

# **PsiloSEEK *P. cubensis* Speciation qPCR Assay with MGC Leaf Punch Lysis or SenSATIVAx<sup>®</sup> DNA Purification**

## **User Guide**

**Real-Time PCR (qPCR) Assay for the detection of *Psilocybe cubensis* species from tissue and spores**

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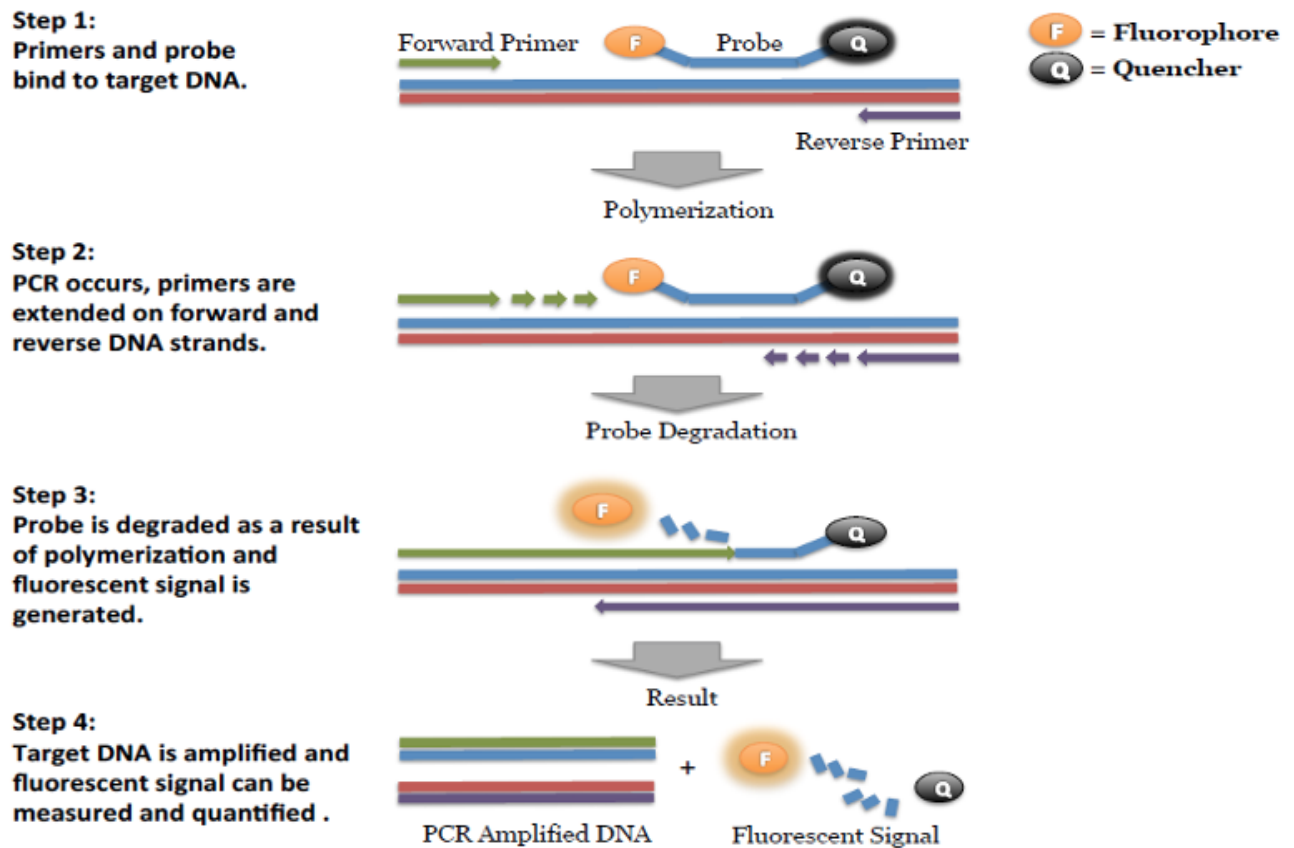
## **Introduction**

*Psilocybe cubensis* is a psilocybin producing fungi that can be easily confused for various other mushrooms in the *Psilocybe* genus. Of particular concern is a class of *Psilocybe* known as Wood Lovers *Psilocybe* that are associated with acute paralysis upon ingestion. This is known as Wood Lovers Paralysis (WLP). The PsiloSEEK Assay with MGC Leaf Punch Lysis or SenSATIVAx® DNA Purification Protocol is designed to detect *Psilocybe cubensis* ITS and Wood Lovers ITS in a single qPCR (Quantitative Polymerase Chain Reaction) from mushroom tissue and spore samples.

## **Process Overview**

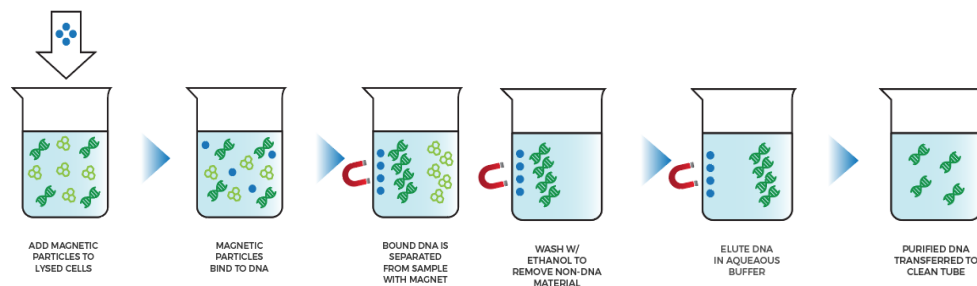
The PsiloSEEK Assay with MGC Leaf Punch Lysis or SenSATIVAx® DNA Purification is a novel, real-time quantitative PCR assay that uses a multiplex system of primers to detect *Psilocybe cubensis* ITS and Wood Lovers ITS in mushroom tissue and spore samples. Figure 1 shows a simplified depiction of the qPCR assay.

Figure 1. Schematic diagram of qPCR assay.



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, fungal or infused product 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 one hour. Hands-on time is less than 45 minutes.

Figure 2. SenSATIVAx DNA Purification Protocol



## **Materials and Methods**

*Test Kit Name:* PsiloSEEK *P. cubensis* Speciation Assay

### **Test Kit Information**

1. MGC Leaf Punch Lysis - P/N 420208 (for spores or  $\leq 1$  mg tissue)
2. SenSATIVAx<sup>®</sup> MIP/Extract DNA Purification Kit - P/N 420004 (for tissue only)
3. Medicinal Genomics qPCR Master Kit v3 - P/N 420201
4. PsiloSEEK *P. cubensis* Speciation Assay - P/N 419001
5. PsiloSEEK Multiplex Positive Control - P/N 419002
6. PsiloSEEK Spike-in Control - P/N 419003

## Test Kit Components

1. MGC Leaf Punch Lysis - P/N 420208 (for spores)
2. SenSATIVAx MIP/Extract DNA Extraction.— P/N 420004 (200 tests). (for tissue)
  - 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. 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
4. PsiloSEEK *P. cubensis* Speciation Assay - P/N 419001(200 reactions).
  - a. Assay — 1 tube (Store kit at -15 to -20°C). Expires 2 Years from Date of Manufacture.
5. PsiloSEEK Positive Control - P/N 419002 (50 reactions)

- a. Control — 1 tube (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture.
- 6. PsiloSEEK Spike-in Control - P/N 419003 (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, HEX, ROX and Cy5.
  - a. Agilent HP Notebook PC option 650
  - b. 96 Well Optical qPCR plate— Agilent #401490 or Fisher Scientific #AB2396
  - c. Adhesive optical seal for qPCR plates— Agilent #401492; USA Scientific TempPlate RT Optical Film #2978-2100
  - 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.**

- 1. Bio-Rad CFX96 Touch™ Real-Time System.
  - a. Bio-Rad Personal PC
  - b. 96 Well Optical qPCR plate — Bio-Rad #HSP-96601 or Fisher Scientific #AB2396

- c. Adhesive optical seal for qPCR plates — Bio-Rad #MSB-1001; USA Scientific TempPlate RT Optical Film #2978-2100
- 2. Adjustable, variable volume pipettes (single or multichannel)—P10, P20, P50, P200 or P300 and P1000
- 3. Adjustable, variable volume filter pipettes tips—For P10, P20, P50, P200, P300 and P1000
- 4. Crushed ice
- 5. 96 Well PCR Cryogenic Rack—VWR #89004-570
- 6. 1.5 mL Tube Benchtop Cryogenic Rack— VWR #89004-558 or equivalent
- 7. Freezer—Capable of maintaining -20°C
- 8. Table Top Mini Plate Centrifuge—Fisher Scientific #14-100-143 or equivalent
- 9. Table Top Mini Centrifuge—VWR #10067-588, #2631-0006 or equivalent
- 10. Vortex-Genie Pulse—Scientific Industries, SKU: SI-0236 or equivalent
- 11. High-Speed centrifuge— to accommodate 1.5mL tubes such as Eppendorf model 5414R or similar with ability to spin up to speeds of 19,300 rcf
- 12. Incubator— capable of maintaining 65°C (VWR® Personal Size Incubator # 97025-630, or similar)
- 13. Beaker or Solo Cup (optional)
- 14. Tryptic Soy Broth—Medicinal Genomics #420205 (Store at 2-8°C)
- 15. 1.5 mL Eppendorf Tubes
- 16. 2.0mL Conical Base Screw Cap Tubes USA Scientific 1420-870 or equivalent



17. 96 Well Plate Magnet—MGC P/N 420202 (*Tissue prep only*)
18. 96 Well Extraction Plate— Medicinal Genomics P/N 100298 (*Tissue prep only*)
19. Eppendorf Tube Rack
20. Scientific Scale—Capable of measuring to milligrams
21. Refrigerator—Capable of maintaining 2–8°C
22. 25 mL Serological Pipette —VWR 89130-890 or 89130-900 or equivalent.
23. 10% bleach.
24. 70% Denatured Ethanol — Medicinal Genomics P/N 420030 (*Tissue prep only*)
25. Chloroform (Ethanol as preservative/ Certified ACS) - Fisher Scientific, C298 (*Tissue prep only*)

## **Safety Precautions and Recommendations for Best Results**

### **Safety Precautions**

The PsiloSEEK Speciation Assay is a qPCR detection assay for the detection of *Psilocybe cubensis* gDNA (HEX), Wood Lovers *Psilocybe* gDNA (CY5), (See list below) from spores or tissue.

Wood Lovers *Psilocybe* species that the primers have been validated on:

- *Psilocybe azurescens*
  - *Psilocybe cyanescens*
  - *Psilocybe allenii*
  - *Psilocybe subaeruginosa*
  - *Psilocybe stunzii*
  - *Psilocybe pelliculosa*
  - *Psilocybe ovoideocystidiata*
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 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. Avoid fingerprints and writing on qPCR reaction strip tube caps because both can interfere with data acquisition.

## Intended User

The PsiloSEEK *Psilocybe cubensis* Speciation Assay with SenSATIVAx<sup>®</sup> 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.

### **Tissue Sample Preparation Pre-Prep (for tissue amounts 1 to 100mg)**

1. Wipe down the workspace with a 10% bleach solution, including the bench top and all equipment being used.
2. Remove the MGC Binding Buffer from the 2-8°C refrigerator (it should come to room temperature, 20-28°C, before use).
3. Prepare consumables. Label all the 1.5 mL centrifuge tubes needed with “[sample name]”.
4. Label the extraction plate with date, and if transferring eluted DNA to a new plate, label the destination plate also.
5. Weigh up to 100 mg of finely ground sample into a 1.5 mL tube.
6. 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.
7. Prepare Dilution of PsiloSEEK Internal Control
  - a. Label new 1.5 mL Eppendorf tube IC (Internal Spike-in Control)
    - i. Determine the volume of diluted control needed for the number of samples being tested

## **DNA Extraction**

DNA Extraction can be done one of two ways depending on the starting material:

1. Leaf Punch Lysis - Used for DNA extraction from spores or  $\leq 1$ mg tissue
2. SenSATIVAx for MIP/Extracts - Used for DNA purification from dried tissue 1 to 100 mg

### **Leaf Punch Lysis DNA Extraction (For spores or $\leq 1$ mg tissue)**

1. Spore Sample Preparation with MGC Leaf Punch Lysis
  - a. Add 50 $\mu$ L of spores to 50 $\mu$ L of MGC Leaf Punch Lysis
  - b. Vortex the tubes and briefly spin
  - c. Dilute PsiloSEEK Spike In Control to 1:50K.
  - d. Add 10  $\mu$ L of prepared dilution of Spike in Internal Control into each boil prep.
  - e. Incubate on a thermocycler 65°C for 6 minutes, 95°C for 2 minutes, and 4°C until ready to use.
  - f. After incubation vortex and spin tube(s) until spores pellet to the bottom.
  - g. Use 5 $\mu$ L of lysis into qPCR
2. Tissue Sample Preparation using MGC Leaf Punch Lysis

*NOTE: This may be an option with small amounts of tissue. Too much tissue will overwhelm the leaf punch lysis tube and small volume of buffer (100  $\mu$ L). If tissue weight is too much, use SenSATIVAx for MIP/Extracts procedure below.*

  - a. Add  $\leq 1$ mg of tissue to 100 $\mu$ L of MGC Leaf Punch Lysis - Using over 1mg will saturate the LPL and lead to internal control failures.
  - b. Vortex the tubes and briefly spin
  - c. Dilute PsiloSEEK Spike-In Control to 1:50k. (Prepare fresh dilutions before each extraction/lysis)
    - i. Prepare a 1:50 dilution
      1. Transfer 1 $\mu$ L stock internal control to 49  $\mu$ L Nuclease free water
      2. Cap, Vortex and spin tube

- ii. Prepare 1:5k dilution
  - 1. Add 1uL of 1:50 dilution to 99 uL of nuclease free water
  - 2. Cap, Vortex and spin tube
- iii. Prepare 1:50k dilution
  - 1. Add 10 uL 1:5k dilution to 90 uL Nuclease free water.
  - 2. Cap, Vortex and spin tube
- d. Add 10 uL of prepared dilution of Spike-in Internal Control into each boil prep.
- e. Set up Lysis on Thermal cycler
  - i. Incubate on a thermocycler 65°C for 6 minutes, 95°C for 2 minutes, and 4°C until ready to use.
- f. After lysis vortex and spin tube(s).
- g. Use 5µL of lysis into qPCR making sure to avoid any tissue that does not pellet.

#### SenSATIVAx<sup>®</sup> for MIP/Extracts DNA Purification

- 1. Add 1mL of Solution A to the 1.5 mL tube containing 1mg to 100mg of sample.
- 2. Vortex vigorously
- 3. Dilute PsiloSEEK Spike-In Control to 1:5k. (Prepare fresh dilutions before each purification/lysis)
  - a. Prepare a 1:50 dilution
    - i. Transfer 1uL stock internal control to 49 uL Nuclease free water
    - ii. Cap, Vortex and spin tube

- h. Prepare 1:5k dilution
  - i. Add 1uL of 1:50 dilution to 99 uL of nuclease free water
  - ii. Cap, Vortex and spin tube
3. Add 10 uL of diluted PsiloSEEK Spike-in control into each tube.
4. Incubate at 65°C for 15 minutes.
5. Vortex tubes to mix then centrifuge for 5 minutes at 14,000 - 19,300 RCF using a bench-top centrifuge.
6. Transfer 600 ul of the solution to a new 1.5mL tube.
7. 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 2 Example of a milky sample with chloroform in it.

8. Centrifuge for 5 minutes at 14,000 - 19,300 RCF using a bench top centrifuge. If no bench top centrifuge is available, centrifuge for 15 minutes using a mini centrifuge.



8. 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.
9. 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.
10. Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in the 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 gently when tip mixing. This is extremely important as to not contaminate the wells in proximity.*

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.

- (a) Add 400µL of 70% Denatured 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.

15. 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.*

16. 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 EtOH wash, as well as the lab environment. Visually inspect the beads for residual EtOH 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.*

17. Remove the extraction plate from the magnet plate and add 50  $\mu$ 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.*

- (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]”.

18. 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. 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	3.75 $\mu$ L	93.75 $\mu$ L	187.5 $\mu$ L
Assay Probe Mix	1 $\mu$ L	25 $\mu$ L	50 $\mu$ L
Reaction Buffer	0.8 $\mu$ L	20 $\mu$ L	40 $\mu$ L
Water	8.2 $\mu$ L	205 $\mu$ L	410 $\mu$ L
Total Assay Probe MM	13.75 $\mu$ L	343.75 $\mu$ L	687.5 $\mu$ L

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 the following dilution:

- a. Prepare a 1:10 dilution of the Assay PositiveControl
  - i. Label 1 new 1.5 mL Eppendorf tubes +C
    1. Add 9  $\mu$ L nuclease free water to tube
    2. Add 1 $\mu$ L stock + Control to 9 $\mu$ L nuclease free water -  
pipette up and down a few times to be sure the 1 $\mu$ L has  
been released into the water.
    3. Vortex well and quick spin

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

ii. Place on ice until use

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  $\mu$ L water then the 1  $\mu$ L of positive control, pipette mix or vortex control dilution to ensure control DNA is in solution.*

7. If SenSATIVAx prep was used, 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. DNA from MGC Leaf Puch Lysis does not need to be placed on the magnet.

8. Use a 96-well optical qPCR plate and label the plate “qPCR Plate\_ [date]”.

9. If SenSATIVAx prep was used, 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.*

10. Transfer 5  $\mu$ L of each sample into the corresponding well on the qPCR plate, keeping the extraction plate on the magnet when aspirating the 5  $\mu$ L.

11. Add 5  $\mu$ L of the diluted Positive Control to the corresponding well.

12. Add 5  $\mu$ L of water to the corresponding negative control well.

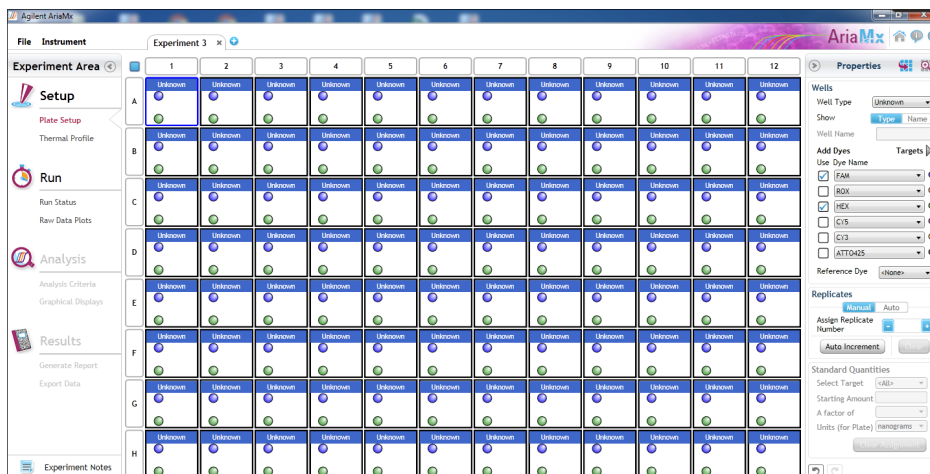
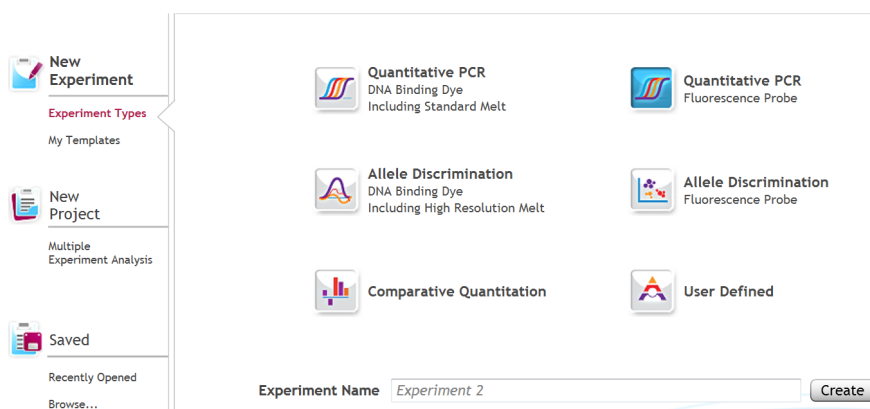
13. Add 13.75  $\mu$ L of PsiloSEEK *P. cubensis* Speciation Assay Probe MM to each corresponding sample well, positive control well, and negative control well in the qPCR plate. Gently tip mix a few times after each addition of qPCR master mix. Be careful not to introduce bubbles during this mix.
14. Seal the plate with strip caps or an 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.

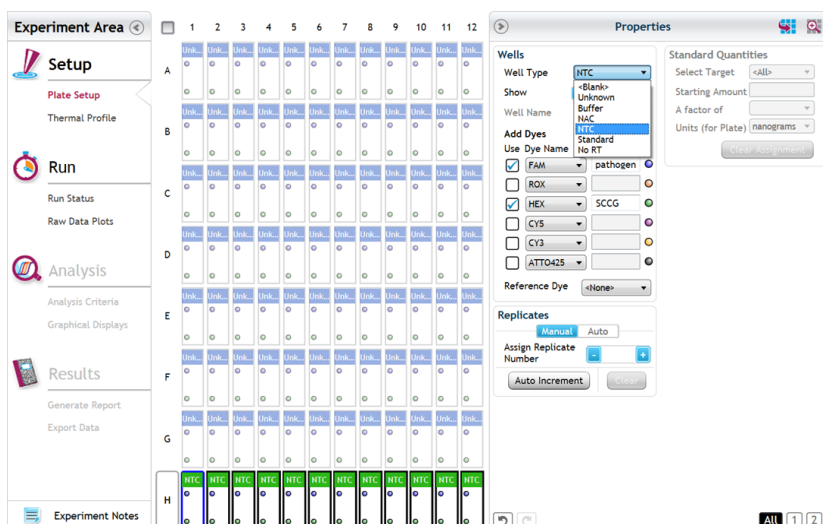
## Running the Agilent AriaMX

The following species will be detected on the following Fluorophores:

- *P. cubensis* ITS: HEX
- *P. cyanescens* (Wood Lovers ITS): Cy5
- Internal Spike-in Control: ROX

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 HEX, ROX and Cy5 under Add Dyes.





3. Change the well types to reflect your plate set up. Add Target names to include “*Psilocybe sp.*”. *P. cubensis* on HEX, *P. cyanescens* on Cy5, and Internal Spike-in Control on ROX.
4. Under Setup > Thermal Profile, create the following PCR thermal profile:
  - a. Hot start at 95°C for 3 minutes, followed by 35 cycles of 95°C for 15 seconds and 64°C for 40 seconds.





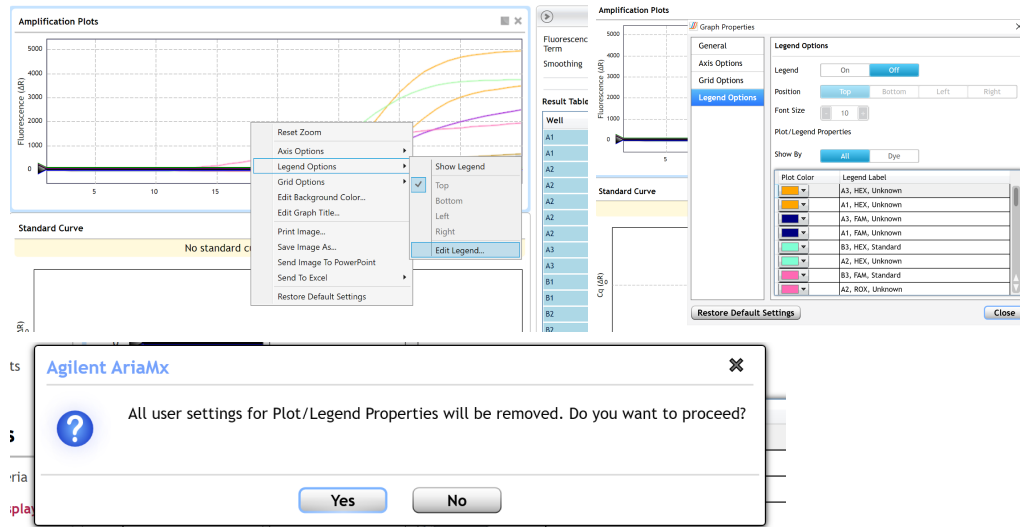
5. Close the lid and click “Start Run”.
6. Save the experiment with the [User] and [date]
7. When the run is complete, dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

### **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<sub>q</sub> 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”

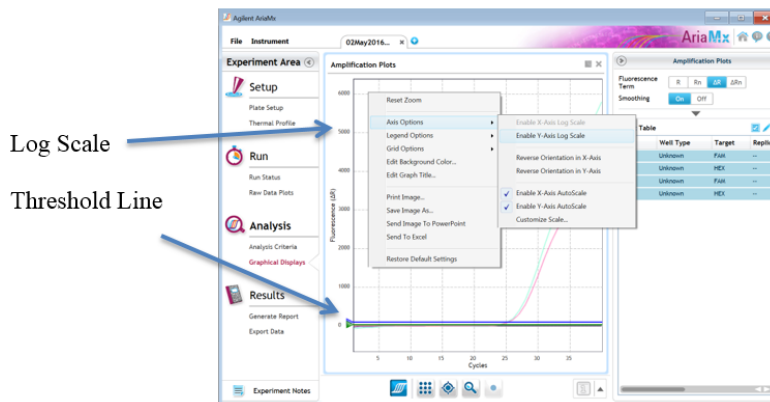
e. All user settings for Plot/Legend Properties will be removed. Do you want to proceed? Select “Yes”.

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

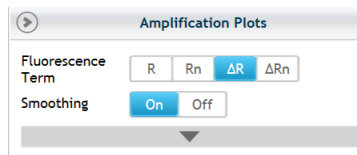


3. To analyze the results:

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 HEX, ROX, and Cy5 fluorophores.

#### 4. Assay Controls

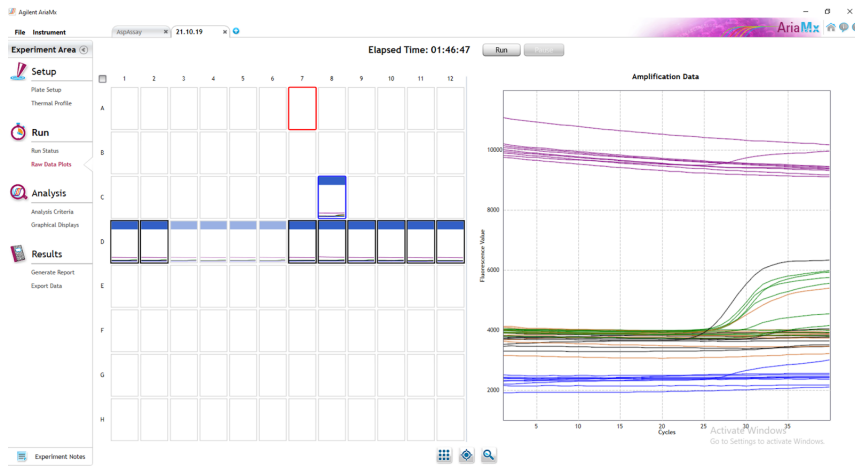
- a. Positive Control, on the HEX, ROX, and Cy5 Fluorophore, has a Cq value  $\leq 33$ .
- i. Visually confirm with the curve on the graph.
- b. Negative Control, on the HEX, ROX and Cy5 Fluorophore, has no Cq value.
- i. Visually confirm with the curve on the graph.

#### 5. Sample Wells

- a. Internal Spike-in Control, on the ROX Fluorophore, has a Cq value  $\leq 33$
- i. Visually confirm with the curve on the graph.
- b. Check for a Cq Value on the HEX and Cy5 Fluorophores.
- c. If there is a Cq value on any of these Fluorophores, visually confirm the 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.

- d. 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.

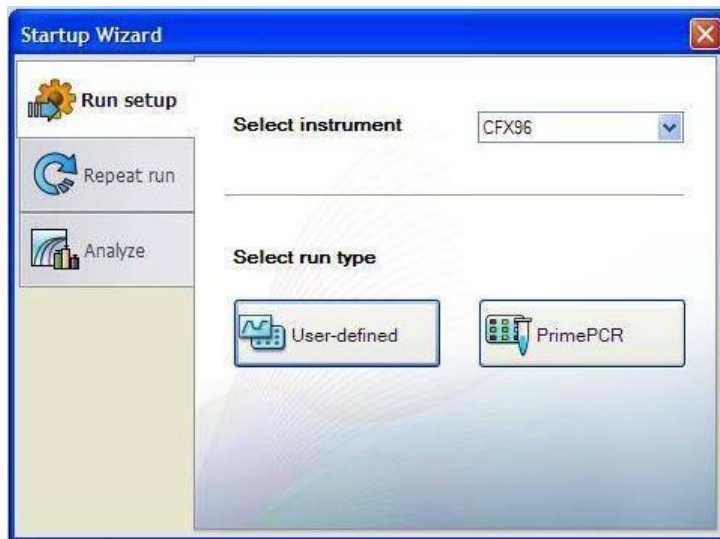


## Running the BioRad CFX96

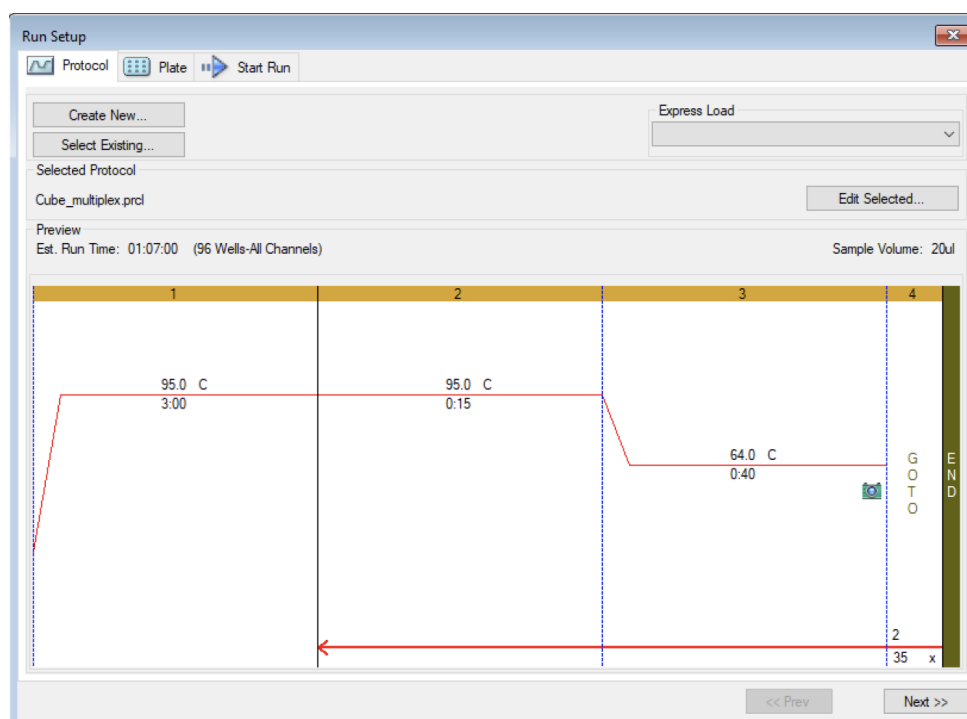
The following targets will be detected on the following fluorophores:

- *P. cubensis* ITS: HEX
- Wood Lovers ITS: Cy5
- Internal Spike-in Control: ROX

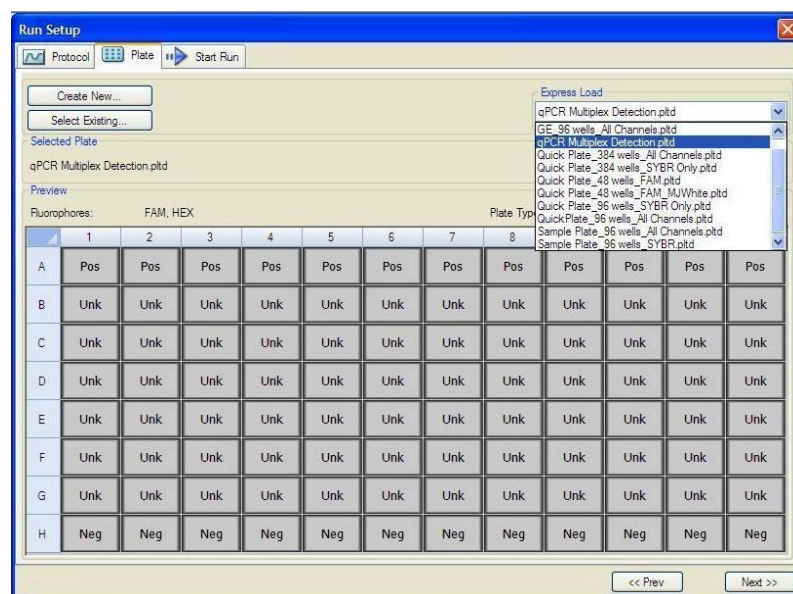
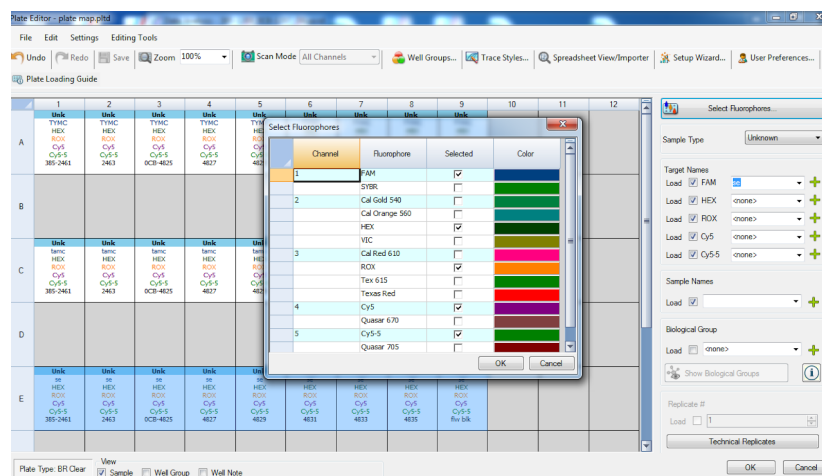
1. Start the qPCR Cycling program.
2. Select User-Defined in the Startup Wizard under Run setup.



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 3 minutes, followed by 35 cycles of 95°C for 15 seconds and 64°C for 40 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 HEX, ROX and Cy5 fluorophores and click “OK”.

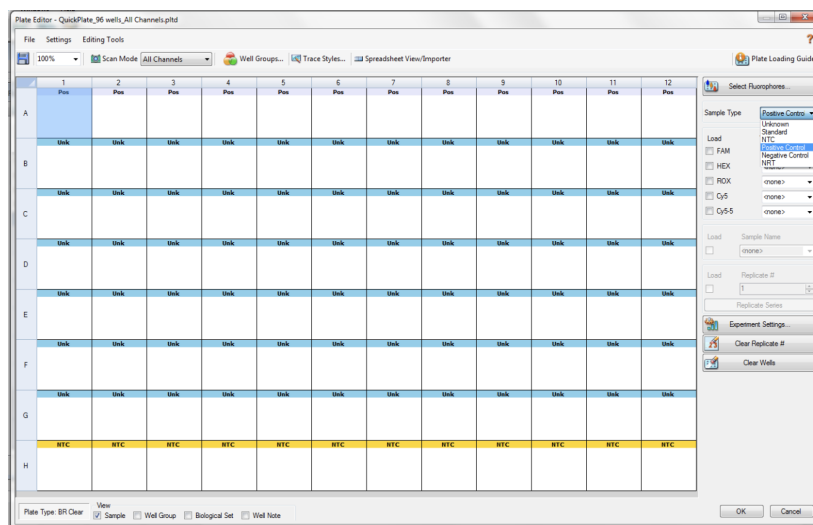


- c. If the plate layout was previously saved, click “Edit Selected” to move to the Plate Editor Screen.
- d. On the Plate Editor Screen, change the Sample Type to correlate with your specific plate setup.

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

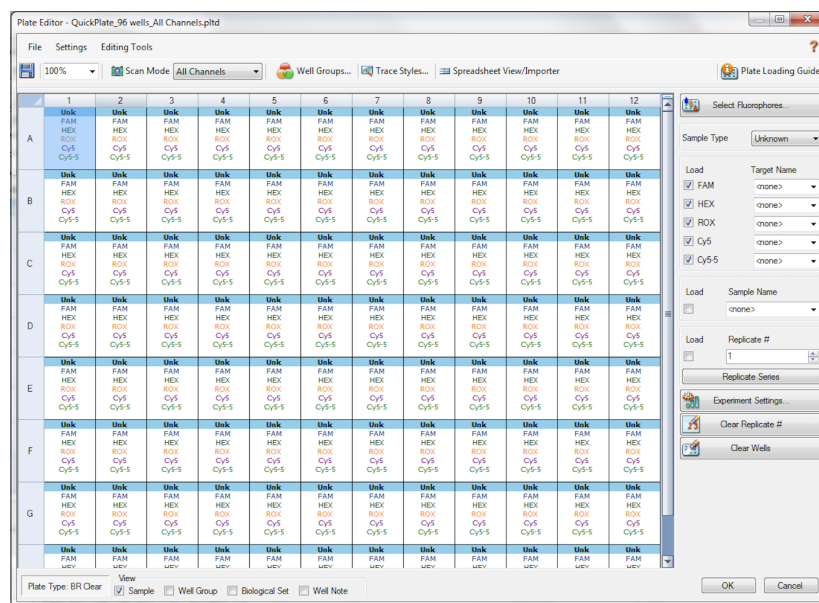
1. *Unknown*
2. *Positive Control*
3. *Negative Control*

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 PsiloSEEK Speciation Assay highlight the well locations and click on **HEX**, **ROX**, 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.*

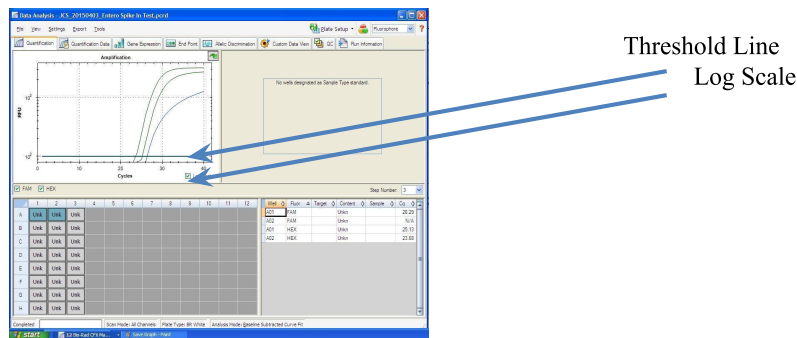
- i. Close the lid and click Start Run.
- j. Save the experiment with the [User] and [date].
- k. When the run is complete, immediately dispose of the plate after qPCR. Do not remove the plate seal after the run to avoid contamination in the lab.

## **Data Analysis: BioRad CFX96**

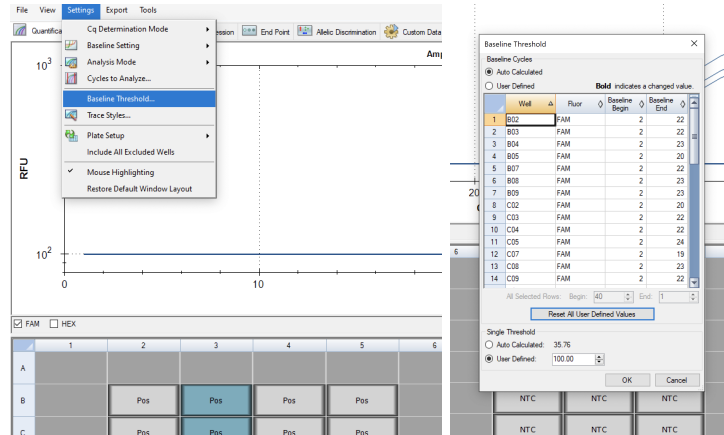
1. The Data Analysis window will open automatically when the run is complete.
2. Highlight the well of interest.

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. Assay Controls

- a. Positive Control, on the HEX, ROX, and Cy5 Fluorophore, has a Cq value  $\leq 33$ .
  - i. Visually confirm with the curve on the graph.
- b. Negative Control, on the HEX, ROX and Cy5 Fluorophore, has no Cq value.
  - i. Visually confirm with the curve on the graph.

## 7. Sample Wells

- a. Internal Spike-in Control, on the ROX Fluorophore, has a Cq value  $\leq 33$ 
  - i. Visually confirm with the curve on the graph.
- b. Check for a Cq Value on the HEX and Cy5 Fluorophores.
- c. If there is a Cq value on any of these Fluorophores, visually confirm the 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).

### **Passing Speciation Test**

Probe/Dye	HEX (Cubensis)	CY5 (Wood Lover)	ROX (Spike in Internal Control)
Presence Result	Cq <33	Cq >33	Cq < 33

## **Troubleshooting Guide**

<b>Symptom</b>	<b>Reason</b>	<b>Solution</b>
Internal spike control failure	Extraction Failure	Repeat 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 CY5, HEX, and ROX. Once completed and the window is closed the analysis should automatically update.
HEX Positive result on a negative sample that do not contain fungal DNA <b>No Cq or a Cq of more than 33 is acceptable. Any Cq lower than 33 constitutes a rerun</b>	Cubensis 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
Negative Control less than Cq of 33	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. Follow this wash with 70% ethanol wipe down. 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 late or 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 the DNA sequence of the Internal spike in control using the ROX Fluorophore.
- A **MIP** is a product infused with Cannabis. In this user guide the SensATIVAx for MIP DNA isolation kit is intended for isolation of DNA from Psilocybe tissue.

## **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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