PathoSEEK® Total Yeast and Mold Count Assay with SenSATIVAx® TLP DNA Purification & Optional Grim Reefer® Free DNA Removal (State of Florida)

User Guide

Real-Time PCR (qPCR) Assay for the detection of Total Yeast and Mold in cannabis flower and MIP matrices

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Introduction

Yeasts and molds have been known to cause deterioration and decomposition of cannabis. Certain species of yeast and mold, such as Aspergillus *fumigatus* can produce toxins and infect immuno-compromised patients with fatal Aspergillosis. The PathoSEEK® Total Yeast and Mold Count (TYM) Detection Assay with SenSATIVAx® Thaumatin Like Protein (TLP) Enzyme Purification Protocol (MGC TYM Method) is a DNA Purification and qPCR method for the rapid enumeration of yeasts and molds in cannabis flower. The Thaumatin Like Protein (TLP) is a beta glucanase that digests the glucan cell wall of some yeasts that are difficult to lyse. In 2020, Candida albicans became a popular TYM-cannabis reference standard. While this organism has never been documented on cannabis it is an excellent example of a yeast with a thick glucan cell wall that is difficult to lyse. While C.albicans has not yet been found on cannabis, it is reasonable to assume other yeast and molds may also produce thick glucan cell walls and more robust lysis methods will be required for concordance with colony forming unit (CFU) based regulations.

Note: The OMMU currently will NOT allow the use of MGCs Grim Reefer Free DNA Removal Enzyme on compliance samples. However, it may be used on R&D samples.

Process Overview

PathoSEEK® Total Yeast and Mold (TYM) Detection Assay with optional Grim Reefer® Free DNA Removal and SenSATIVAx® TLP Enzyme Purification Protocol is a novel, real-time quantitative PCR assay that uses a multiplex system of primers to enumerate yeast and molds in cannabis matrices. Figure 1 shows a simplified depiction of the qPCR assay.

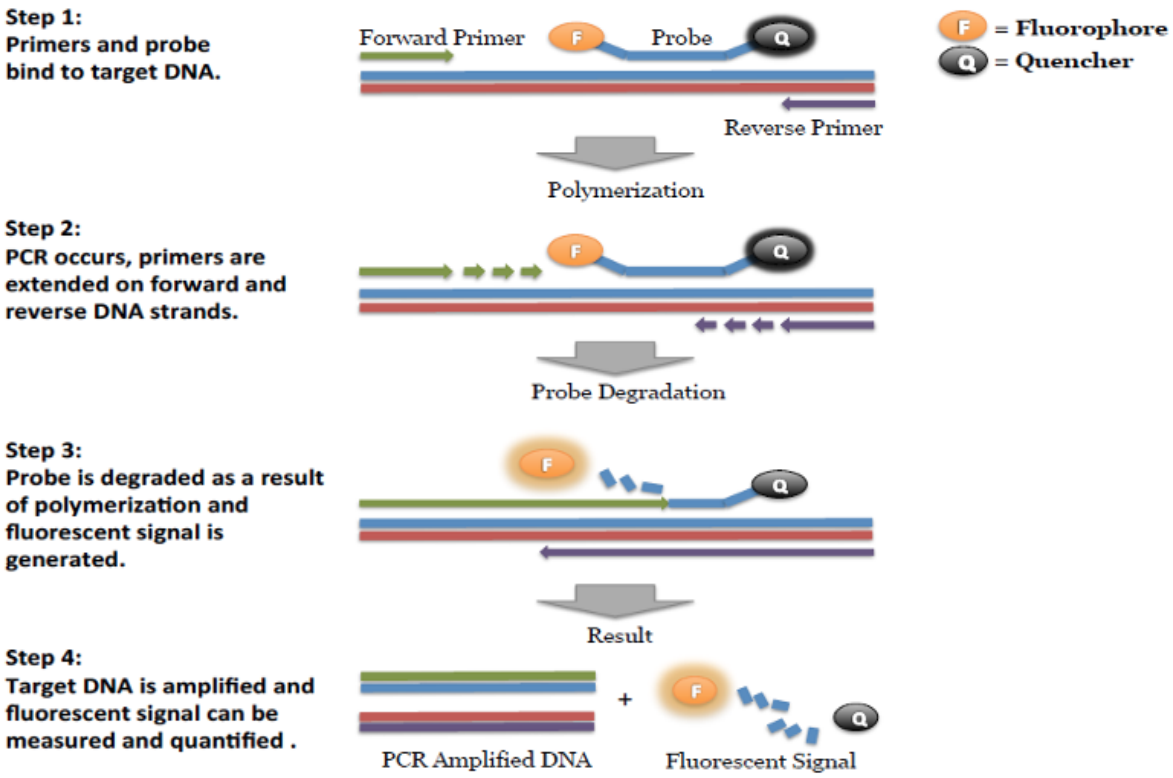
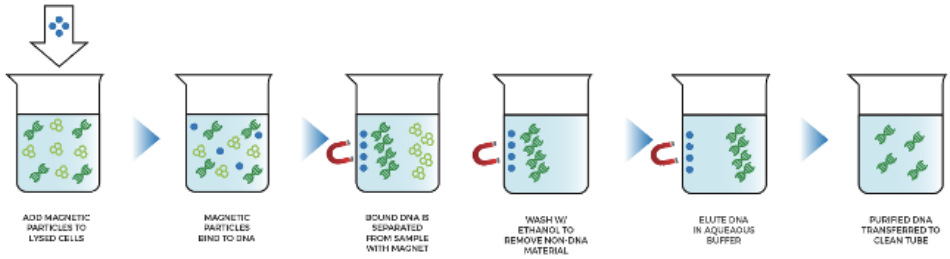


Figure 1: Overview of qPCR

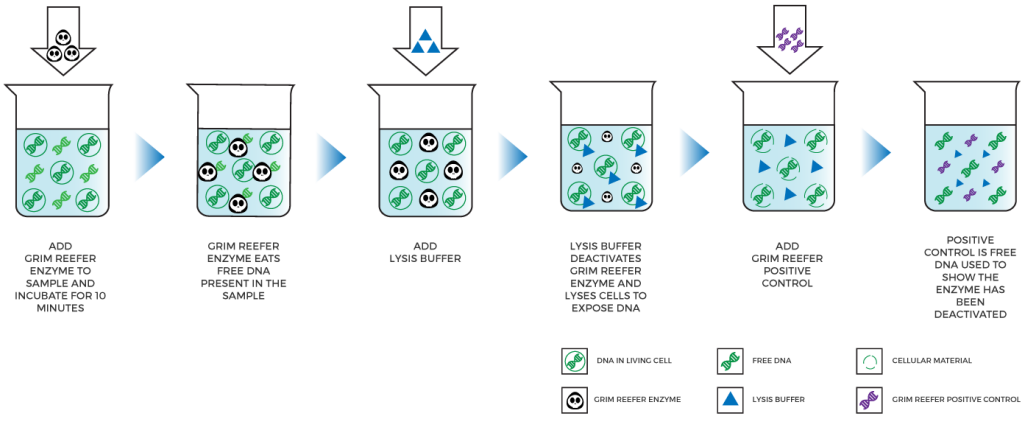
SenSATIVAx® is a proprietary DNA isolation process that uses magnetic particles to isolate and purify both plant and microbial DNA from a raw, homogenized plant or MIP sample. The use of magnetic particles affords 8 or 96-tip automation, enabling high throughput applications. DNA can be isolated from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

Figure 2: SenSATIVAx DNA Purification Protocol

without Grim Reefer®



with Grim Reefer®



Materials and Methods

Test Kit Name: PathoSEEK® Total Yeast and Mold Count Assay

Test Kit Information

1. SenSATIVAx® Flower/Leaf DNA Purification Kit - P/N 420001
2. SenSATIVAx® TLP Purification Enzyme - P/N 420206
3. SenSATIVAx® MIP/Extract DNA Purification Kit - P/N 420004/420005
4. PathoSEEK® qPCR Master Kit v3 - P/N 420201
5. PathoSEEK® Total Yeast and Mold Count Detection Assay - P/N 420103
6. PathoSEEK® Total Yeast and Mold Count Assay Positive Control - P/N 420303
7. Grim Reefer® Free DNA Removal Kit - P/N 420145
8. Grim Reefer® Free DNA Removal Control - P/N 420144
9. Grim Reefer® Free DNA Removal Assay - P/N 420143

Test Kit Components

1. SenSATIVAx® Flower/Leaf DNA Purification 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® TLP Purification Enzyme - P/N 420206 (50 Purifications). Store at -15°C to -20°C. (for flower Purifications ONLY)
    a. 2.0mL clear top - 1 vial

3. SenSATIVAx® MIP/Extract DNA Purification.— P/N 420004/420005 (200 tests).
    a. MGC Binding Buffer — 1 bottle. Store at 2-8°C. Expires 1 Year from Date of Manufacture
    b. MGC 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

4. PathoSEEK® 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® Total Yeast and Mold Assay - P/N 420103 (200 tests)
    a. Assay — 1 tube (Store kit at -15 to -20°C). Expires 2 Years from Date of Manufacture.
6. PathoSEEK® Total Yeast and Mold Count Assay Positive Control - P/N 420303 (50 reactions)

a. Control — 1 tube (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture.

7. Grim Reefer® Reagents (Optional Step)
   a. Grim Reefer Free DNA Removal Kit— P/N 420145 - 1 kit (Store at -20°C). Expires 2 Years from Date of Manufacture.
   b. Grim Reefer Free DNA Removal Control— P/N 420144 - 1 tube (Store at -20°C). Expires 2 Years from Date of Manufacture.
   c. Grim Reefer Free DNA Removal Assay— P/N 420143 - 1 tube (Store at -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, HEX, and Cy5 (if using optional Grim Reefer).
   a. Agilent HP Notebook PC (Optional)
   b. 96-Well Optical qPCR plate— Medicinal Genomics P/N 100164
   c. Adhesive optical seal for qPCR plates— Medicinal Genomics P/N 100177
   d. Optical Strip Caps (Optional) —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. Medicinal Genomics will provide at no charge if AriaMx is purchased through Medicinal Genomics.

2. Bio-Rad CFX96 Touch™ Real-Time System
    a. Bio-Rad Personal PC (Optional)
    b. 96-well Optical qPCR plate — Bio-Rad #HSP-96601 or Medicinal Genomics P/N 100164
    c. Adhesive optical seal for qPCR plates — Bio-Rad #MSB-1001 or Medicinal Genomics P/N 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—VWR #89004-570
7. 1.5 mL Tube Benchtop Cryogenic Rack— VWR #89004-558 or equivalent
8. Freezer—Capable of maintaining -20°C
9. Tabletop Mini Plate Centrifuge—Fisher Scientific #14-100-143 or equivalent
10. Tabletop 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 37ºC (VWR® Personal Size Incubator # 97025-630, or similar)

14. MIPs only - Heat Block capable of maintaining 65ºC (VWR® Advanced Mini Dry Block Heater with 1.5 mL tube adapter #10153-364)
15. MIPs only - Mini Block insert for 15 x 1.5 mL tubes (VWR #10153-364)
16. Filter Bags—Whirl Pak #B01385WA or Medicinal Genomics P/N 100008
17. Beaker or Solo Cup (optional)
18. Tryptic Soy Broth—Medicinal Genomics P/N 420205 (Store at 2-8°C)
19. 1.5 mL Eppendorf Tubes
20. 15 mL or 50 mL conical tubes (for MIP)
21. 96-well Plate Magnet— Medicinal Genomics P/N 420202
22. 96-well extraction plate— Medicinal Genomics P/N 100298
23. Eppendorf Tube Rack
24. Scientific Scale—Capable of measuring to milligrams
25. Refrigerator—Capable of maintaining 2–8 °C
26. 25 mL Serological Pipette —VWR 89130-890 or 89130-900 or equivalent
27. 10% bleach
28. 70% Ethanol — Medicinal Genomics P/N 420030
29. Chloroform (Ethanol as preservative/ Certified ACS) - Fisher Scientific, C298

Safety Precautions and Recommendations for Best Results

Safety Precautions

The PathoSEEK® Total Yeast and Mold Count Assay is a qPCR detection assay for the rapid detection and enumeration of yeast and mold 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% 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 pipettes, 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. If using qPCR reaction strip tubes instead of plates, avoid fingerprints and writing on caps because both can interfere with data acquisition.

Intended User

The Total Yeast and Mold Count Detection Assay with SenSATIVAx® Purification 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 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. **OPTIONAL:** Dilute GR Pos control to 1:10:000

a. Label a new 1.5 mL Eppendorf tube (GRC 1:100), add 1 µl of GR positive control into 99 µl of dH2O. Vortex to mix thoroughly and quickly spin tube. Label another 1.5 mL Eppendorf tube (GRC 1:10,000), add 99 µl of dH2O, then add 1 µl of the GRC 1:100 dilution. Vortex to mix thoroughly and quickly spin tube. This will result in a 1:10,000 dilution of the Grim Reefer Control.

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 the liquid is in the tip before adding it to the tube for dilutions 1 and 2.

b. Place on ice until use.

8. 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. Homogenize for 1 minute by hand.

Figure 3: Homogenized cannabis flower and TSB.

9. *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 7 x ***n*** mL of Solution A to each test portion. Vortex or homogenize sample with Solution A.
10. 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.
11. Allow a 1.5 mL tube rack to come to temperature in a 37 ºC incubator.

SenSATIVAx® for Flower/Leaf DNA Purification

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 14,000 - 19,300 RCF for 5 minutes.
3. Remove and discard 950 µL of the supernatant without disturbing the pellet.
4. Resuspend the pellet by adding 200 uL of nuclease free water.
5. Pipette mix and vortex well to resuspend the pellet.
6. (Optional) If Performing the Grim Reefer free DNA removal step, perform the following steps. Otherwise, proceed directly to Step 7.
    a. Add 28 µL of MGC Grim Reefer Buffer and 5 µL of MGC Grim Reefer Enzyme to the resuspended pellet.
    b. Vortex for 10 seconds and incubate tubes in a 37 °C Incubator for 10 minutes.
    c. Remove tubes from the incubator, add 12.5 µL of MGC Lysis buffer and vortex for 30 seconds.

d. Allow tubes to incubate for 5 minutes at room temperature.

Note: The addition of MGC Lysis buffer deactivates the Grim Reefer Enzyme and should be done as quickly as possible.

e. Add 2.5 µL of prepared 1:10,000 dilution of Grim Reefer Positive Control to the tube.

f. Proceed to step 8.

7. If NOT performing Grim Reefer steps, add 12.5 µL of MGC lysis buffer.
8. Add 12 µL of MGC-TLP Purification Enzyme and vortex for 30 seconds.
9. Incubate tubes in a 37 °C incubator for 30 minutes.
10. Remove tubes from the incubator and vortex for 30 seconds.
11. Let incubate on the bench for 2-5 minutes.

a. After 2-5 minute incubation, spin for at least 1-3 minutes in a benchtop mini centrifuge or high-speed centrifuge to pellet cellular debris.

Figure 4: Picture of clear lysate after centrifugation

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

12. 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.

13. Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer for at least 30 seconds.
14. 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.

15. Incubate the plate on the bench for at least 5 minutes.
16. Place the extraction plate onto the 96-well plate magnet plate for at least 5 minutes.
17. 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.
18. Add 400 µL of 70% ethanol (EtOH) with the extraction plate still on the magnet plate.
19. 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.

20. 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.
21. After all the EtOH has been removed, let the beads dry at room temperature on the magnet plate for up to 15 minutes. Necessary drying time will vary based on complete removal of the second ethanol wash, as well as the lab environment. Visually inspect beads for residual ethanol before the elution step.

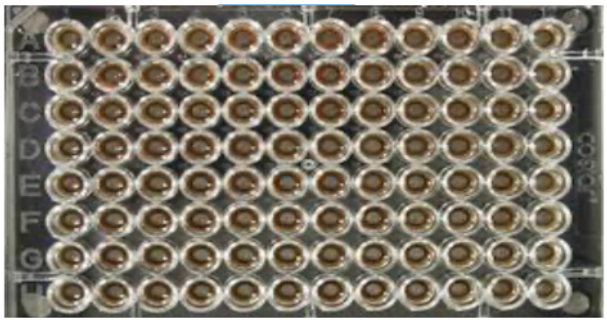


Figure 5: Extraction plate during wash step on magnetic plate.

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.

22. 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. It may be helpful to allow the elution buffer to soak the beads prior to tip mixing.

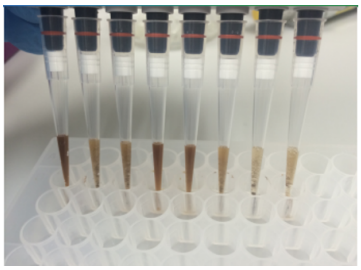


Figure 6: Multichannel pipette tips showing magnetic beads resuspended in elution buffer.

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 eluant 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 Purification

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

    b. Place on ice until use.

    Note: The 100 µL dilution prepared above is enough diluted SCCG for approximately 10 Purifications. If more Purifications 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.

2. Add initial sample weight x 7 mL SenSATIVAx Solution A to conical tube with sample. 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 SCCG internal control (1:5,000) to the 1.5 mL tube and vortex to mix well.
5. Heat tube at 65 °C for 15 minutes in a preheated heat block.

6. Vortex again and centrifuge for 10 minutes at 14,000 - 19,300 RCF using a high-speed benchtop centrifuge.
    a. If no benchtop 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.

7. 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.
8. Add 600 µL 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

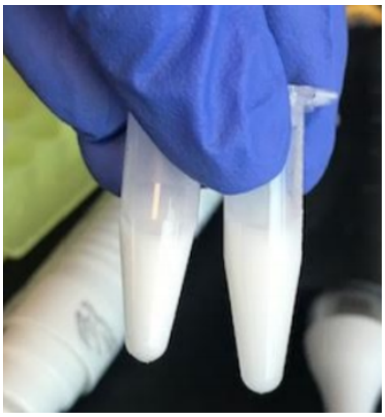


Fig 7. Example of a milky sample with chloroform in it.

9. Centrifuge for 5 minutes at 14,000 - 19,300 RCF using a benchtop centrifuge.
10. If no benchtop 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.

11. Transfer 100 µL 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.
12. Add 100 µL of SenSATIVAx Solution B to the 100ul sample in the 96-well extraction plate and mix by pipetting up and down 3 times.
13. Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer for at least 30 seconds.
14. 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.
15. Incubate the plate on the bench for at least 5 minutes.
16. Place the extraction plate onto the 96-well plate magnet plate for at least 5 minutes.
17. 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.
    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.

18. 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.

19. 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.

20. 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. It may be helpful to allow the elution buffer to soak the beads prior to tip mixing.

    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 eluant to a new extraction plate labeled with "Final Extract [date]".

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

    Note: Do not vortex the qPCR Master Mix at any point during the protocol.
3. Make a separate master mix in a 1.5 mL 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.

Table 1: qPCR Reagent Volumes - No Grim Reefer

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

Table 2: 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 Grim Reefer 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.

4. 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.
5. For the positive control, make a 1:10 dilution of stock
    a. Vortex the stock positive control tube and pulse spin-down. Add 1 µL of Positive Control to 9 µL nuclease free water (found in the kit) and vortex to mix.
6. 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 Purification 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.
10. If frozen, let the DNA thaw completely and spin the plate before removing the seal to avoid cross-contamination between samples. Pipette mix the DNA and place the extraction plate on the magnet plate for one minute.

    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 µL of Total Yeast and Mold 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 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.

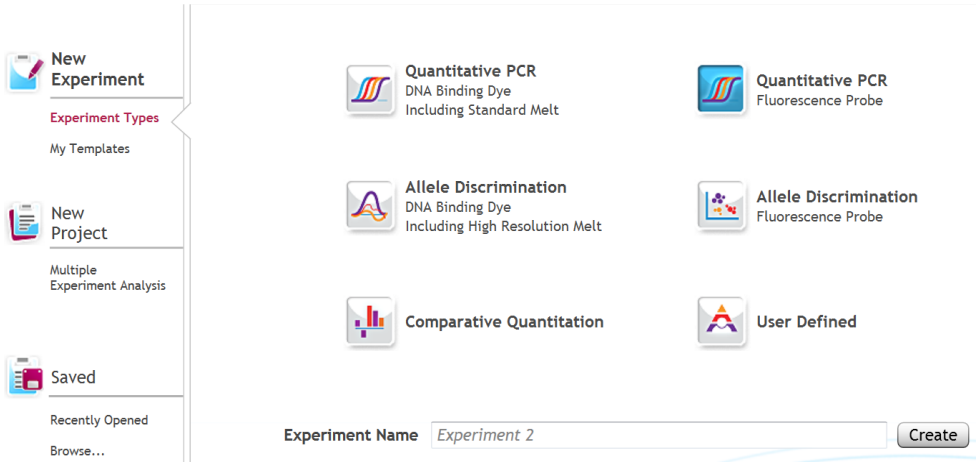
    Note: Check for bubbles at the bottom of the wells (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the bottom of the wells, spin down for another minute.
17. For the Agilent Aria: If using an adhesive seal; place the reusable compression pad (gray side down) on the 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

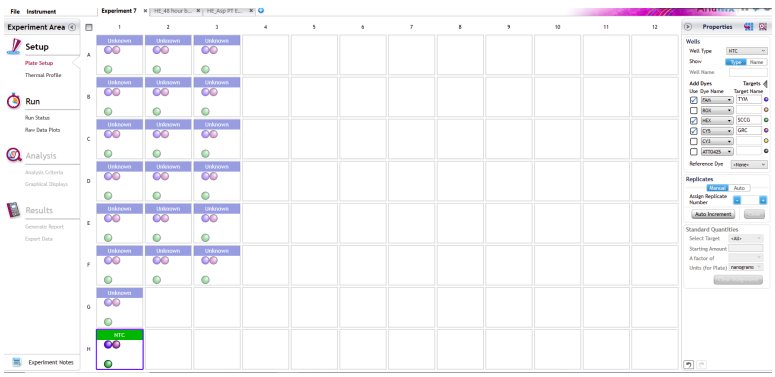
The following species will be detected on the following Fluorophores:

- Total Yeast and Mold: FAM
- Cannabis DNA: HEX
- Optional Grim Reefer Control: Cy5

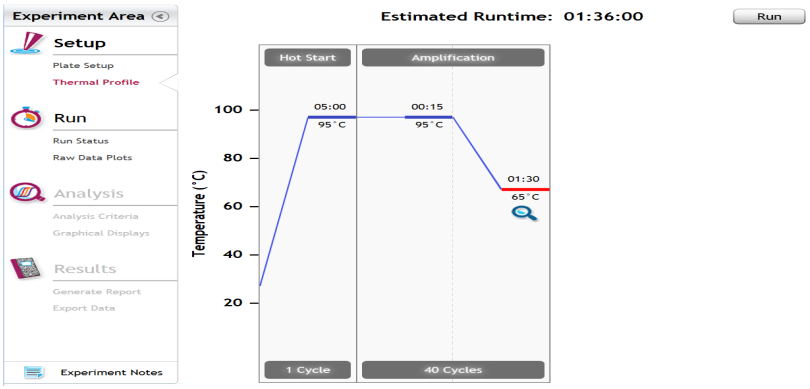
1. Create a New Experiment on the Agilent qPCR instrument.



2. Under Setup > Plate Setup, highlight **only** the wells that contain reactions and select FAM and HEX (and Cy5 if using Grim Reefer) under **Add Dyes**. Having fluorescence detection turned on in empty wells can affect the calculations which the software makes to obtain the corrected data, ΔR log.



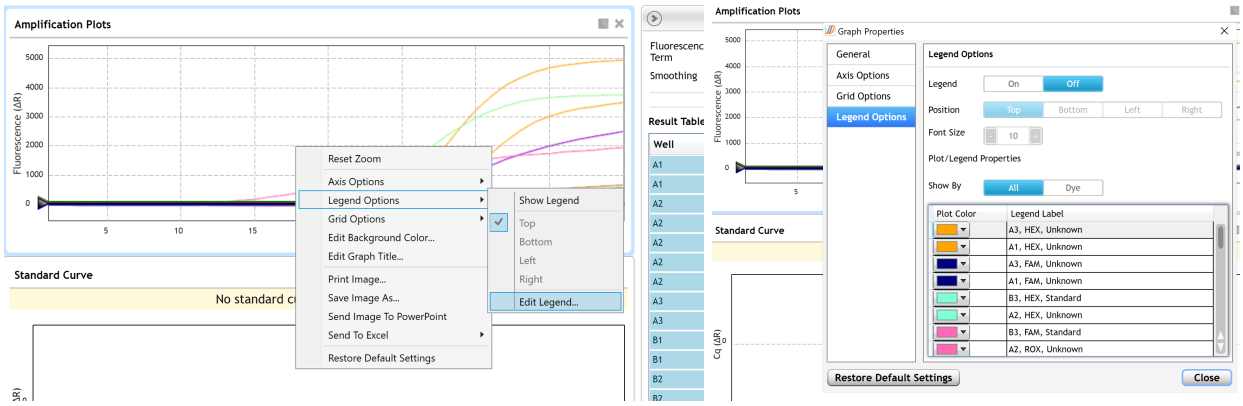
3. Change the well types to reflect your plate setup. Add Target names to include “pathogen name” for FAM and SCCG (single copy control gene) for HEX and GRC (Grim Reefer Control) for Cy5.
4. Under Setup>Thermal Profile, create the following PCR thermal profile:
    a. Hot start at 95ºC for 5 minutes, 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.

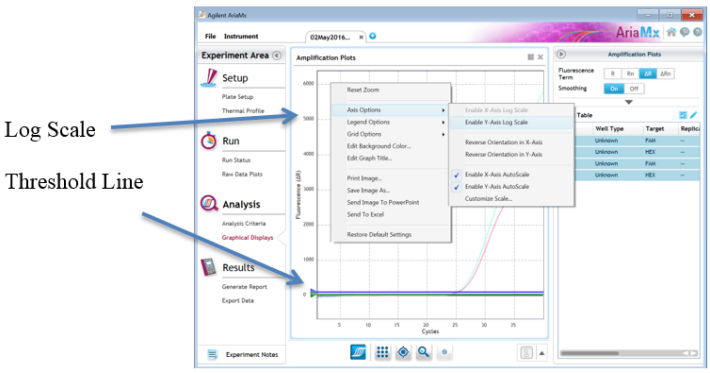
Data Analysis: Agilent AriaMX

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.

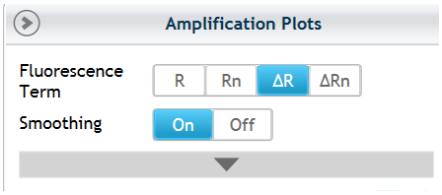


3. To analyze the results:

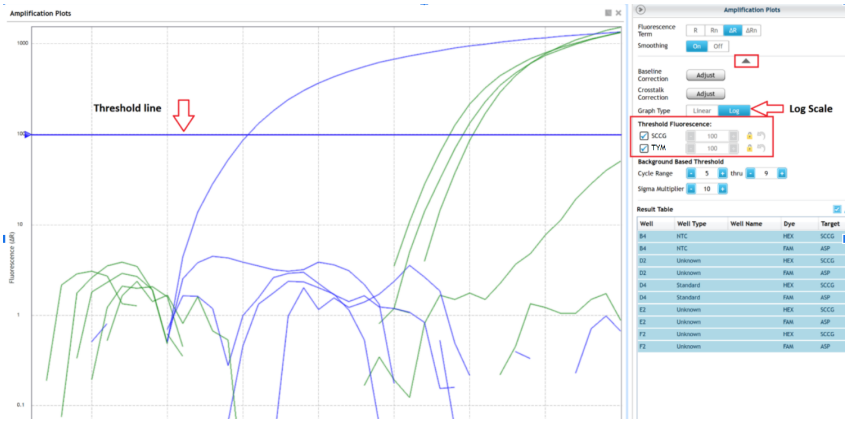
   a. Start by turning the graph to Log Scale by right-clicking on the chart and selecting Axis options. Enable y-axis log scale.



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



   c. Manually adjust thresholds to 100 RFU for the FAM, HEX and Cy5 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 and <40 for all other matrices.
        i. Visually confirm with the curve on the graph.
        ii. If no HEX signal or HEX signal greater than 35 is observed for flower samples, the Purification should be repeated. This result is an indication that the Purification was not successful due to processing error or that the sample type contains very little cannabis DNA.
5. Unknown Total Yeast and Mold Targets
    a. A high CFU count or **failing** result for the unknown Total Yeast and Mold target(s).
        i. Failing Sample Result: Check Cq Value on the FAM Fluorophore. Use Cq to CFU conversion equation to determine approximate CFU/g. See Table 3.
        ii. Visually confirm with the curve on the graph. It is very important to confirm with the amplification curve when a high CFU count occurs. Sometimes the background amplification will give a false positive reading,

especially when Cq reading is less than 15 (See troubleshooting guide below for more details).

b. A low CFU count or **passing** result for the unknown Total Yeast and Mold targets.

i. Passing Sample Result: Check Cq Value on the FAM Fluorophore. Use Cq to CFU conversion equation to determine approximate CFU/g. See Table 3.

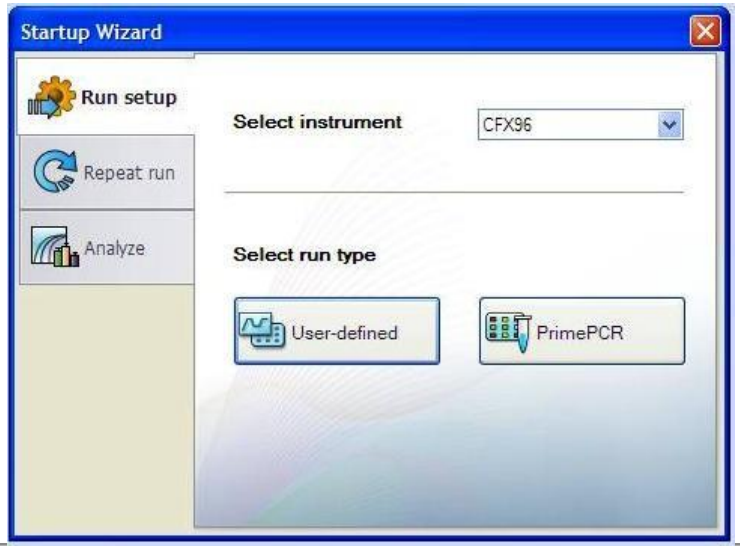
ii. Visually confirm with the curve on the graph.

Running the BioRad CFX96

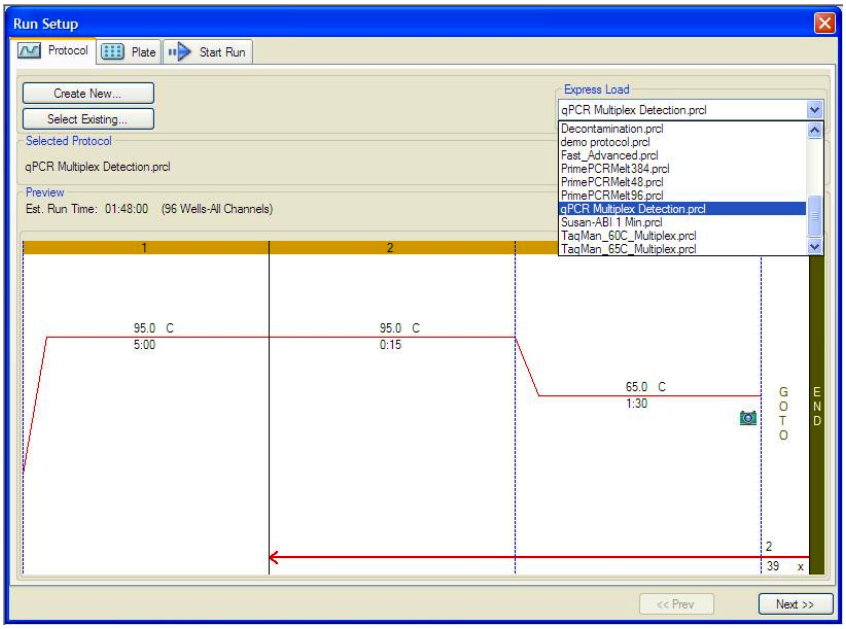
The following targets will be detected on the following fluorophores:

- Total Yeast and Mold: FAM
- Cannabis DNA: HEX
- Grim Reefer Control: Cy5

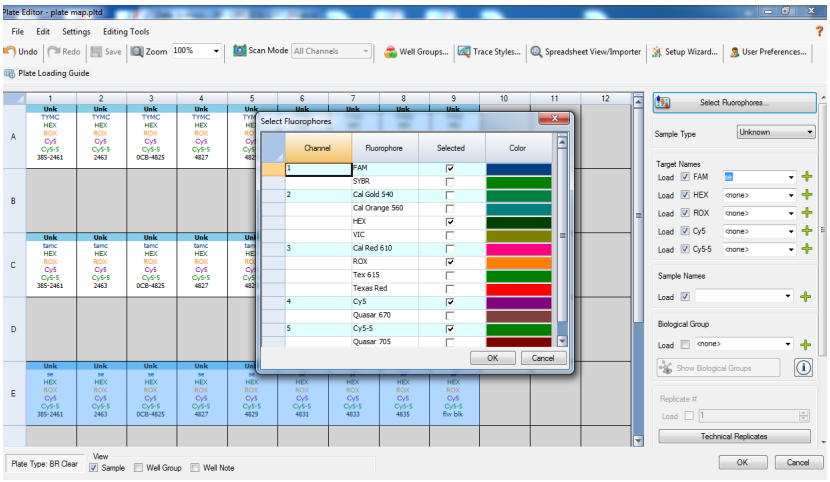
1. Start the qPCR Cycling program.
2. Select User-Defined in the Startup Wizard under Run setup.

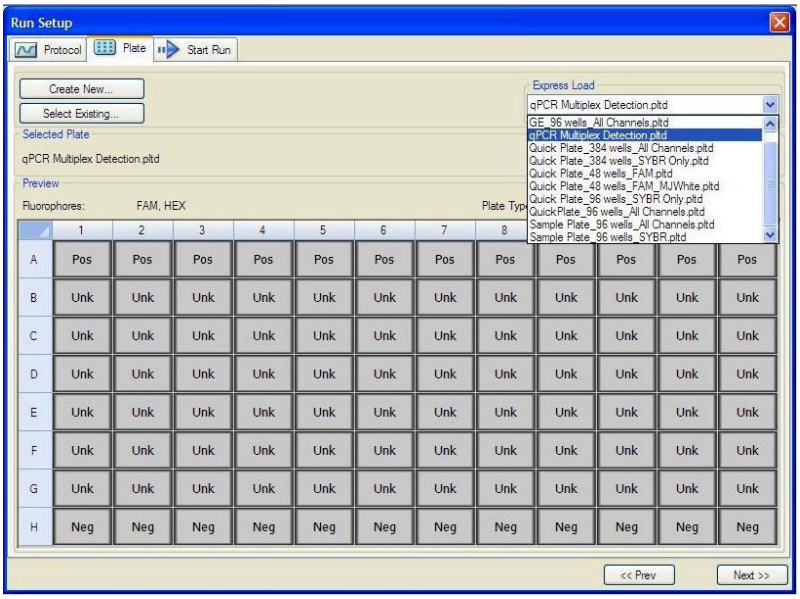


3. Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click “Next”.
4. If not already pre-programmed, create a cycling program with the following specifications and save as “qPCR Multiplex Detection”:
    a. Hot start at 95ºC for 5 minutes, followed by 40 cycles of 95ºC 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, HEX, and Cy5 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:

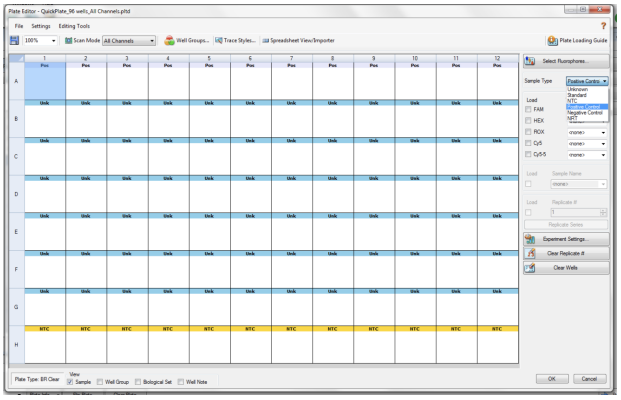
1. **Unknown**
2. **Positive Control**
3. **Negative Control**

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

f. Attach the fluorophores to the wells being used.

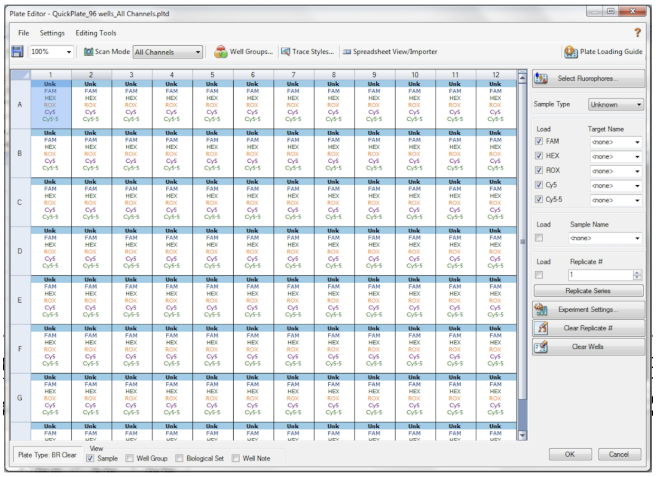
   i. Highlight all the wells being used.

   ii. For the Total Yeast and Mold Count Assay highlight the well locations and click on **FAM, HEX, and Cy5**.



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.

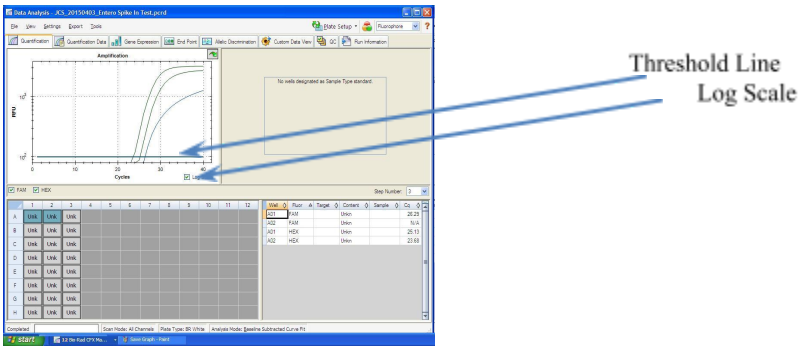
i. Close the lid and click Start Run.

j. Save the experiment with the [User] and [date].

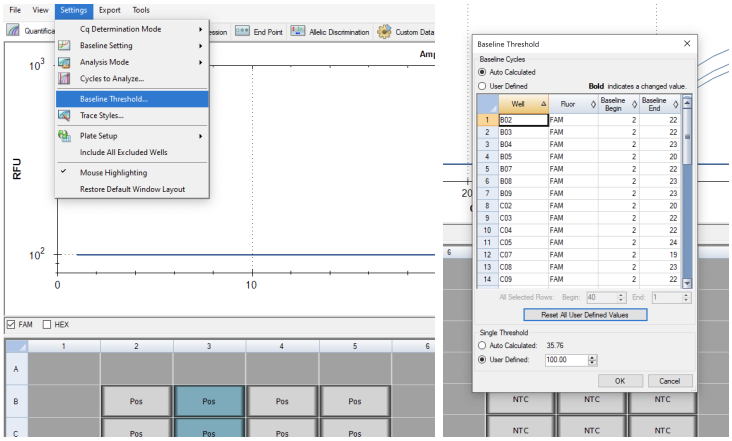
k. When the run is complete, immediately dispose of the plate after qPCR. Do not remove the plate seal after the run to avoid contamination in the lab.

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.

        i. Visually confirm with the curve on the graph.

    b. Assay-specific 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.

        ii. If no HEX signal or HEX signal greater than 35 is observed for flower samples, the Purification should be repeated. This result is an indication that the Purification was not successful due to processing error or that the sample type contains very little cannabis DNA.

    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.

e. A high CFU count or **failing** result for the unknown Total Yeast and Mold target(s).

i. Failing Sample Result: Check Cq Value on the FAM Fluorophore. Use Cq to CFU conversion equation to determine approximate CFU/g. See Table 3.

ii. Visually confirm with the curve on the graph. It is very important to confirm with the amplification curve when a high CFU count occurs. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15 (See troubleshooting guide below for more details).

f. A low CFU count or **passing** result for the unknown Total Yeast and Mold targets.

i. Passing Sample Result: Check Cq Value on the FAM Fluorophore. Use Cq to CFU conversion equation to determine approximate CFU/g. See Table 3.

ii. Visually confirm with the curve on the graph.

Cq to CFU Conversion Equation

Table 3: Cq to CFU Conversion Equation for Flower and MIP matrices

Matrix	Microbial Test	Cq to CFU Conversion Equation
Flower*	Total Yeast and Mold Count	CFU/g =10^((-0.1896*Cq)+8.5798) Multiply resulting CFU x 20 to account for upfront dilution factor
Non Flower*	Total Yeast and Mold Count	CFU/g = 10^((-0.096*Cq)+7.1104)) Multiply resulting CFU x 8 to account for upfront dilution factor

**** In the state of Florida the OMMU requires that testing labs pass a proficiency test (PT) twice a year. For this reason, the conversions above were confirmed using Proficiency Tests from NSI Lab Solutions and not real world samples. Commercially available Proficiency Tests and Certified Reference Materials are created using live organisms. When testing real samples, especially non flower matrices where Grim Reefer cannot be employed, if the resulting Cq to CFU results in a failure (>100,000 cfu/g) we recommend plating the sample for enumeration and viability.***

Troubleshooting Guide

Symptom	Reason	Solution
Internal control (SCCG Primer) failure	Purification Failure	Repeat SenSATIVAx™ and PathoSEEK™ by following the protocol.
	Residual ethanol in elution	Ethanol is an inhibitor to PCR. Return to the SenSATIVAxTM 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, and thoroughly clean all pipettes and bench areas with 10% bleach solution.
	qPCR bench too close to Purification area	Designate separate benches, pipettes etc. for Purifications 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, ROX and Cy5 Fluorophores.

Amplification of the **Internal Control** is expected in every reaction containing cannabis DNA. It ensures the DNA isolation procedure was successful. 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.

Revision History

Version	Date	Description
v0	June 2021	1. New SOP - Full process in one document 2. Inclusion of v3 Master Kit for flower 3. Addition of TLP enzyme to SenSATIVAx protocol 4. Update Cq to CFU equation for flower
v1	June 2022	1. Update to User Guide Format 2. Inclusion of v3 Master Kit for MIPs 3. Changes to upfront dilution for flower 4. Addition of heating step in MIPs 5. Updates to Cq to CFU equations for flower and MIPs
v2 Florida	April 2023	1. Add Grim Reefer Free DNA Removal as an optional step in flower prep. 2. Update Cq to CFU equation for Flower 3. Update Cq to CFU equation for MIP (Enumeration for non-flower allowed in Florida only due to high action limit of 100,000 CFU/g)
V2.1 Florida	May 2023	1. Revert Cq to CFU equation for Flower to v1 iteration to better match 100,000 cfu/g action limit equivalence vs plating techniques.
V2.2 Florida	June 2023	1. Revert Cq to CFU equation for MIP to v1 iteration to better match 100,000 cfu/g action limit equivalence vs PTs and plating techniques. 2. OMMU restriction of Grim Reefer use to R&D samples only

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 Agilent AriaMX or BIO-RAD CFX96 Touch® Real-Time PCR Detection System.

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