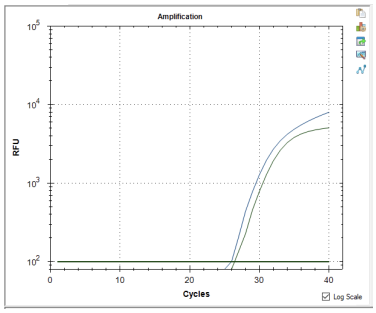


6. Controls
    a. Assay-specific Positive Control, on the FAM fluorophore, has a Cq value ≤ 35.
    b. Visually confirm with the curve on the graph.
    c. Assay-specific Negative Control, on the FAM fluorophore has a Cq value of > 30 or no Cq value.
        i. Visually confirm with the curve on the graph.

d. Internal Control, on the HEX Fluorophore, has a Cq value ≤ 35 for flower samples, < 40 for all other matrices.
    i. Visually confirm with the curve on the graph.

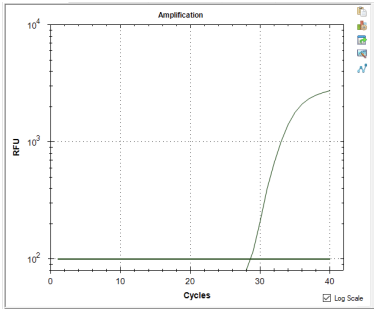
7. Unknown TAC Targets
    a. Check for a Cq Value on the FAM Fluorophore.
    b. If there is a Cq value, visually confirm Cq value with the curve on the graph to confirm amplification. Sometimes background amplification will give a false positive reading, especially when Cq reading is less than 15 (See troubleshooting guide below for more details).

Example of a presence result:



    i. Flower: Determine resulting CFU/g by using the equation in the Cq to CFU conversion equation table. Assess the resulting CFU/g value against your local regulations allowable limits to determine if the sample has passed or failed.
    ii. Non-Flower: If this test results in a Cq value indicating the presence of the target organisms, an unenriched sample should be plated on culture plates of your choice to determine enumeration.

Example of an absence result:



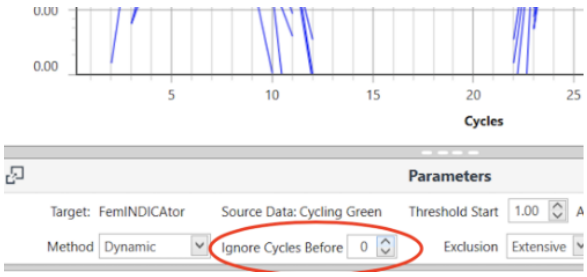
Running the Bio Molecular Systems Mic Real-Time PCR Instrument

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: Hot start at 95 ºC for 5 minutes, followed by 40 cycles of 95 ºC for 15 seconds and 65 ºC 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 with BioMolecular System Mic

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 TAC.
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. The software will determine the samples as TAC detected, TAC not detected, or low IC rerun sample.
5. Data may be exported by selecting the "Report" tab, then clicking the Export icon. Use the FAM Cq values to determine CFU/g using the equation below.

Table I: Cq to CFU Conversion Equation Table

Matrix	Microbial Test	Cq to CFU Conversion Equation
Flower	Total Aerobic Count	CFU/g =10^((-0.2383*Cq)+10.005) Multiply resulting CFU x 20 to account for upfront dilution factor
Non Flower	Total Aerobic Count	IF Cq<40 after enrichment, Plate confirm for enumeration

Conversion Equation Example

A resulting Cq value for a flower sample should be plugged into the equation in the table above. See step by step instructions below. The following example assumes a resulting FAM Cq value of 31.0.

1. Multiply the Cq value by -0.2383
   = (31 x -0.2383) = -7.3873
2. Add 10.005 to result in step 1
   = -7.3873 + 10.005 = 2.6177
3. Raise 10 to the power of the result in step 2.
   = 10^2.6177 = 414.67
4. Multiply the result in step 3 by 20 = 414.67 x 20
   = Approximately 8,293 CFU/gram TAC detected in cannabis flower
5. Determine if the resulting CFU/g result is above or below the action limit set by the regulatory body.

Troubleshooting Guide

Symptom	Reason	Solution
Internal Control Failure	DNA purification failure	Repeat SenSATIVAx and PathoSEEK by following the protocol.
	Residual ethanol in elution	Ethanol is an inhibitor to PCR. Return to the SenSATIVAx protocol and repeat all steps.
	Mix up in qPCR Reaction Setup	Repeat the qPCR by following the protocol.
	Missing Fluorophore on plate set up	Check plate setup in qPCR platform data file and ensure the correct fluorophores were chosen for the assay being run.
	Matrix related (ex: age, remediation, ground)	Repeat SenSATIVAx and PathoSEEK following the protocol. If unacceptable internal control results are obtained a second time, Internal Cannabis Control may be spiked into samples during extraction for the third run. Please visit our Help Center for more information.
	qPCR inhibition	Dilute Elution 1:10 with nuclease free water and re-perform qPCR
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	Cannabis 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 DNA purification area	Designate separate benches, pipettes etc. for DNA purification and qPCR setup
Positive Negative Control	Low 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	Ensure correct well location was chosen for assay positive control. If yes, 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 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 and Rox Fluorophores.

Amplification of the **Internal Cannabis 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.

STEC is short for Shiga Toxin producing E coli.

REVISION HISTORY

Version	Date	Description
v1	September, 2021	1. Update to User Guide Format 2. Updated sample to media ratio used for homogenization of flower 3. Update to qPCR Master Mix v3 4. Update to conversion equations
v2	November 2022	1. Updated sample to media ratio used for homogenization of flower 2. Update to conversion equation for flower matrix 3. Update MIP detection to a presence absence, removal of conversion equation for MIP matrices.
v3	August 2025	1. New kitted packaging format 2. New assay version (v2) to include use with BMS Mic and Myra 3. BMS Mic and Myra data analysis section 4. Update to Internal Control from SCCG to IC

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 factors known to affect the growth of bacterial and fungal species.

LIMITED USE LABEL LICENSE

This product is covered by at least one or more claims of US patent applications, which are exclusively licensed to Medicinal Genomics Corporation. This product is sold strictly for the use of the buyer, and the buyer is not authorized to transfer this product [or any materials made using this product] to any third party.