

PathoSEEK[®] Salmonella & STEC Multiplex Assay with SenSATIVAx[®] Extraction

User Guide

Real Time PCR (qPCR)

**Assay for the detection of Salmonella sp. and Shiga Toxin producing *E. coli* (STEC) in
cannabis flower and MIP matrices**

Table of Contents

Introduction	3
Process Overview	3
Limit of Detection	5
Materials and Methods	6
Test Kit Information	6
PathoSEEK Salmonella & STEC E. coli Multiplex Detection Assay v2	6
Test Kit Components	6
Supplies, Reagents, and Equipment	8
Safety Precautions and Recommendations for Best Results	10
Environment	10
Safety Precautions	11
Intended User	12
Sample Preparation	13
DNA Extraction	15
SenSATIVAx for Flower/Leaf DNA Extraction	15
SenSATIVAx® for MIP/Extracts DNA Extraction	18
Real-Time Quantitative PCR (qPCR) Setup Protocol	23
Running the Agilent AriaMX	27
Data Analysis Agilent AriaMX	30
PathoSEEK® Salmonella/STEC Multiplex Data Analysis Quick Reference Table: All Matrices	30
Running the BioRad CFX96	37
Data Analysis BioRad CFX96	42
PathoSEEK® Salmonella/STEC Multiplex Analysis Quick Reference Table: All Matrices	42
Confirmation of Presumptive Positives	44
Alternative Confirmation	46
AOAC Performance Tested MethodSM Applicability Claim	47
Troubleshooting Guide	48
Glossary and Definitions	49
LIMITED USE LABEL LICENSE	50

Introduction

Current regulations require cannabis flower and cannabis products to be free of select species of *Salmonella* species and shiga toxin producing *E. coli*. The PathoSEEK® *Salmonella* and STEC *E. coli* Multiplex Assays with SenSATIVAx® Extraction Protocol are designed to detect multiple species of *Salmonella* and Shiga Toxin producing *E. coli* in a single qPCR (Quantitative Polymerase Chain Reaction) in cannabis flower, hemp flower, cannabis concentrates, infused edibles and infused non-edibles.

Process Overview

The *Salmonella* and STEC Multiplex microbial detection assays use a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of multiple species of *Salmonella*, shiga toxin producing *E. coli* and cannabis DNA in every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting pathogens, resulting in the minimization of false negative results due to reaction set-up errors or failing experimental conditions. Below is a simplified depiction of the qPCR assays.

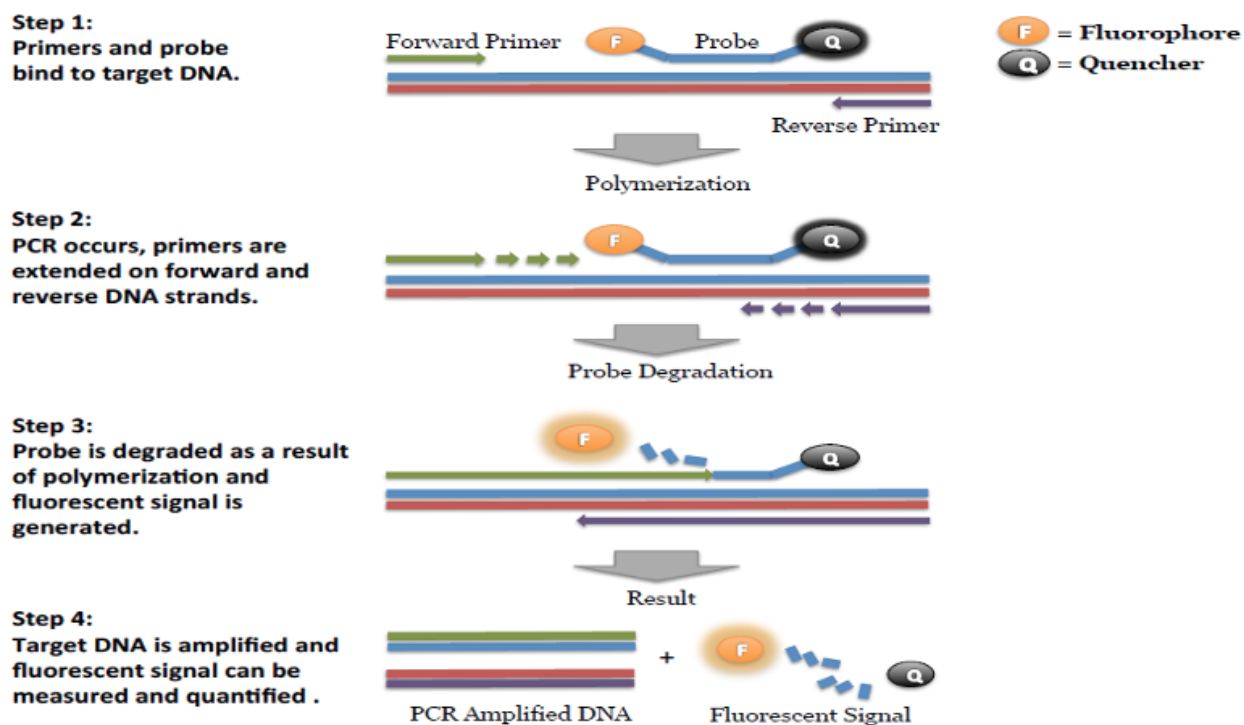
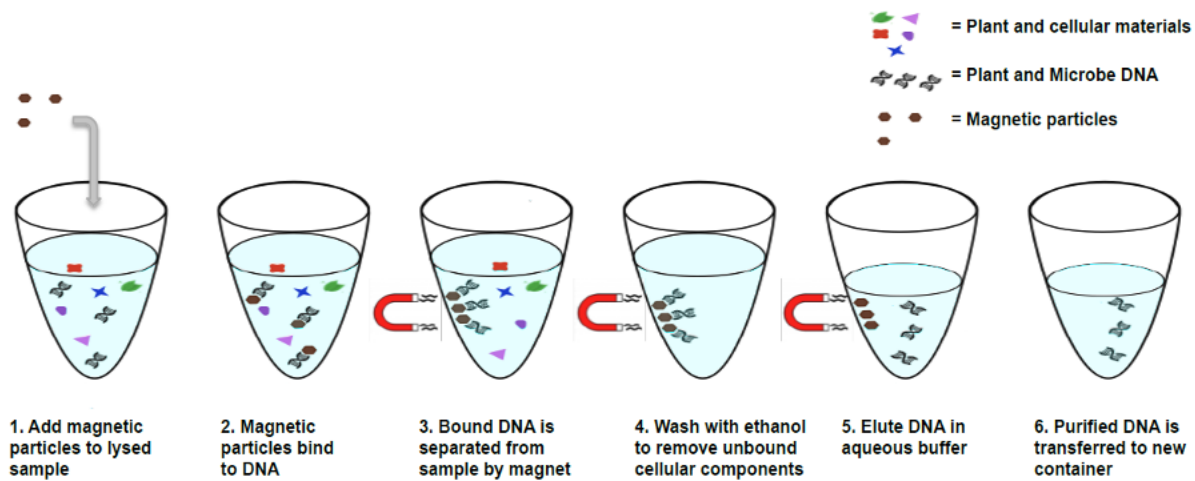


Figure 1: Overview of qPCR

SenSATIVax is a proprietary DNA 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



Limit of Detection

The method is able to detect down to 1 CFU per test portion.

Materials and Methods

Test Kit Name: PathoSEEK Salmonella and STEC Multiplex Assay with SenSATIVAx Extraction

Test Kit Information

1. *PathoSEEK Salmonella & STEC E. coli Multiplex Detection Assay v2*
 - a. *P/N 420120*
2. *SenSATIVAx Flower/Leaf DNA Extraction Kit.*
 - a. *P/N 420001*
3. *SenSATIVAx MIP/Extract DNA Extraction Kit.*
 - a. *P/N 420004*
4. *PathoSEEK Salmonella & STEC E. coli multiplex Positive Control.*
 - a. *P/N 420322*
5. *Medicinal Genomics qPCR Master Kit v3*
 - a. *P/N 420201*

Test Kit Components

1. *PathoSEEK Salmonella & STEC E. coli Multiplex Detection Assay v2* - — P/N 420120
(200 tests).

- a. *Assay* — 1 tube. Store kit at -15 to -20°C. Expires 2 Years from Date of Manufacture.
-
- 2. *SenSATIVAx Flower/Leaf DNA Extraction*.— 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
-
- 3. *SenSATIVAx MIP/Extract DNA Extraction*.— P/N 420004 (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 Salmonella & STEC E. coli multiplex Positive Control* — P/N 420322. (50 reactions)
 - a. Control - 1 tube. Store at -15 to -20°C. Expires 2 Years from Date of Manufacture
5. *qPCR Master Kit v3*— P/N 420201. Kit. Store at -15 to -20°C. Expires 2 Years from Date of Manufacture
 - (1) *Reaction Buffer (10x)*.— 1 tube.
 - (2) *Nuclease Free Water*.—2 tubes.
 - (3) *qPCR Master Mix* - 1 tube

Supplies, Reagents, and Equipment

(a) Agilent AriaMx Real-Time PCR System G8830A—Containing the following Optical

Channels: FAM, ROX, HEX

- (1) Agilent HP Notebook PC option 650.
- (2) 96 Well Optical qPCR plate.— Agilent #401490 or Fisher Scientific #AB2396
- (3) Adhesive optical seal for qPCR plates.— Agilent #401492; USA Scientific TempPlate RT Optical Film #2978-2100.
- (4) 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.

(b) Bio-Rad CFX96 Touch™ Real-Time System.

- (1) Bio-Rad Personal PC.
- (2) 96 Well Optical qPCR plate.— Bio-Rad #HSP-96601 or Fisher Scientific #AB2396
- (3) Adhesive optical seal for qPCR plates.— Bio-Rad #MSB-1001; USA Scientific TempPlate RT Optical Film #2978-2100.
- (c) Adjustable, variable volume pipettes (single or multichannel).—P10, P20, P50, P200, P300 and P1000.
- (d) Adjustable, variable volume filter pipettes tips.—For P10, P20, P50, P200, P300 and P1000.
- (e) Crushed ice.
- (f) 96 Well PCR Cryogenic Rack.—VWR #89004-570.
- (g) 1.5 µL Tube Benchtop Cryogenic Rack.— VWR #89004-558 or equivalent.
- (h) Freezer.—Capable of maintaining -20°C.
- (i) Table Top Mini Plate Centrifuge.—Fisher Scientific #14-100-143 or equivalent.
- (j) Table Top Mini Centrifuge.—VWR #10067-588, #2631-0006 or equivalent.
- (k) Vortex-Genie Pulse.—Scientific Industries, SKU: SI-0236 or equivalent.
- (l) 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 rpm.
- (m) Filter Bags.—Whirl Pak #B01385WA
- (n) Beaker or Solo Cup. (optional)
- (o) Tryptic Soy Broth.—Medicinal Genomics #420205. Store at 2-8°C.

- (p) 1.5 mL Eppendorf Tubes.
- (q) *15 mL or 50 mL conical tubes.*
- (r) 96 Well Plate Magnet.—MGC P/N 420202
- (s) 96 Well Extraction Plate.— Perkin Elmer P/N 6008290
- (t) Eppendorf Tube Rack.
- (u) Scientific Scale.—Capable of measuring to milligrams.
- (v) Refrigerator.—Capable of maintaining 2–8°C.
- (w) Incubator.—Capable of maintaining $37 \pm 2^{\circ}\text{C}$, VWR #97025-630 or equivalent.
- (x) 25mL Serological Pipette.—VWR 89130-890 or 89130-900 or equivalent.
- (y) 10% bleach.
- (z) 70% Ethanol.— MGC P/N 420030

Safety Precautions and Recommendations for Best Results

Environment

The quality of results depends on the strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR:

- Never circulate lab equipment from one workstation to another
- Always use a positive and negative control for each series of amplification reactions
- Periodically verify the accuracy and precision of pipette, as well as correct functioning of the instruments
- Change gloves often, especially if you suspect contamination
- Clean workspaces periodically with 10% bleach and other decontaminating agents

- Use powder-free gloves and avoid fingerprints and writing on tube caps. Both can interfere with data acquisition

Safety Precautions

(a) *Escherichia coli* is a bacterium that is commonly found in the human gastrointestinal tract. Some strains, however, can be pathogenic to humans: STEC, EPEC, EHEC, ect. STEC are *E. coli* that produce shiga toxins encoded by *stx* genes. STEC may be, but are not always necessarily associated with human disease. *Salmonella* has been recognized as a primary cause of foodborne illness worldwide. STEC and *Salmonella* are considered biological safety level 2 organisms and only trained individuals should be involved in their manipulation.

(b) 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.

(c) It is the responsibility of each laboratory to handle waste and effluents processed according to their nature and degree of hazardness and to treat and dispose of them in accordance with applicable local, state, and federal regulations.

(d) Hazard Statement: Chloroform

- (1) Harmful if inhaled or swallowed.
- (2) 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.
- (3) Keep the container tightly closed and sealed until ready for use. Wash thoroughly after handling.
- (4) Please refer to the Manufacturer Safety Data Sheet (SDS) for more information and proper disposal



(e) Hazard Statement: 70% Ethanol

- (1) Highly flammable liquid and vapor May cause respiratory irritation
- (2) May cause drowsiness or dizziness Causes damage to organs
- (3) May cause damage to organs through prolonged or repeated exposure
- (4) Please refer to the Safety Data Sheet (SDS) for more information and proper disposal



Intended User

The PathoSEEK[®] Salmonella & STEC Multiplex 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

(a) Aliquot Tryptic Soy Broth (TSB).

Note: TSB is a very good growth medium for microbes. Due to this, it is best to transfer the approximate amount of TSB into another sterile tube or container so as to not contaminate the whole bottle. Return it to the 2-8°C refrigerator immediately after use.

(b) Wipe down the workspace with a 10% bleach solution, including the bench top and all equipment being used.

(c) Remove the MGC Binding Buffer and TSB from the 2-8°C refrigerator (it should come to room temperature, 20-28°C, before use).

(d) Prepare consumables. Label all the filter bags with “[sample name] [date]”.

(e) Prepare consumables. Label all the 1.5mL centrifuge tubes needed with [sample name]

(f) Label extraction plate with date, and if transferring eluted DNA to new plate.

(g) Before weighing out the sample to be tested, make sure that the entire sample is broken up and thoroughly homogenized. A well-homogenized sample will ensure more accurate testing.

(h) *Cannabis flower, n grams* —Weigh flower sample material into one side of the mesh liner inside the Whirl-Pak bag. Add $9 \times n$ mL of TSB to each test portion. Close the Filter bag by folding the top over three times. Mix for 1 minute by hand. Incubate for 16 - 24 h at $37 \pm 1^\circ\text{C}$.

(i) *MIP, 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 or homogenize sample and TSB. Incubate for 16 - 24 h at $37 \pm 1^\circ\text{C}$.

Note* During the AOAC Research Institute *Performance Tested Method*SM validation study, 10 g of dried cannabis flower (THC) >0.3%), 5 g of cannabis concentrate and 25 g of THC-infused chocolate were evaluated.

Note* For optimal performance of the method, end users should verify minimum incubation times on the strains of matrix evaluated at their facility.

(j) If processing multiple samples, be sure to change gloves between each, to ensure there is no cross contamination between samples during the weighing process. See flower example in Figure 3.



Figure 3: Homogenized cannabis flower and TSB.

DNA Extraction

SenSATIVAx for Flower/Leaf DNA Extraction

- a) Aspirate **1 mL** from the side of the filter bag, free of plant debris, and dispense into the 1.5mL tube.
- b) Add 50 μ L of MGC Lysis buffer and vortex for 10 seconds then let incubate on the bench for 2 minutes.
- c) After 2 minute incubation, spin for at least 1-3 minutes in a bench top mini centrifuge.

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. See Figure 4.

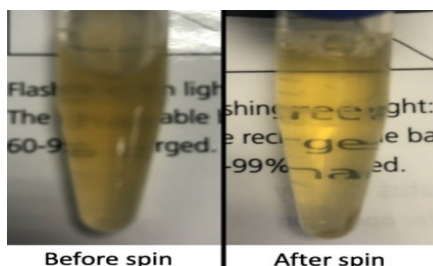


Figure 4: Example of translucent lysate after spinning.

- d) 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. Place the 200 μ L in a labeled 96 well extraction plate labeled with Extraction Plate [date]”
- Note: Pellet size will vary depending on trichome density.*
- e) Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer for at least 30 seconds.

f) 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.

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

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

i) 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. See Figure 5.

(1) Add 400µL of 70% 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 pipet tip at the bottom center of the well to remove all liquid.

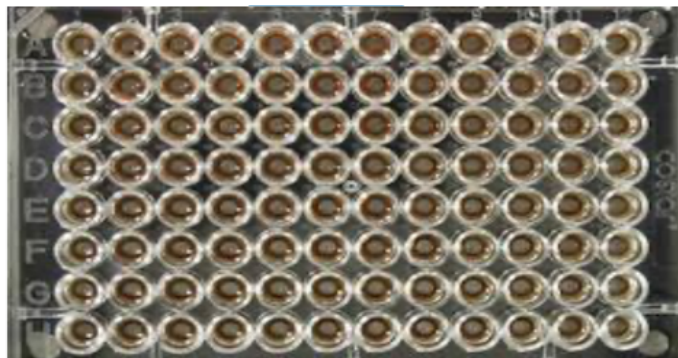


Figure 5: Extraction plate during wash step on magnetic plate.

j) 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 pipet tip to remove the excess. Leftover EtOH can inhibit qPCR efficiency.

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

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.

l) Remove the extraction plate from the magnet plate and add 50 μ L 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. See Figure 6.

(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]”.

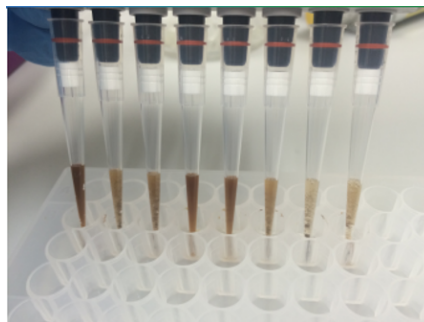


Figure 6: Multichannel pipette tips showing magnetic beads resuspended in elution buffer.

m) 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

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.

- b) 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 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 14,000 rpm 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 ul of the solution to a new tube. Push pipet tip through the top solid layer (if one exists), without disturbing the pellet on the bottom to aspirate the sample.
- g) Add 600 µL chloroform and vortex vigorously until 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 14, 000 rpm 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.
- 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% 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 pipet 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 pipet 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 on the magnet plate for 15 minutes.

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

a) 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.

b) Before preparing the reaction, invert or vortex and spin-down the reagents.

(1) Assay probe mix tubes, reaction buffer, positive controls and water – Vortex quickly followed by a pulse spin-down in a microcentrifuge.

(2) qPCR Master Mix – Invert the tube 5 times (do not vortex), followed by a pulse spin-down in a microcentrifuge.

(3) Return all reagents to the ice.

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

c) 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.

Table 1: PCR Reagent Volumes

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

d) Once combined gently, tip mix or invert the tube 5 times to combine the assay master mix.

(1) Pulse spin-down tube in microcentrifuge.

(2) Place qPCR Master Mix tubes on ice until used.

(3) For the positive control, make a 1:10 dilution

i. Add 1 µL of Positive Control to 9 µL nuclease free water (found in the kit)

ii. 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.

e) Place the Extraction Plate on the magnet. This is to ensure no magnetic beads are transferred into the qPCR reactions if there are some left over from the extraction elution process.

f) Use a 96-well optical qPCR plate and label the plate “qPCR Plate_ [date]”.

g) Carefully remove the seal from the Extraction Plate. If frozen, let the DNA thaw completely and spin the plate to avoid cross contamination between samples. Transfer 5 µL of each sample into the corresponding well on the qPCR plate. Keep the extraction plate on the magnet when aspirating the 5 µL.

(1) Add 5 µL of the diluted Positive Control to the corresponding well. Then add 5 µL of water to the corresponding negative well.

Note: ALWAYS use a fresh tip for every liquid transfer into the qPCR plate

h) Add 13.75 µL of specific 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.

Note: It may be helpful to label each of the corresponding wells to accurately dispense the correct sample.

i) Seal the plate with strip caps or an adhesive seal.

j) 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.

k) 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.

l) Place the sealed plate onto the PCR instrument, positioning the A1 well in the top left corner.

m) Follow the software specific instructions to initiate the run.

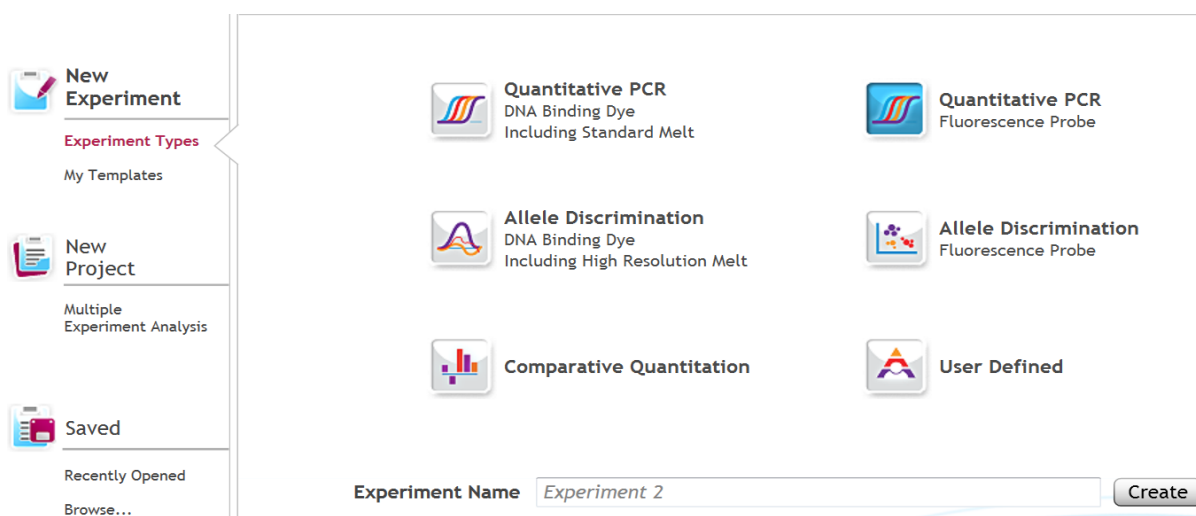
Running the Agilent AriaMX

The following species will be detected on the following Fluorophores:

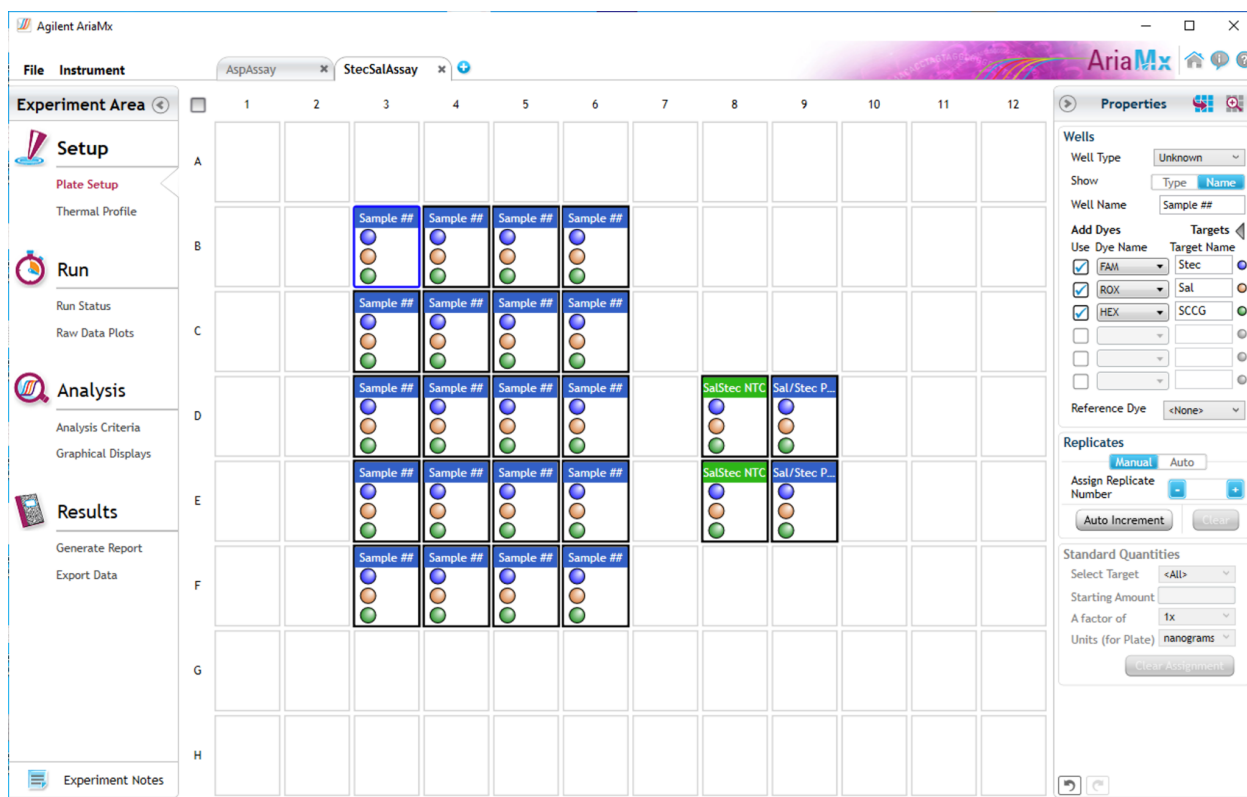
- STEC: FAM
- Salmonella: ROX
- Cannabis DNA: HEX

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

i) Select “Quantitative PCR: Fluorescence Probe” from Experiment Types.



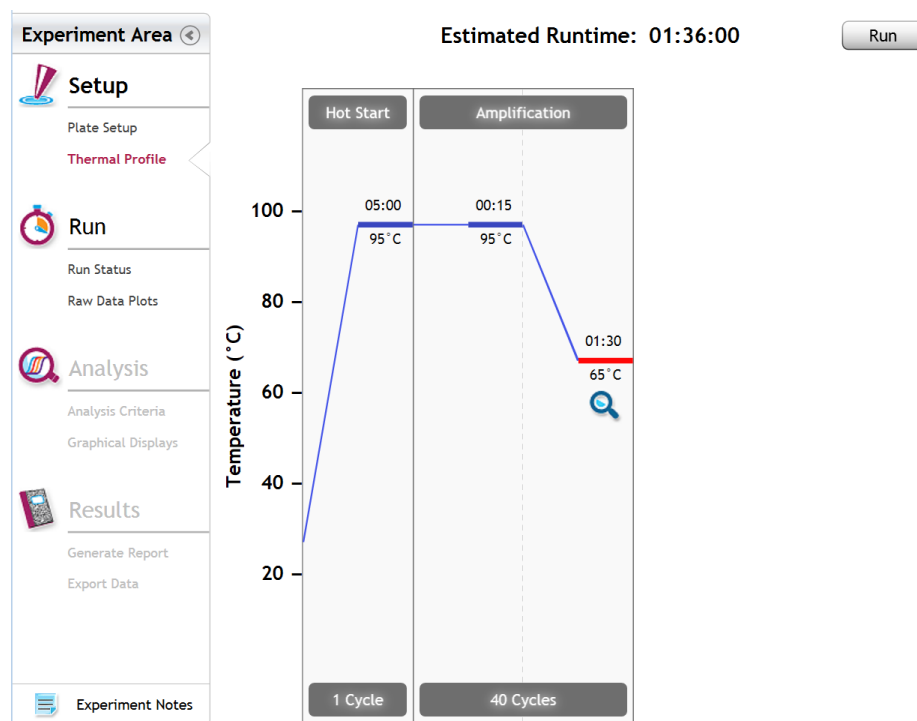
ii) Under Setup>Plate Setup, highlight wells that contain reactions and select FAM, HEX, and ROX under **Add Dyes**. Having fluorescence detection turned on in empty wells can affect the calculations which the software makes to obtain the corrected data, ΔR log.



- b) Change the well types to reflect your plate set up. All wells should be Unknown except the negative control can be NTC well type. Add Target names to the dyes under **Targets**:
- i) FAM : STEC
 - ii) ROX : Salmonella
 - iii) HEX : Single Copy Cannabis Gene (SCCG)

- c) Under Setup>Thermal Profile, create the following PCR thermal profile

(1) Hot start at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 65°C for 90 seconds.

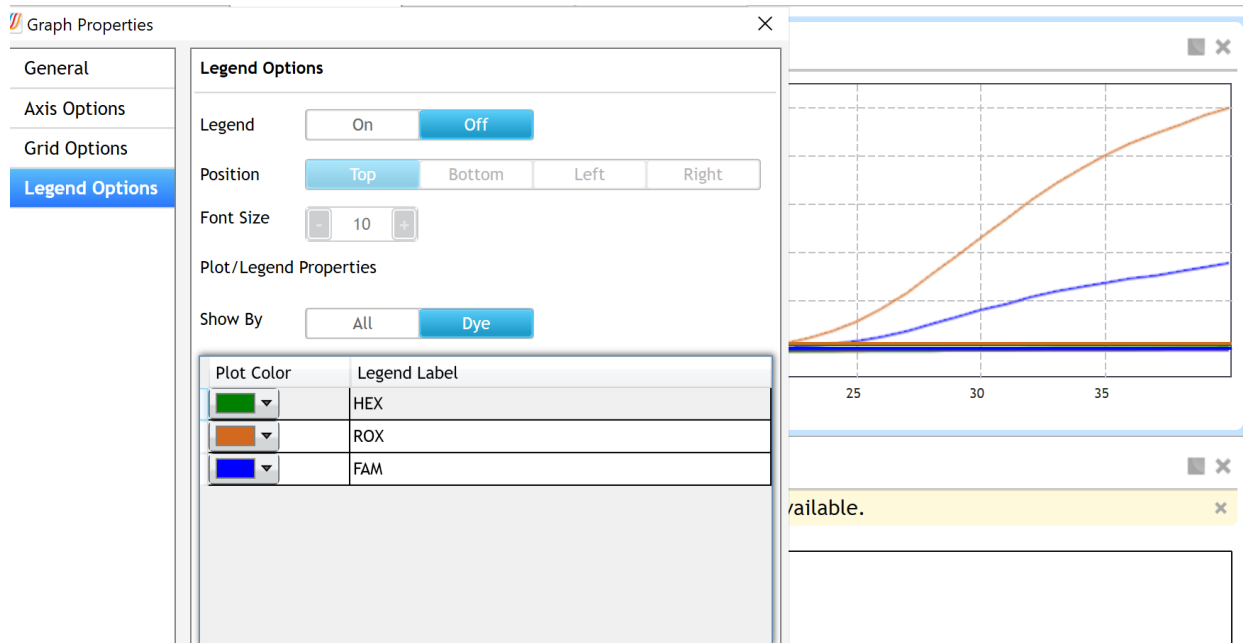
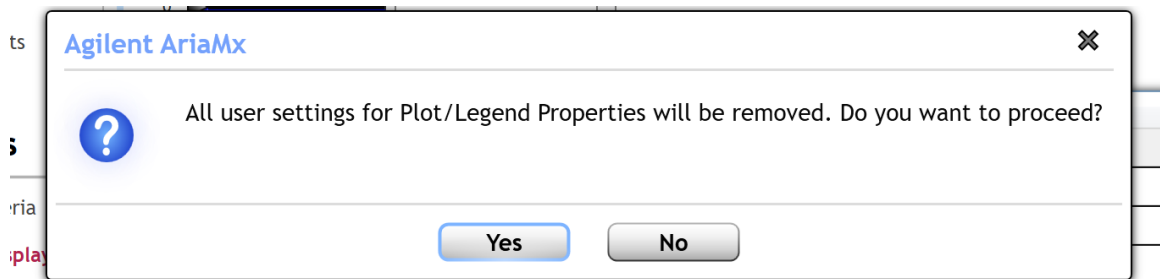
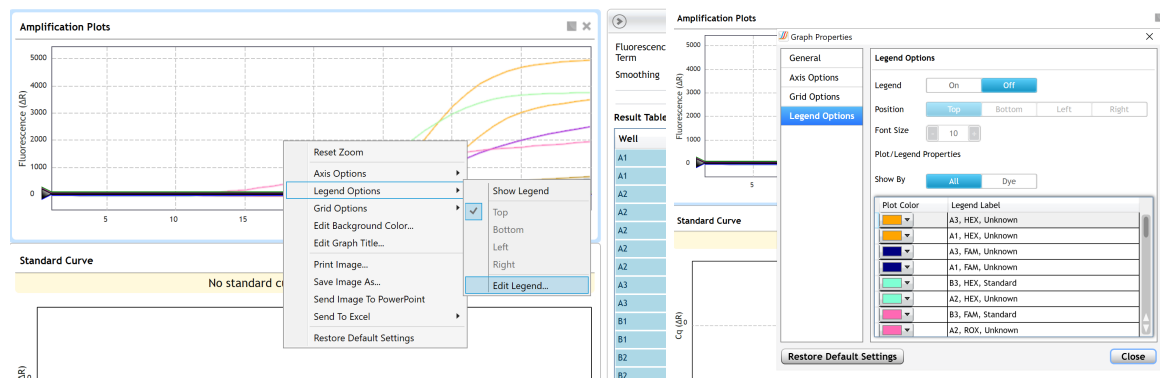


- d) Close the lid and click “Start Run”
- e) Save the experiment with the [User] and [date]
- f) When the qPCR run is complete, immediately dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

PathoSEEK® Salmonella/STEC Multiplex Data Analysis Quick Reference Table: All Matrices

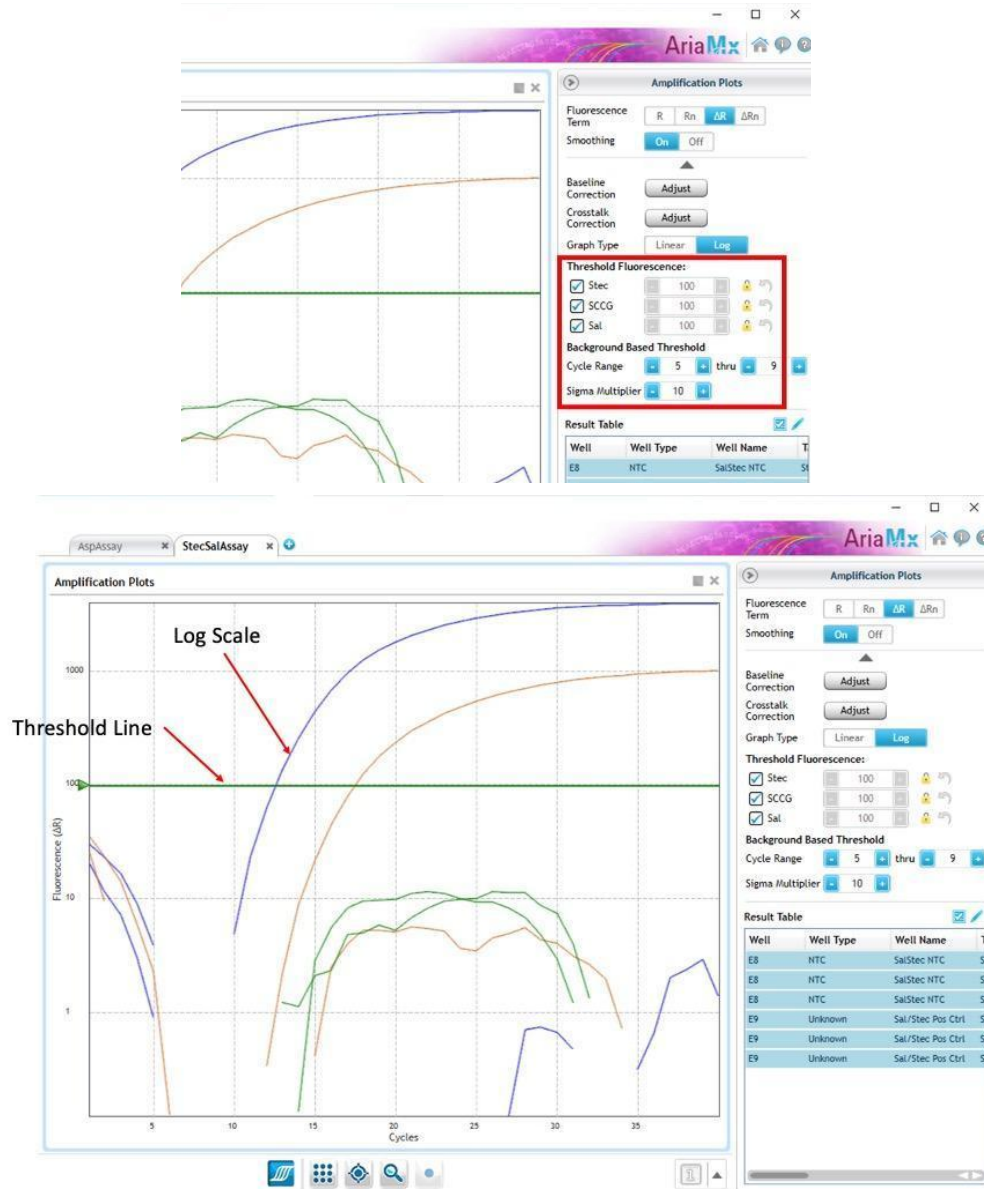
PathoSEEK® Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g) (This may vary by state)
<i>Salmonella species</i>	≤ 40	ROX	No Cq	Presence/Absence
<i>STEC E.coli species</i>	≤ 40	FAM	No Cq	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA and has a ≤ 35 value for flower and ≤ 40 for all other matrices.	
Assay Positive Control	≤35	FAM/ROX/HEX		

- a) Open the Data Analysis window when the run is complete.
- b) Highlight the wells of interest in the Analysis Criteria under Analysis, then select Graphical Display
 - i) Amplification plots will be available for viewing
 - ii) The Cq values will appear to the right in the table
 - iii) Right click inside the graph, select Edit Legend under Legend Options
 - iv) Change “All” to “Dye”
 - v) All user settings for Plot/Legend Properties will be removed. Do you want to proceed? Select “Yes”.
 - vi) This will assign a single color to each fluorophore.



c) To analyze the results

- i) Start by turning the graph to Log Scale with a right click on the chart, select Axis options, enable y-axis log scale. Expand the amplification plots settings by clicking on the triangle
- ii) Manually adjust thresholds to 100 RFU for the HEX, FAM, and ROX



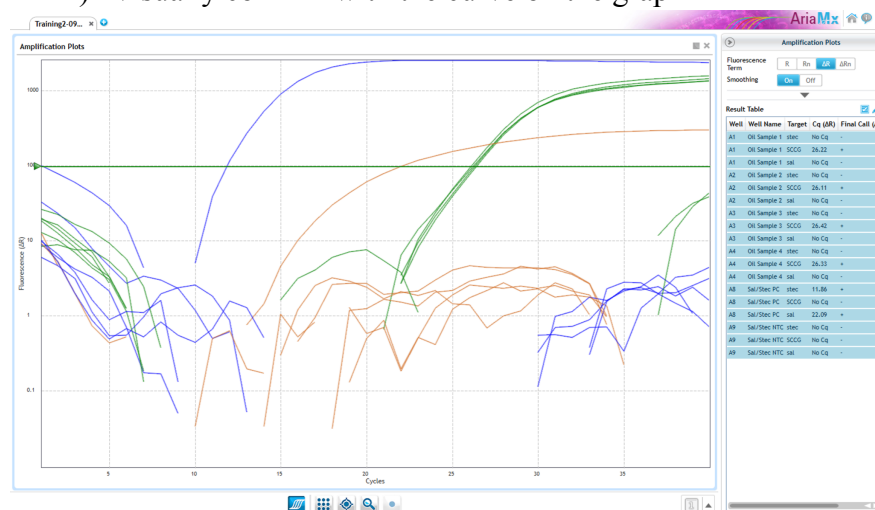
d) Controls

- i) Positive Control, on the FAM and ROX Fluorophores, have Cq values ≤ 35 .
 - 1) Visually confirm with the curve on the graph.

- ii) Negative Control, on the FAM and ROX Fluorophores, have no Cq Value.
 - 1) Visually confirm with the curve on the graph.

e) Unknown Salmonella and STEC *E.coli* Targets

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



- ii) A “presence” or failing result for the unknown samples Salmonella and or STEC target(s).
 - 1) Any Cq value for the FAM or ROX Fluorophores ≤ 40 .
 - 2) Visually confirm with the curve on the graph. It is very important to check the R to confirm with the amplification curve when a presence result occurs.

(a) 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.

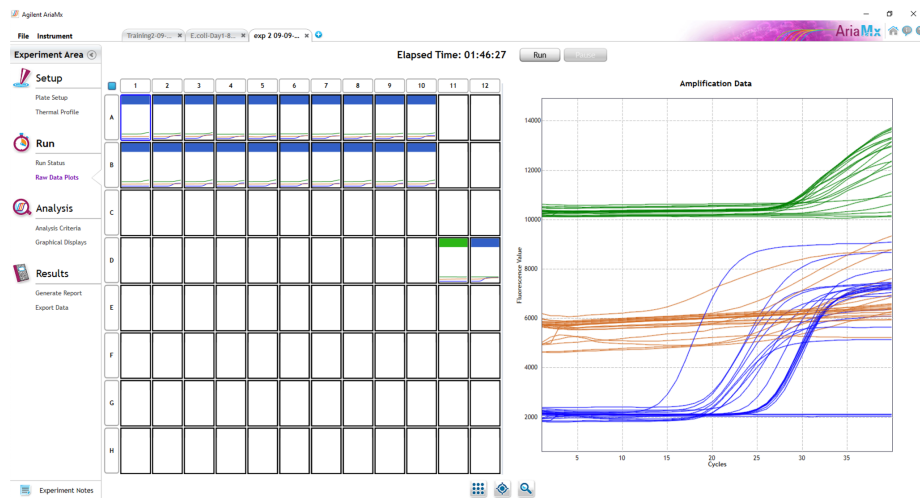


Figure: Raw data (R) plots for which the cycle quantification (Cq) will be generated.

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

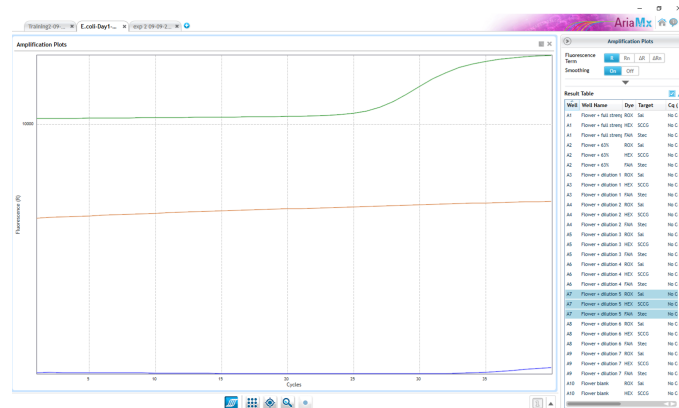


Figure: Example of a good baseline with sigmoidal shaped curve increase in the internal control fluorescence.

- 3) Check the amplification plots for a semi-logarithmic curve with two distinct phases that crosses the threshold:
 - (a) Select the ΔR Fluorescence Term and look at the shape of the amplification plot. The graph should have the classic semi-logarithmic shape with two visually distinct phases.
 - (b) Phase 1 shows exponential growth in the fluorescence, in a span of around 5 cycles.
 - (c) Phase 2 is a plateau where the amplification signal growth ends but remains level.



iii) An “absence” or passing result for the unknown Salmonella and or STEC target.

- 1) No Cq value for the FAM or ROX Fluorophores.
- 2) Visually confirm that there is no curve on the graph.

Running the BioRad CFX96

The following targets will be detected on the following fluorophores:

- Salmonella species: ROX
- STEC *E.coli* species: FAM
- Cannabis DNA: HEX

a) Start the qPCR Cycling program

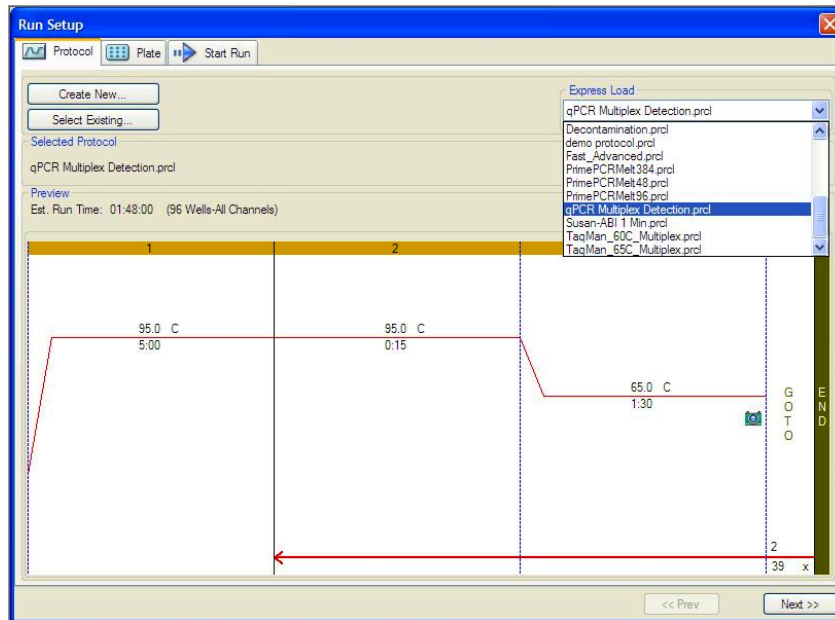
(1) Select User-Defined in the Startup Wizard under Run setup



b) Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click “Next”.

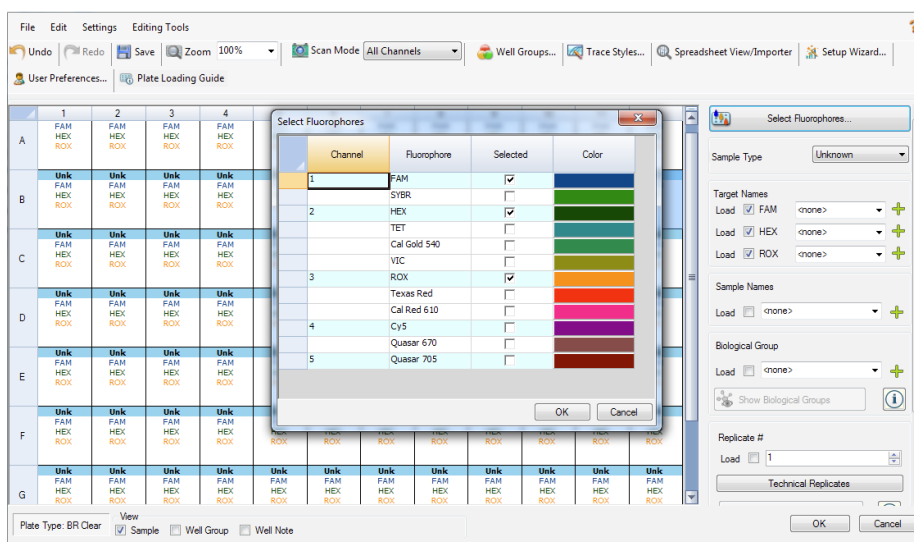
(1) If not already pre-programmed, create a cycling program with the following specifications and save as “qPCR Multiplex Detection”

- i. 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.

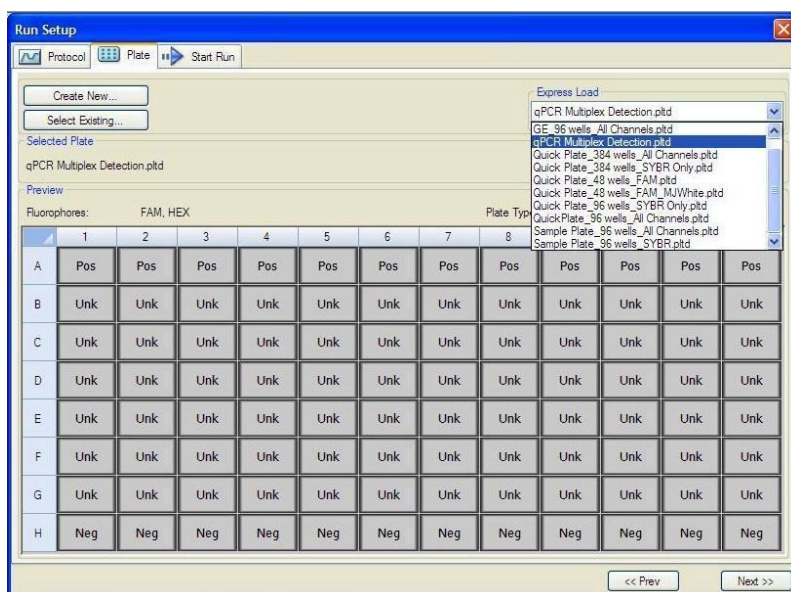


c) Design your plate under the plate tab in the Run Setup

- (1) Select the qPCR Multiplex Detection from the dropdown menu. If not already present, click “Create New”
- (2) Plate editor window will appear. Choose FAM, HEX, and ROX Fluorophores and click “OK”.



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

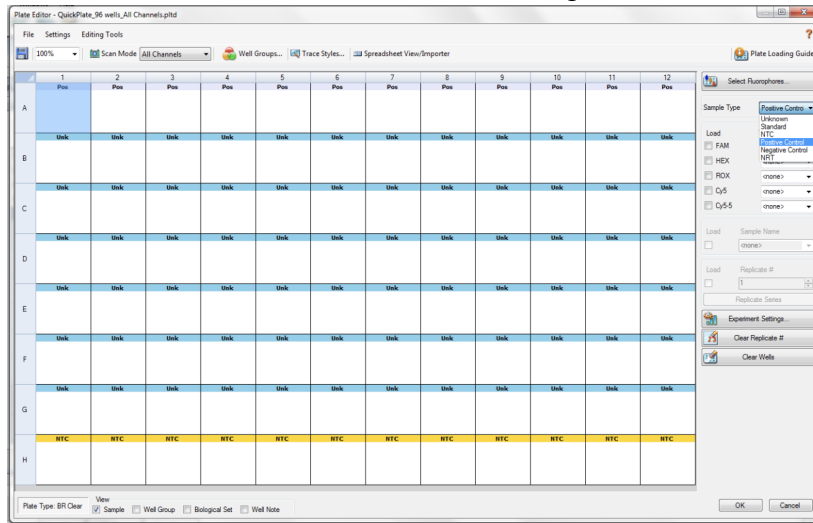


(4) On the Plate Editor Screen, change the Sample Type to correlate with your specific plate setup.

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

Positive Control
Negative Control

(5) Make sure All Channels is selected from the dropdown menu at the top.



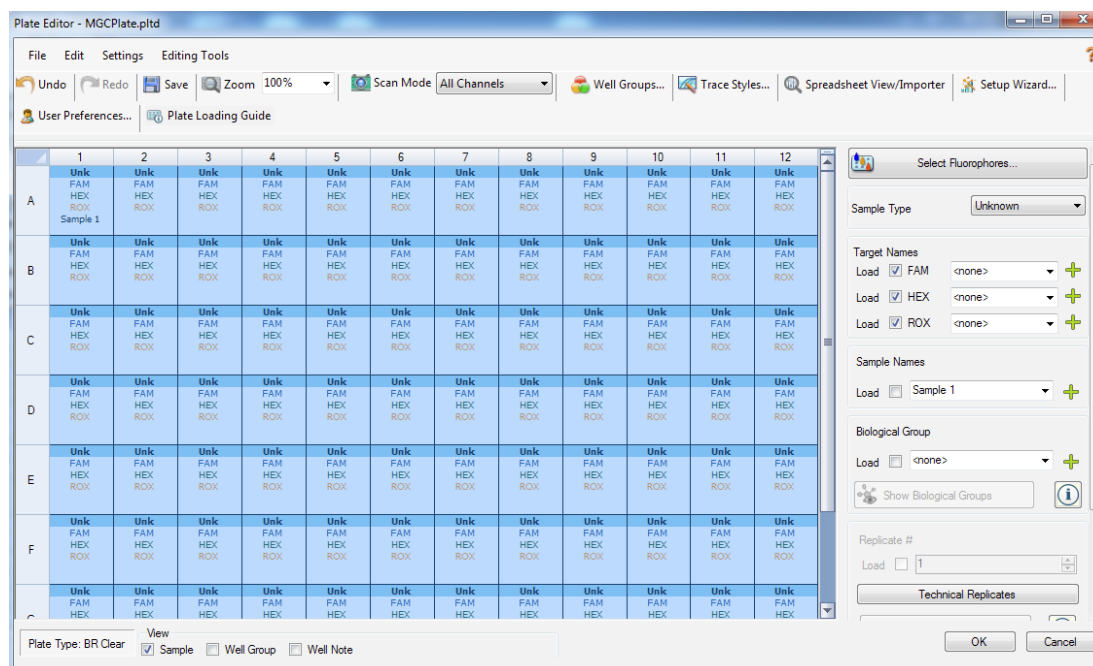
(6) Attach the fluorophores to the wells being used.

i. Highlight all the wells being used

ii. For Salmonella/STEC Multiplex Assay, **Highlight the well locations and click on FAM, HEX, and ROX**

(7) When the plate is designed correctly, click OK.

(8) 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 (that is fine).

(9) Close the lid and click Start Run.

(10) Save the experiment with the [User] and [date]

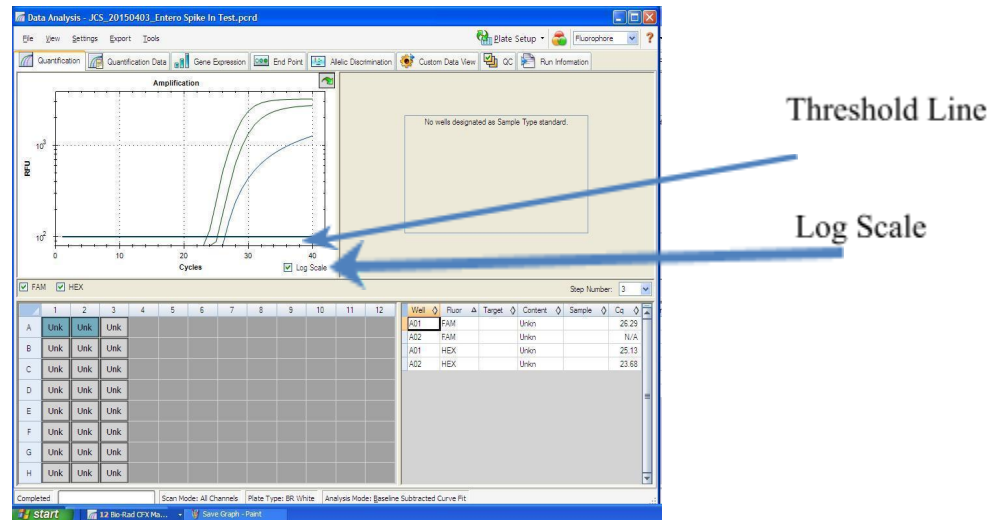
(11) When the run is complete, immediately dispose of the plate after qPCR. **Do not remove the plate seal after the run to avoid contamination in the lab.**

Data Analysis BioRad CFX96

PathoSEEK® Salmonella/STEC Multiplex Analysis Quick Reference Table: All Matrices

PathoSEEK® Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g) (This may vary by state)
<i>Salmonella species</i>	≤ 40	ROX	No Cq	Presence/Absence
<i>STEC E.coli species</i>	≤ 40	FAM	No Cq	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA and has a ≤ 35 value for flower and ≤ 40 for all other matrices.	
Assay Positive Control	≤35	FAM/ROX		

- a) The Data Analysis window will open automatically when the run is complete.
- b) Highlight the well of interest.
 - (1) The graph will appear above.
 - (2) The Cq values will appear to the right.
- c) To analyze the results
 - (1) Start by turning the graph to Log Scale and manually moving the threshold to 10² 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.



(2) Controls

i. Assay-specific Positive Control, on the FAM and ROX fluorophore, has a

Cq value ≤ 35 .

a. Visually confirm with the curve on the graph.

ii. Assay-specific Negative Control, on the FAM, ROX, and HEX fluorophore,

has no Cq value.

a. Visually confirm with the curve on the graph.

(3) Unknown Salmonella and STEC Targets: The following fluorophores detect the following organisms:

i. Salmonella species: ROX

ii. STEC *E.coli*: FAM

iii. Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, ≤ 40 for all other matrices.

- a. Visually confirm with the curve on the graph
- iv. A “presence” or failing result for the unknown Salmonella/STEC targets.
 - a. Any Cq value for the FAM or ROX fluorophores ≤ 40 .
 - b. Visually confirm with the curve on the graph. It is very important to confirm with the amplification curve when a presence result occurred. Sometimes the background amplification will give a false positive reading.
- vi. An “absence” or passing result for the unknown Salmonella & STEC targets.
 - a. No Cq value for the FAM or ROX fluorophores.
 - b. Visually confirm no curve on the graph.

Confirmation of Presumptive Positives

All positive results should be confirmed following guidance in AOAC SMPR 2020.002, 2020.0012 or the laboratory's confirmation protocol and state and/or regional requirements.

Salmonella spp.

- (a) Mix enriched sample thoroughly by hand. Transfer 0.1 mL of each test portion to 10 mL Rappaport Vassiliadis (RV) medium and 1 mL to 10 mL Tetrathionate (TT) broth. Incubate RV medium at $42 \pm 0.2^{\circ}\text{C}$ for 22–26 h in a circulating water bath. Incubate TT broth at $37 \pm 1^{\circ}\text{C}$ for 22–26 h.

- (b)** From the secondary enrichments, streak a loopful to Xylose Lysine Deoxycholate (XLD) and CHROMagar *Salmonella*. Incubate at $37 \pm 2^{\circ}\text{C}$ for 22–26 h.
- (c)** Pick up to 2 colonies from each agar plate and stab/streak to triple sugar iron (TSI) and lysine iron agar (LIA). Incubate at $37 \pm 2^{\circ}\text{C}$ for 22–26 h.
- (d)** Refer to US FDA BAM Chapter 5 [6] or USDA FSIS MLG 4.11 [7] for guidance on reading TSI and LIA slants. If further confirmation is required based on reactions in TSI and LIA, streak growth from TSI onto a tryptic soy agar (TSA) plate. Incubate for 18–24 h at $35 \pm 2^{\circ}\text{C}$.
- (e)** Pick one well isolated colony and perform a spot polyvalent O and polyvalent H serology test.
- (f)** Biochemically analyzing using an AOAC PTM or OMA approved method or alternatively confirm via sequencing.

STEC

- (a)** Mix enriched sample thoroughly by hand. From the primary enrichment, perform an isolation streak to CHROMagar STEC and either MacConkey Agar with Sorbitol, Cefixime and Tellurite (CT SMAC) or Levine’s Eosin Methylene Blue (L-EMB) agar. Incubate at $37 \pm 2^{\circ}\text{C}$ for 18–24 h.

- (b) Screen typical colonies with antigen specific latex agglutination test. Pick presumptive positive colony and streak to tryptic soy agar with yeast (TSAYE). Place a ColiComplete (CC) disc into the heaviest streak area. Incubate at $37 \pm 2^{\circ}\text{C}$ for 18–24 h.
- (c) STEC will produce a blue color (galactopyranosidase; X-gal positive) and not fluorescence under ultraviolet (UV) light (glucuronidase, 4-methylumbelliferyl- β -D-glucuronide (MUG) negative).
- (d) Wet filter paper with Kovac's reagent and perform a spot indole test using growth from the TSAYE plate.
- (e) Confirm the presence of *Stx* in the isolate by reprocessing an isolated colony with the PathoSEEK Salmonella and STEC E. coli assay.
- (f) Biochemically analyzing using an AOAC PTM or OMA approved method or alternatively confirm via sequencing.

Alternative Confirmation

Using the purified DNA from step (l)(3) or (s)(3) from the DNA Extraction section, reanalyze the sample following the Real-Time Quantitative PCR (qPCR) Setup Protocol starting on page 23. *This confirmation protocol is outside the scope of the AOACRI PTM certification.

AOAC Performance Tested MethodSM Applicability Claim

The PathoSEEK® Salmonella & STEC *E.coli* Multiplex Assay with SenSATIVAx® Extraction has been validated according to the AOAC *Performance Tested MethodSM* Program for the detection of *Salmonella* species and Shiga toxin producing *E.coli* (STEC) in dried cannabis flower (>0.3% THC, 10 g), cannabis concentrates (5 g) and THC-infused chocolate (25 g) on the AriaMx and CFX-96 instruments. Based on the results of the independent laboratory validation and the variability in cannabinoid profile of flower matrix and the impact it has on the growth rates of the target organisms, and it may benefit end users to analyze samples beyond the minimum validation time point for optimal results. The validation study met the requirements as set forth in the AOAC *Standard Method Performance RequirementsSM* 2020.002 for the detection of *Salmonella* species and 2020.012 for the detection of Shiga toxin producing *E.coli* (STEC) in cannabis and cannabis infused products.

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.

LIMITED USE LABEL LICENSE

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

© 2022 Medicinal Genomics Corporation. All rights reserved.

* All Trademarks are property of their respective owners.