

PathoSEEK[®] Bile Tolerant Gram Negative Detection Assay with SenSATIVAx[®] DNA Extraction

User Guide

Real Time PCR (qPCR) Assay for the detection of Bile Tolerant Gram Negative Bacteria in cannabis flower and MIP matrices

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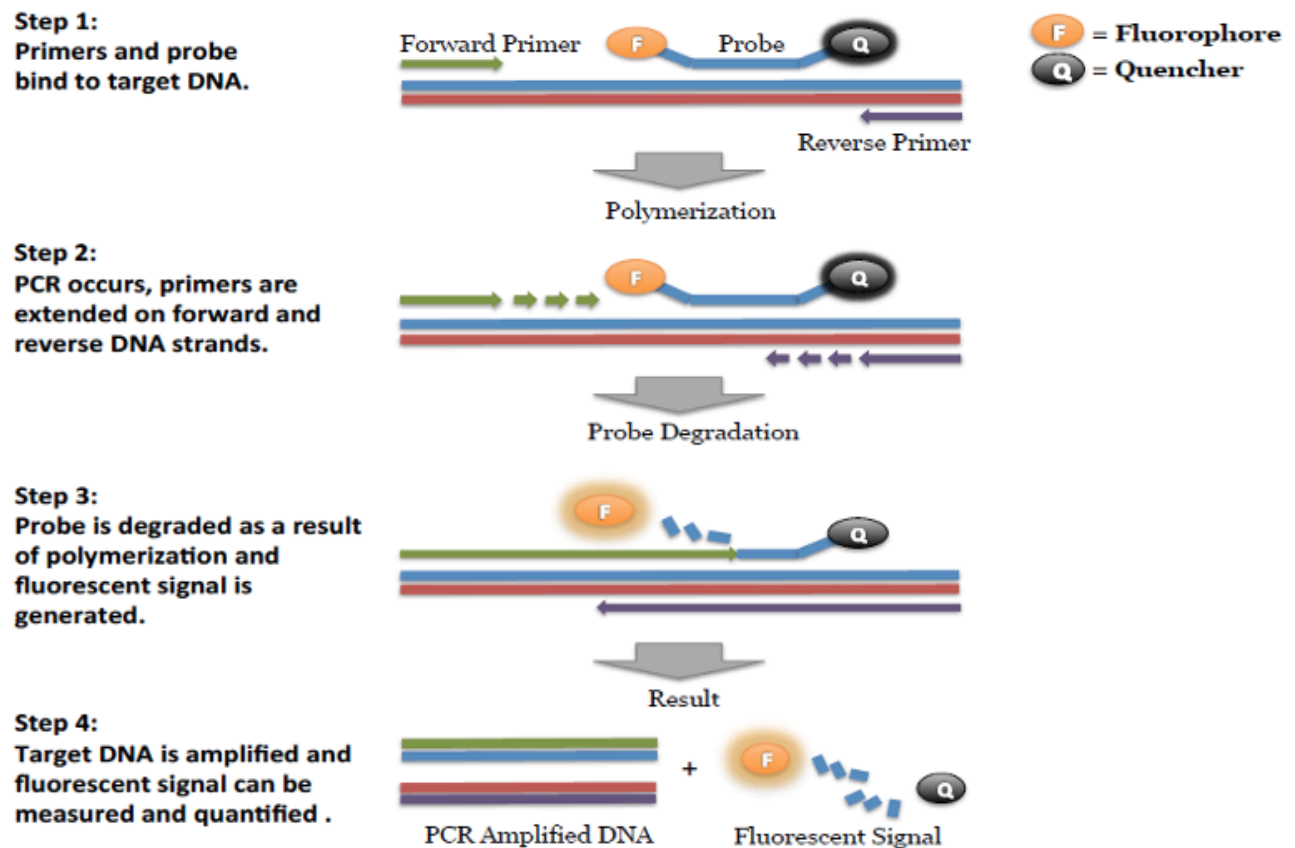
Introduction

Bacteria that fall into the Bile Tolerant Gram Negative (BTGN) families encompass many species. Bacteria can cause deterioration and decomposition of cannabis, and certain species of 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 BTGN bacteria. The PathoSEEK® Bile Tolerant Gram Negative Assay with SenSATIVAx® Extraction Protocol is designed to detect all BTGN bacteria in a single qPCR (Quantitative Polymerase Chain Reaction). The PathoSEEK® Bile Tolerant Gram Negative Detection Assay with SenSATIVAx® Extraction Protocol is a DNA extraction and qPCR method for the rapid detection of all BTGN bacteria in cannabis flower and non flower matrices.

Process Overview

PathoSEEK® Bile Tolerant Gram Negative Detection Assay with SenSATIVAx® Extraction Protocol is a novel, real-time quantitative PCR assays that uses a multiplex system of primers to detect Bile Tolerant Gram Negative bacteria in cannabis flower and non flower matrices. Figure 1 shows a simplified depiction of the qPCR assay.

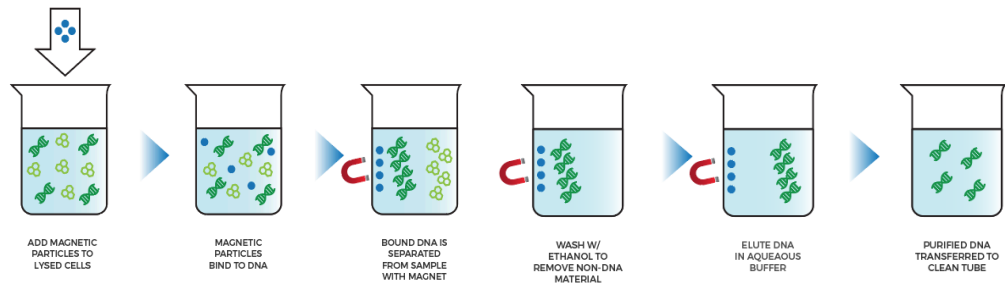
Figure 1: Overview of qPCR



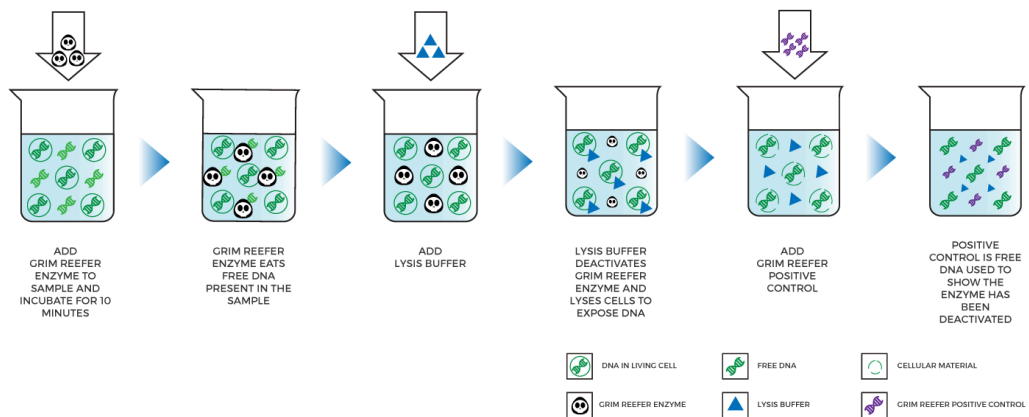
SenSATIVAx is a proprietary DNA isolation process that uses magnetic particles to isolate and purify both plant and microbial DNA from a raw, homogenized plant or MIP sample. The use of magnetic particles affords 8 or 96 tip automation, enabling high throughput applications. DNA can be isolated from a single sample or a large batch in under 1 hour. Hands-on time is less than 45 minutes.

Figure 2: SenSATIVAx Extraction Protocol

NO Grim Reefer



+ Grim Reefer



Materials and Methods

Test Kit Name: PathoSEEK® Bile Tolerant Gram Negative Assay with SenSATIVAx® Extraction

Test Kit Information

1. SenSATIVAx® Flower/Leaf DNA Extraction Kit - P/N 420001
2. SenSATIVAx® MIP/Extract DNA Extraction Kit - P/N 420004
3. Medicinal Genomics qPCR Master Kit v3 - P/N 420201
4. PathoSEEK® Bile Tolerant Gram Negative Detection Assay v2 - P/N 420149
5. PathoSEEK® Bile Tolerant Gram Negative / Coliform / Enteric Multiplex Positive Control
- P/N 420353
6. Grim Reefer Free DNA Removal Kit - P/N 420145
7. Grim Reefer Free DNA Removal Control - P/N 420144
8. Grim Reefer Free DNA Removal Assay - P/N 420143

Test Kit Components

1. SenSATIVAx® Flower/Leaf DNA Extraction Kit - P/N 420001 (200 tests).
 - a. MGC Lysis Buffer— 1 bottle (Store at 20-28°C). Expires 1 Year from Date of Manufacture.
 - b. MGC Binding Buffer—1 bottle (Store at 2-8°C). Expires 1 Year from Date of Manufacture.
 - c. MGC Elution Buffer—1 bottle (Store at 20-28°C). Expires 1 Year from Date of Manufacture.

2. SenSATIVAx MIP/Extract DNA Extraction.— P/N 420004 (200 tests).
 - a. Binding Buffer — 1 bottle. Store at 2-8°C. Expires 1 Year from Date of Manufacture
 - b. Elution Buffer — 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture
 - c. Solution A — 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture
 - d. Solution B — 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture
3. Grim Reefer Reagents (Optional Step)
 - a. Grim Reefer Free DNA Removal Kit - 1 kit (Store at -20°C). Expires 2 Years from Date of Manufacture.
 - b. Grim Reefer Free DNA Removal Control - 1 tube (Store at -20°C). Expires 2 Years from Date of Manufacture.

Grim Reefer Free DNA Removal Assay - 1 tube (Store at -20°C). Expires 2 Years from Date of Manufacture.
4. qPCR Master Kit v3 - P/N 420201. Kit (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture
 - a. Reaction Buffer (10x).— 1 tube
 - b. Nuclease Free Water.—2 tubes
 - c. qPCR Master Mix — 1 tube

5. PathoSEEK[®] Bile Tolerant Gram Negative Detection Assay v2 - P/N 420149 (200 reactions).
 - a. Assay — 1 tube (Store kit at -15 to -20°C). Expires 2 Years from Date of Manufacture.
6. PathoSEEK[®] Bile Tolerant Gram Negative / Coliform / Enteric Multiplex Positive Control - P/N 420353 (50 reactions)
 - a. Control — 1 tube (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture.

Supplies, Reagents, and Equipment

1. Agilent AriaMx Real-Time PCR System G8830A—Containing the following Optical Channels: FAM and HEX and Cy5 (if using optional Grim Reefer).
 - a. Agilent HP Notebook PC option 650
 - b. 96 Well Optical qPCR plate - Medicinal Genomics #100164
 - c. Adhesive optical seal for qPCR plates - Medicinal Genomics #100177
 - d. Optical Strip Caps.—Agilent #401425

Note: If using adhesive seals instead of strip caps, use Applied Biosystems MicroAmp Optical Film Compression Pad, Fisher Scientific, #43-126-39 to prevent evaporation and cross contamination between wells.
2. Bio-Rad CFX96 Touch[™] Real-Time System.
 - a. Bio-Rad Personal PC
 - b. 96 Well Optical qPCR plate — Medicinal Genomics 100164

- c. Adhesive optical seal for qPCR plates — Medicinal Genomics 100177
3. Adjustable, variable volume pipettes (single or multichannel)—P10, P20, P50, P200 or P300 and P1000
 4. Adjustable, variable volume filter pipettes tips—For P10, P20, P50, P200, P300 and P1000
 5. Crushed ice
 6. 96 Well PCR Cryogenic Rack (optional) —VWR #89004-570
 7. 1.5 mL Tube Benchtop Cryogenic Rack (if no ice)— VWR #89004-558 or equivalent
 8. Freezer—Capable of maintaining -20°C
 9. Table Top Mini Plate Centrifuge—Fisher Scientific #14-100-143 or equivalent
 10. Table Top Mini Centrifuge—VWR #10067-588, #2631-0006 or equivalent
 11. Vortex-Genie Pulse—Scientific Industries, SKU: SI-0236 or equivalent
 12. High Speed centrifuge— to accommodate 1.5mL tubes such as Eppendorf model 5414R or similar with ability to spin up to speeds of 14,000 - 19,300 RCF
 13. Incubator— capable of maintaining 37°C (VWR® Personal Size Incubator # 97025-630, or similar)
 14. Filter Bags—Whirl Pak #B01385WA
 15. Beaker or Solo Cup(optional)
 16. Tryptic Soy Broth—Medicinal Genomics #420205 (Store at 2-8°C)
 17. 1.5 mL Eppendorf Tubes
 18. 15 mL or 50 mL conical tubes (for MIP)

19. 96 Well Plate Magnet—Medicinal Genomics P/N 420202
20. 96 Well Extraction Plate— Medicinal Genomics P/N 100298
21. Eppendorf Tube Rack
22. Scientific Scale—Capable of measuring to milligrams
23. Refrigerator—Capable of maintaining 2–8°C
24. 25 mL Serological Pipette —VWR 89130-890 or 89130-900 or equivalent.
25. 10% bleach.
26. 70% Ethanol — MGC P/N 420030
27. Chloroform (Ethanol as preservative/ Certified ACS) - Fisher Scientific, C298

Safety Precautions and Recommendations for Best Results

Safety Precautions

The PathoSEEK[®] Bile Tolerant Gram Negative Detection Assay 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: 70% Denatured 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.



4. 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



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

The Bile Tolerant Gram Negative Detection Assay with SenSATIVAx[®] Extraction and all components required to perform the method are intended for use 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 bench top 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 1.5mL 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 $19 \times n$ 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. Homogenize for 1 minute by hand.

Homogenized cannabis flower and TSB.

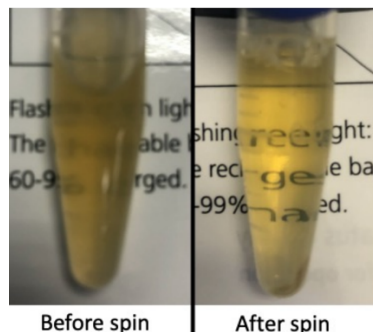


8. *MIP and concentrates, n grams.* — Weigh the MIP matrix into a 15 mL conical tube, 50 mL conical tube, or Whirl-Pak bag depending on MIP volume. Add $2.4 \times n$ mL of TSB to each test portion. Vortex to homogenize the sample with TSB and enrich for 16-24 hours at 37°C.
9. If processing multiple samples, be sure to change gloves between each sample to ensure that there is no cross contamination between samples during the weighing process.

DNA Extraction

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 1.5mL tube.
2. Spin tubes at 19,300 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 950ul of the supernatant without disturbing the pellet.
4. Resuspend the pellet by adding 200uL of nuclease free water.
5. Pipette mix and vortex well to resuspend pellet.
6. (Optional) If Performing the Grim Reefer extracellular DNA removal step, perform the following steps. Otherwise proceed directly to Step 7.
 - a. Add 28μL of 10X GR Buffer and mix well by vortexing
 - b. After vortexing add 5μL of GR Enzyme and mix well by vortexing.
 - i. After addition of the GR Enzyme immediately incubate at 37C for 10 minutes then proceed to step 7.

7. Add 12.5 μ L of MGC Lysis buffer and vortex for 30 seconds then let incubate on the bench for 2 minutes.

- a. If processing with Grim Reefer Free DNA Removal add 2.5 μ L of the diluted GR Positive control (1:10,000) after 2 minute incubation and mix by vortexing.

Note: The GR Positive Control is used to show the GR enzyme was completely deactivated during the lysis step.

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 μ L of supernatant from the 1.5ml tube containing the centrifuged sample, being careful not to disturb the pellet at the bottom of the tube and dispense the 200 μ L 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 for at least 30 seconds.

11. Add 200µL of MGC Binding Buffer to each sample, and pipette tip mix 15 times.

Note: Be careful to avoid adding too many bubbles by pipetting 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 400µL of the supernatant as possible.

Be careful not to disturb or aspirate the beads.

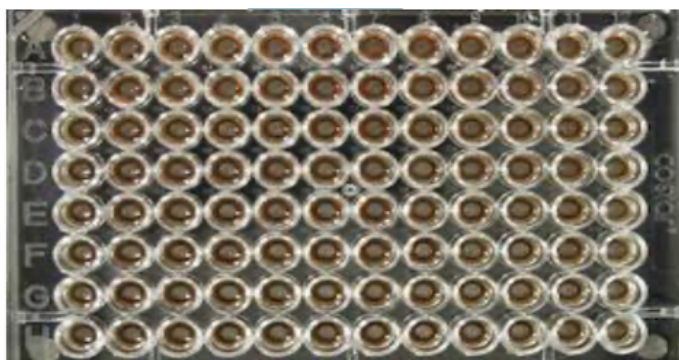
15. Add 400µL of 70% denatured ethanol (EtOH) with the extraction plate still on the magnet plate.

16. Wait at least 30 seconds and remove all the EtOH.

Note: Place the 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.

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



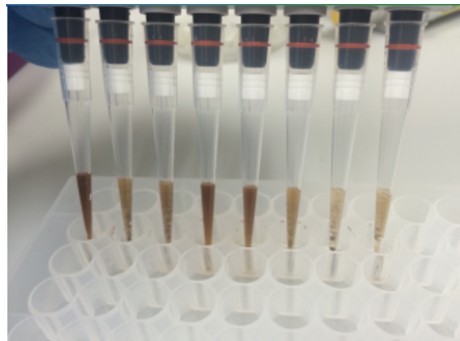
Extraction plate during wash step on magnetic plate.

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. 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 μ L 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

- 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. Store at -20°C until ready to perform the qPCR protocol.

SenSATIVAx[®] for MIP/Extracts DNA Extraction

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

a) Prepare a SCCG positive control dilution of 1:5,000 (internal control)

(1) Label a new 1.5mL Eppendorf tube (SCCG 1:50), add 1µl of SCCG positive control into 49µl of dH₂O. Vortex to mix thoroughly and quick spin tube. Label another 1.5mL Eppendorf tube (SCCG 1:5,000), add 99µl of dH₂O, then add 1µl of the SCCG 1:50 dilution. Vortex to mix thoroughly and quick spin tube. This will result in a 1:5,000 dilution of SCCG.

Note: It's easy to mis-pipette when trying to pipette only 1 µL of liquid. Visually check your pipette tip after aspirating 1 µL to ensure it is in the tip before adding it to the tube for dilutions 1 and 2.

(2) Place on ice until use.

Note: The 100 µL dilution prepared above is enough diluted SCCG for approximately 10 extractions. If more extractions are going to be prepared at the same time, the initial 1:50 dilution can be used to make multiple 1:5,000 dilutions of SCCG.

c) Add initial sample weight x 4.6 mL SenSATIVAx Solution A to conical tube with enriched sample/TSB. Vortex the sample vigorously until homogenized.

c) Transfer 1 mL of the homogenized sample and Solution A into a 1.5 mL tube.

d) Add 10µL of the SCCG internal control (1:5,000) to 1.5 mL tube and vortex to mix well.

e) Centrifuge for 10 minutes at 19,300 RCF using a high-speed bench top centrifuge.

(1) 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.

f) Transfer 600 μ L 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.

g) Add 600 μ L chloroform and vortex vigorously 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



Fig 7. Example of a milky sample with chloroform in it.

h) Centrifuge for 5 minutes at 19,300 RCF using a bench top centrifuge.

i) If no bench top centrifuge is available, centrifuge for 15 minutes using a mini centrifuge.

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.

j) Transfer 100µL of aqueous layer (TOP LAYER) from Step 7 to a well of the labeled 96 well extraction plate. Be careful not to disturb the lower chloroform layer.

k) Add 100µL of SenSATIVAx Solution B to the 100ul sample in the 96 well extraction plate and mix by pipetting up and down 3 times..

l) Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer for at least 30 seconds.

m) Add 200µL of MGC Binding Buffer to each sample, and pipette tip mix 15 times.

Note: Be careful to avoid adding too many bubbles by pipetting gently when tip mixing. This is extremely important as to not contaminate the wells in proximity.

n) Incubate the plate on the bench for at least 5 minutes.

o) Place the extraction plate onto the 96 well plate magnet plate for at least 5 minutes.

p) After the 5 min incubation, remove as much of the 400µL of the supernatant as possible. Be careful not to disturb or aspirate the beads.

(1) Add 400µL of 70% denatured ethanol (EtOH) with the extraction plate still on the magnet plate.

(2) 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.

q) 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.

r) 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.

s) Remove the extraction plate from the magnet plate and add 50 µL of MGC Elution Buffer.

(1) 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.

(2) Incubate the plate for at least 1 minute on the bench, before returning the plate to the magnetic plate.

(3) Let the plate sit on the magnet for at least 1 minute before transferring the eluant to a new extraction plate labeled with “Final Extract [date]”.

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

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

3. Make a separate master mix in a 1.5mL tube for each assay type being run. All probe mixes contain the internal plant control, SCCG 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 - No Grim Reefer

| Reagents | 1 Reaction | 24 Reactions (Plus 1 excess rxn) | 48 Reactions (Plus 2 excess rxn) |
|----------------------|------------|-------------------------------------|-------------------------------------|
| qPCR Master Mix | 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 |

5. qPCR Reagent Volumes - With Grim Reefer

| Reagents | 1 Reaction | 24 Reactions (Plus 1 excess rxn) | 48 Reactions (Plus 2 excess rxn) |
|--------------------------------|------------|-------------------------------------|-------------------------------------|
| qPCR Master Mix | 3.75 µL | 93.75 µL | 187.5 µL |
| Assay Probe Mix | 1 µL | 25 µL | 50 µL |
| Grim Reefer Assay Probe Mix | 0.5 µL | 12.5 µL | 25 µL |
| Reaction Buffer | 0.8 µL | 20 µL | 40 µL |
| Water | 7.7 µL | 192.5 µL | 385 µL |
| Total Assay Probe MM | 13.75 µL | 343.75 µL | 687.5 µL |

Note: The GR Assay is detected in the Cy5 Channel of the qPCR instrument so be sure to select the Cy5 channel when setting up the detection plate.

- 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.
7. For the positive control, make a 1:10 dilution of stock
 - a. Add 1 μL of Positive Control to 9 μL nuclease free water (found in the kit) and vortex to mix.
 - b. For the negative control, use water (found in the kit).

Note: It is best to add the largest volume reagent first, in this case the 9 μL water then the 1 μL of positive control, pipette mix or vortex control dilution to ensure control DNA is in solution.
8. 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.
9. Use a 96-well optical qPCR plate and label the plate “qPCR Plate_ [date]”.
10. 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.

Note: ALWAYS use a fresh tip for every liquid transfer into the qPCR plate.
11. 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 .
12. Add 5 μL of the diluted Positive Control to the corresponding well.
13. Add 5 μL of water to the corresponding negative control well.

14. Add 13.75 μ L of BTGN Assay Probe MM to each corresponding sample well, positive control well, and negative control well in the qPCR plate. Gently tip mix a few times after each addition of qPCR master mix. Be careful not to introduce bubbles during this mix.
15. Seal the plate with strip caps or an adhesive seal.
16. 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.

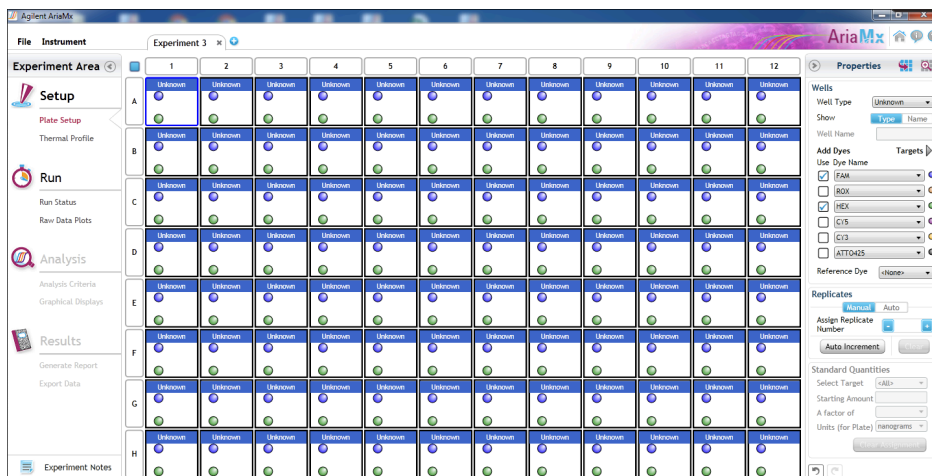
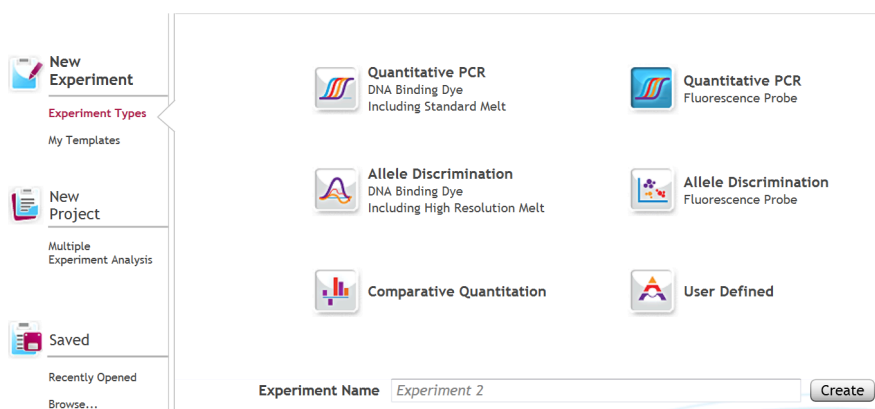
Note: Check for bubbles at the bottom of the wells (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the bottom of the wells, spin-down for another minute.
17. For the Agilent Aria: If using an adhesive seal; place the reusable compression pad (gray side down) on the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

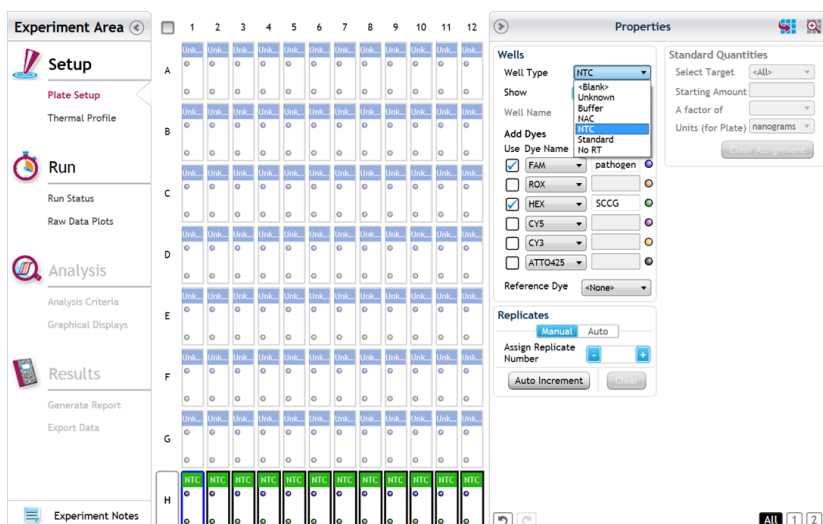
Running the Agilent AriaMX

The following species will be detected on the following Fluorophores:

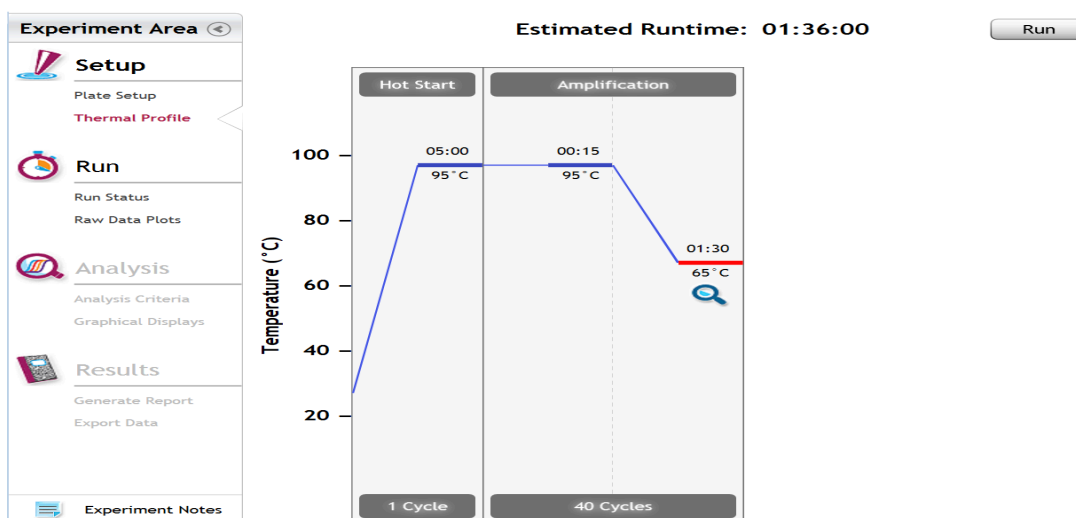
- BTGN: FAM
- Cannabis DNA: HEX
- Optional Grim Reefer Control: Cy5

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, HEX and Cy5 (if using GR) under Add Dyes.





3. Change the well types to reflect your plate set up. Add Target names to include “pathogen name” for FAM and SCCG (single copy control gene) for HEX.
4. Under Setup>Thermal Profile, create the following PCR thermal profile:
 - a. Hot start at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 65°C for 90 seconds.



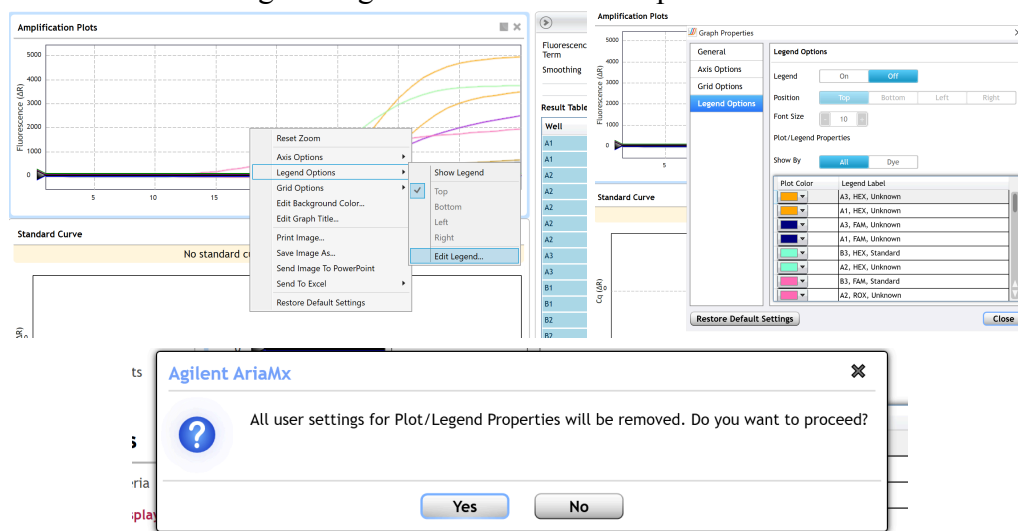
5. Close the lid and click “Start Run”.
6. Save the experiment with the [User] and [date]
7. When the run is complete, dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

Data Analysis: 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

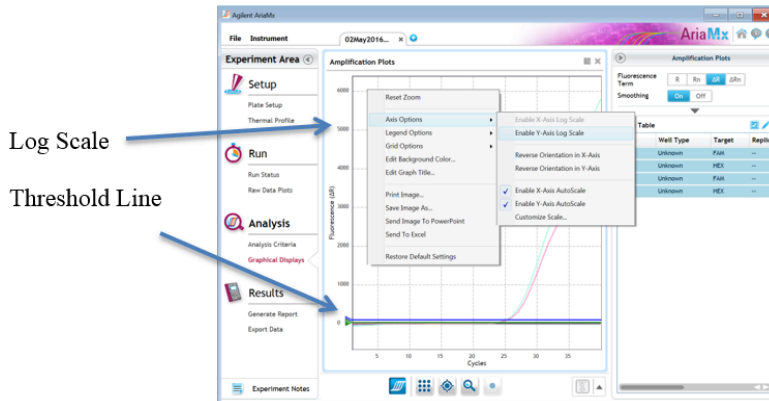
Graphical Display

- a. Amplification plots will be available for viewing
- b. The C_q 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.

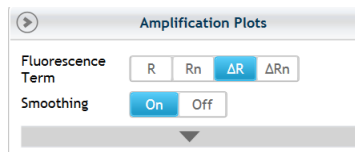


3. To analyze the results:

- 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 flower samples, < 40 for all other matrices.
 - i. Visually confirm with the curve on the graph.
 - d. Optional Grim Reefer Control on the Cy5 fluorophore ≤ 35
 - i. Visually confirm with the curve on the graph.
 - ii. If no Cy5 signal or Cy5 signal greater than 35 is observed this is an indication that the lysis buffer was not added in a timely manner after the GR incubation, or that the purification was not successful due to processing error.
5. Unknown BTGN 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).
 - 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.

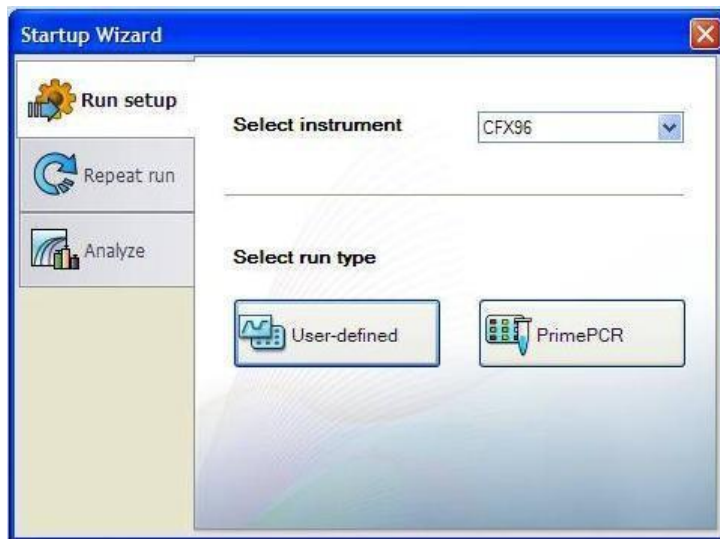
- ii. Non-Flower: If this test results in a Cq value indicating the presence of the target organisms, an unenriched sample should be plated on culture plates of your choice to determine enumeration.

Running the BioRad CFX96

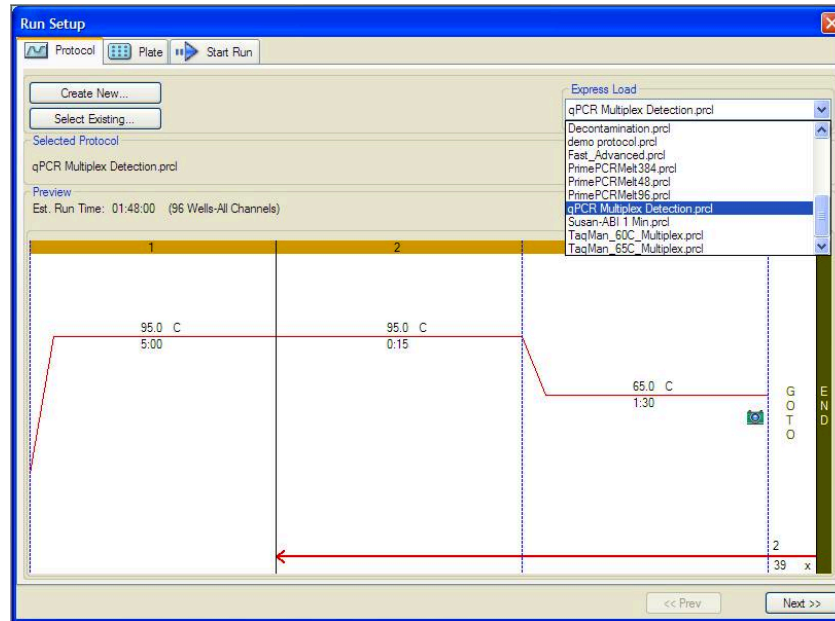
The following targets will be detected on the following fluorophores:

- BTGN: FAM
- Cannabis DNA: HEX
- Optional Grim Reefer Control: Cy5

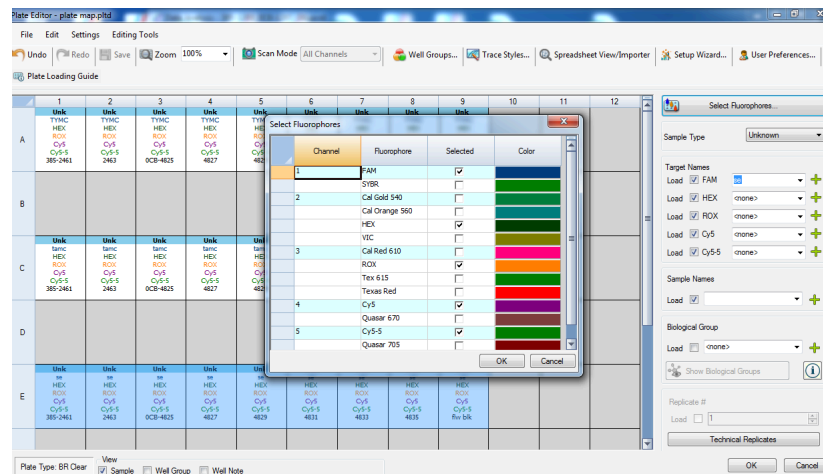
1. Start the qPCR Cycling program.
2. Select User-Defined in the Startup Wizard under Run setup.

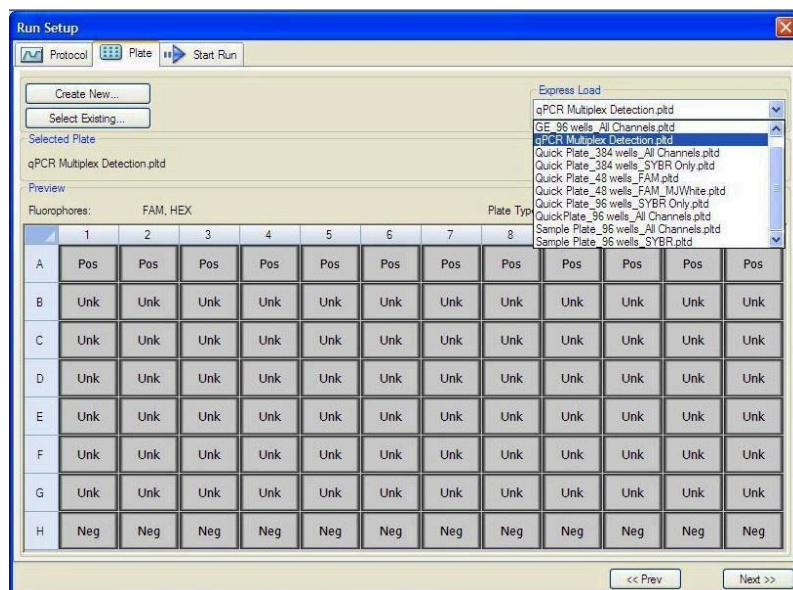


3. Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click “Next”.
4. If not already pre-programmed, create a cycling program with the following specifications and save as “qPCR Multiplex Detection”:
 - a. Hot start at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 65°C 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. Plate editor window will appear. Choose FAM HEX and Cy5 (if using GR) fluorophores and click “OK”.



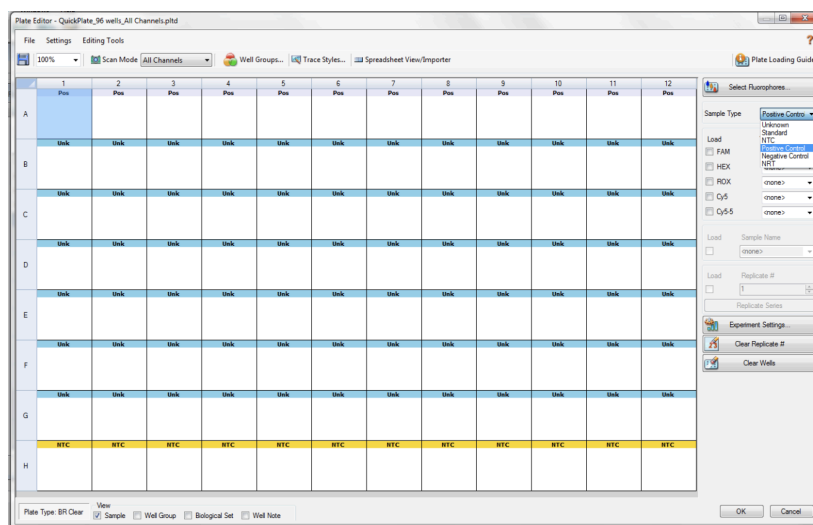


- c. If plate layout previously saved, click “Edit Selected” to move to the Plate Editor Screen.
- d. On the Plate Editor Screen, change the Sample Type to correlate with your specific plate setup.

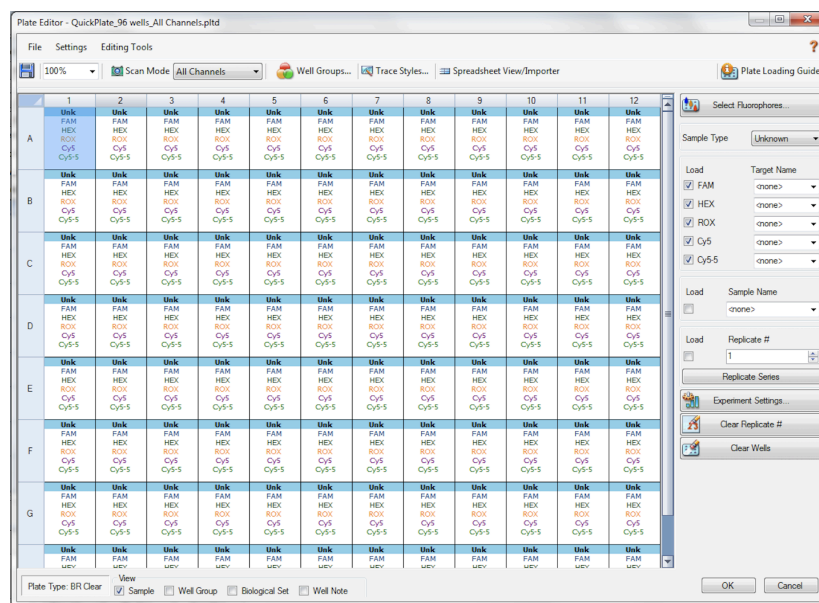
NOTE: To select the Sample Type, highlight the wells you would like to define, then choose from the dropdown menu one of three types:

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 BTGN Assay highlight the well locations and click on **FAM**, **HEX** and **Cy5** (if Grim Reefer was used).
- 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.

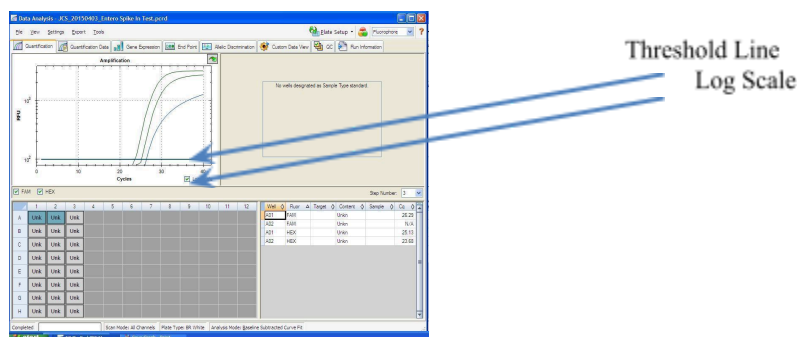


Note: Saving will override the template but will not cause any issues.

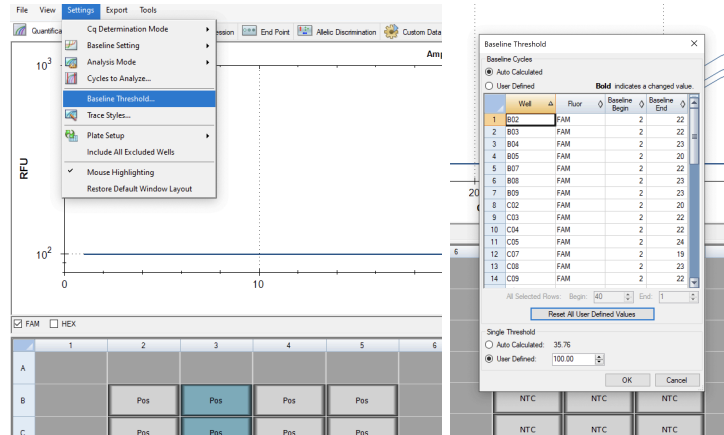
- i. Close the lid and click Start Run.
- j. Save the experiment with the [User] and [date].
- k. When the run is complete, immediately dispose of the plate after qPCR. Do not remove the plate seal after the run to avoid contamination in the lab.

Data Analysis: 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. 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 flower samples, < 40 for all other matrices.
 - i. Visually confirm with the curve on the graph.
- e. Optional Grim Reefer Control on the Cy5 fluorophore ≤ 35 .
 - i. Visually confirm with the curve on the graph.
 - ii. If no Cy5 signal or Cy5 signal greater than 35 is observed this is an indication that the lysis buffer was not added in a timely manner after the GR incubation, or that the purification was not successful due to processing error.

7. Unknown BTGN 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).
 - 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.

Cq to CFU Conversion Equation Table

| Matrix | Microbial Test | Cq to CFU Conversion Equation |
|---|----------------|--|
| Flower | BTGN Count | $CFU/g = 10^{((-0.2855 * Cq) + 11.698)}$ Multiply resulting CFU x 20 to account for upfront dilution factor |
| All Non Flower Action Limit = 100 CFU/g | BTGN | IF Cq < 40 after enrichment, Plate confirm for enumeration |

Troubleshooting Guide

| Symptom | Reason | Solution |
|---|---|--|
| Internal control (SCCG Primer) failure | Extraction 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 Reaction Setup | Repeat the qPCR by following the protocol. |
| | Missing Fluorophore on plate set up | In the Data Analysis window click on View/Edit Plate Setup from the Settings drop down. All wells should have both FAM and HEX. Once completed and the window is closed the analysis should automatically update. |
| Internal Control (SCCG) Positive result on positive or negative control samples or samples that do not contain plant DNA No Cq or a Cq of more than 35 is acceptable. Any Cq lower than 35 constitutes a rerun | Plant DNA contamination in a reagent | Troubleshoot which reagent was contaminated; use new reagents, thoroughly clean all pipettes and bench areas with 10% bleach solution. |
| | qPCR bench too close to extraction area | Designate separate benches, pipettes etc. for extractions and qPCR setup |
| Positive Negative Control | Small Cq value <15 | Visually confirm that there is an amplification curve. If not, this is a low level background and is to be expected. |
| | Contamination | Repeat the qPCR by following the protocol. |
| | Insufficient pre-setup bleaching | Wipe down the lab workspace and all equipment with 10% Bleach. Repeat qPCR. |
| Negative Positive Control | Mix up in Reaction Setup | Repeat the qPCR by following the protocol. |
| Total run failure | Excessive vortex of the qPCR Master Mix | Repeat the qPCR by following the protocol. |
| Background Amplification | Unclear | This is usually seen with a very low 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 Fluorophore.

The **Internal Control** is added to every sample reaction where a Cq is expected. It ensures the effectiveness and efficiency of each reaction. The internal control targets plant DNA, or more specifically, a Single Copy Control Gene (SCCG), using the HEX Fluorophore.

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

DISCLAIMER

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

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

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