# PathoSEEK® *Salmonella*, *E. coli,* and Shiga Toxin-Producing *E. coli* (STEC) Detection Assays with MaGiC Lysis Kit

## User Guide

**Real Time PCR (qPCR) assays for the detection of *Salmonella* sp., total *E. coli* and Shiga Toxin-producing *E. coli* (STEC) in cannabis flower and infused products matrices**

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## Introduction

In some locations, regulations require cannabis flower and cannabis products to be free of select species of *E. coli*, *Salmonella*, and Shiga Toxin-producing *E. coli* (STEC). Medicinal Genomics offers several PathoSEEK® qPCR detection assays designed to detect these targets. This user guide will address the following three assays:

- PathoSEEK® E. coli Detection Assay v2
- PathoSEEK® E. coli (STEC) Detection Assay v2
- PathoSEEK® Salmonella Detection Assay v3

When used in combination with MaGiC Lysis, the PathoSEEK Salmonella, E. coli, and STEC Detection Assays can detect multiple species of *Salmonella, E. coli* and STEC in a single qPCR (Quantitative Polymerase Chain Reaction) in cannabis flower, hemp flower, cannabis concentrates, infused edibles and infused non-edibles.

Note: The following multiplex assays are available from MGC and have their own dedicated user guides:

- PathoSEEK® Salmonella & E. coli Multiplex Detection Assay v3
- PathoSEEK® Salmonella & STEC E. coli Multiplex Detection Assay v3 (AOAC PTM)

## Process Overview

PathoSEEK *Salmonella, E. coli*, and STEC Detection Assays use a multiplexing strategy with an internal control (IC) to ensure accurate detection of the target species and cannabis DNA in each 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 MaGiC Lysis and the qPCR assays.

Figure 1: Overview of MaGiC Lysis

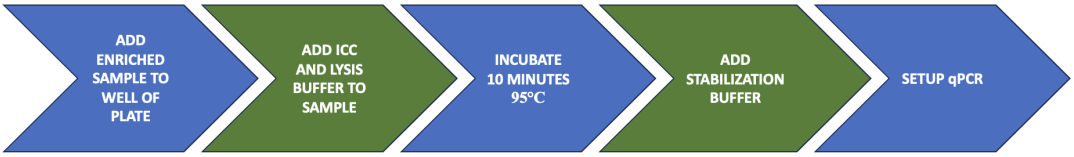
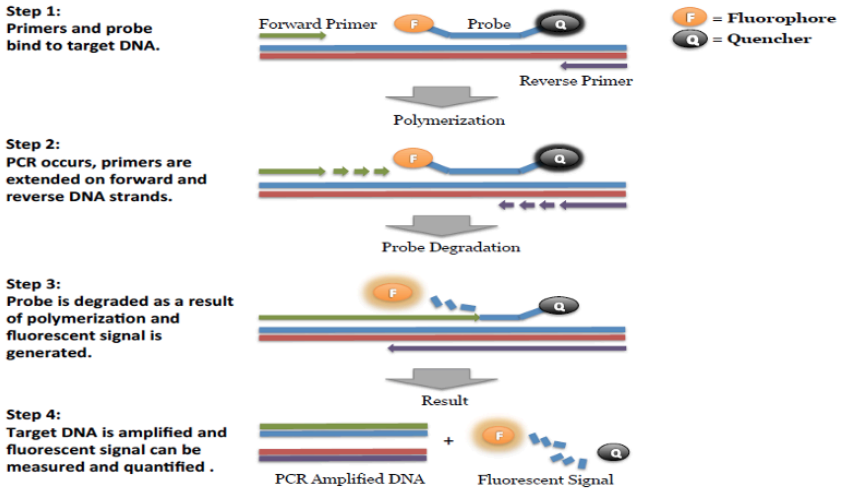



Figure 2: Overview of qPCR




## Limit of Detection

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

Kit Components

**PathoSEEK® Salmonella Detection Assay v3 with MaGiC Lysis Kit, P/N 420532**

(Kit contains sufficient reagents for 200 reactions). Please note some components are stored at different temperatures. Actual fill volumes include overage.

| Component Name | Qty Provided | Storage Conditions |
|---|---|---|
| MaGiC Lysis Reagent | 1 Bottle (12 mL) | RT (20-28 ºC) |
| MaGiC Stabilization Buffer | 1 Bottle (24 mL) | RT (20-28 ºC) |
| PathoSEEK Amplification Mix<br>*Includes 2 tubes nuclease free water for resuspension* | 4 Vials (50 rxns/each) | RT (20-28 ºC)/ -15 to -20 ºC* |
| PathoSEEK® Salmonella Detection Assay v3 | 1 Tube (200 µL) | -15 to -20 ºC |

**PathoSEEK® E. coli STEC Detection Assay v2 with MaGiC Lysis Kit, P/N 420534**

(Kit contains sufficient reagents for 200 reactions). Please note some components are stored at different temperatures. Actual fill volumes include overage.

| Component Name | Qty Provided | Storage Conditions |
|---|---|---|
| MaGiC Lysis Reagent | 1 Bottle (12 mL) | RT (20-28 ºC) |
| MaGiC Stabilization Buffer | 1 Bottle (24 mL) | RT (20-28 ºC) |
| PathoSEEK Amplification Mix<br>*Includes 2 tubes nuclease free water for resuspension* | 4 Vials (50 rxns/each) | RT (20-28 ºC)/ -15 to -20 ºC* |
| PathoSEEK® E. coli STEC Detection Assay v2 | 1 Tube (200 µL) | -15 to -20 ºC |

* The PathoSEEK Amplification Mix can be stored lyophilized at Room Temperature for up to 2 years. Once re-hydrated it must be stored at -15 to -20 ºC for up to 3 months.

**PathoSEEK® E. coli Detection Assay v2 with MaGiC Lysis Kit, P/N 420533**

(Kit contains sufficient reagents for 200 reactions). Please note some components are stored at different temperatures. Actual fill volumes include overage.

| Component Name | Qty Provided | Storage Conditions |
|---|---|---|
| MaGiC Lysis Reagent | 1 Bottle (12 mL) | RT (20-28 ºC) |
| MaGiC Stabilization Buffer | 1 Bottle (24 mL) | RT (20-28 ºC) |
| PathoSEEK Amplification Mix<br>*Includes 2 tubes nuclease free water for resuspension* | 4 Vials (50 rxns/each) | RT (20-28 ºC) /<br>-15 to -20 ºC* |
| PathoSEEK® E. coli Detection Assay v2 | 1 Tube (200 µL) | -15 to -20 ºC |

* The PathoSEEK Amplification Mix can be stored lyophilized at Room Temperature for up to 2 years. Once re-hydrated it must be stored at -15 to -20 ºC for up to 3 months.

**Additional Required Reagents Not in Kit**

| Item P/N | Item Name | Qty Provided | Storage Conditions |
|---|---|---|---|
| 420337 | Internal Control | 1 Tube (50 µL) | -15 to -20 ºC |
| 420301 | PathoSEEK Salmonella Positive Control | 1 Tube (50 µL) | -15 to -20 ºC |
| 420302 | PathoSEEK® E. coli Positive Control | 1 Tube (50 µL) | -15 to -20 ºC |
| 420304 | PathoSEEK® STEC Positive Control | 1 Tube (50 µL) | -15 to -20 ºC |
| 420205 | Tryptic Soy Broth | CS/10 x 500mL bottles | 2-25°C |
| 420184 | PCR Grade Water | 500 mL Bottle | 2-25°C |

Shelf Life and Storage

Once received, each kit component must be stored at its designated storage condition. Reagents stored properly can be used until the expiration date indicated on each component label.

Required Equipment and Supplies

Equipment:

- Agilent AriaMx Real-Time PCR System G8830A, containing the following Optical Channels: FAM and HEX - **Medicinal Genomics P/N 420387**
    - Agilent HP Notebook PC option 650 or lab supplied Windows PC
    - Optical Strip Caps - Agilent #401425. **Note: If using adhesive seals instead of strip caps, use Applied Biosystems MicroAmp Optical Film Compression Pad, Fisher Scientific, #43-126-39 to prevent evaporation and cross contamination between wells.**
- Bio-Rad CFX96 Touch™ Real-Time System.
    - Bio-Rad supplied or own Windows PC
- Bio Molecular Systems Mic 4-Channel PCR Instrument - **Medicinal Genomics P/N 420241**
    - BMS supplied or lab supplied Windows PC
    - Mic Tubes and Racked Caps - **Medicinal Genomics P/N 420244**
    - Mic Tubes and Caps (Bulk) - **Medicinal Genomics P/N 420243**
- Adjustable, variable volume pipettes (single or multichannel).—P10, P20, P200, and P1000
- Adjustable, variable volume filter pipettes tips.—For P10, P20, P200, and P1000
- 1.5 mL Tube Benchtop Cryogenic Rack— VWR #89004-558 or equivalent
- Freezer—Capable of maintaining -20 ± 2 °C
- Tabletop Mini Plate Centrifuge—Fisher Scientific #14-100-143 or equivalent
- Tabletop Mini Centrifuge—VWR #10067-588 or equivalent
- Vortex-Genie Pulse—Scientific Industries, SKU: SI-0236, or equivalent
- Incubator—Capable of maintaining 37 ± 2 °C, VWR #97025-630 or equivalent.
- 96-Well PCR Plate Cryogenic Rack (optional) —VWR #89004-570

- 1.5 µL Tube Benchtop Cryogenic Rack (optional if crushed ice is not an option) — VWR #89004-558 or equivalent
- Eppendorf Tube Rack
- Scientific Scale—Capable of measuring to milligrams
- Refrigerator—Capable of maintaining 2–8 °C

Supplies:

- 96-well Optical qPCR plate — **Medicinal Genomics P/N 100164**
- Adhesive optical seal for qPCR plates — **Medicinal Genomics P/N 100177**
- Filter Bags—**Medicinal Genomics P/N 100008** (Whirl-Pak #B01385WA)
- Crushed ice
- Beaker or Solo Cup. (optional)
- 1.5 mL Eppendorf Tubes
- 15 mL or 50 mL conical tubes
- Pipette - Aid Portable Pipetting device for serological pipettes, VWR 89166-464 or equivalent
- 25mL Serological Pipette—VWR 89130-890 or 89130-900 or equivalent
- 10% bleach

## 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

- *Escherichia coli* is a bacterium that is commonly found in the human gastrointestinal tract. Some strains, however, can be pathogenic to humans, e.g., STEC, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), etc. STEC are *E. coli* that produce Shiga toxins encoded by the 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.
- 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.
- It is the responsibility of each laboratory to handle waste and effluents processed according to their nature and degree of hazardousness and to treat and dispose of them in accordance with applicable local, state, and federal regulations.

## Intended User

The PathoSEEK Assays 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. Therefore, it is best to pour the approximate amount of TSB into another sterile tube or container, to not contaminate the stock bottle.**
2. Wipe down the workspace with a 10% bleach solution, including the bench top and all equipment being used.

3. If not already at room temperature, remove TSB from the 2-8 °C refrigerator, the TSB should come to room temperature, 20–28 °C, before use.
4. Prepare consumables. Label all the filter bags with "[sample name] [date]".
5. Label lysis plate with date.
6. 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.
7. *Dried Cannabis flower,* ***n*** *grams* —Weigh flower sample material into one side of the mesh liner inside the Whirl-Pak bag. Add 9 x *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 ± 2 °C.
8. *Infused products,* ***n*** *grams.* — Weigh the Infused Product matrix into a 15 mL conical tube, 50 mL conical tube, or Whirl-Pak bag depending on Infused Product volume. Add 9 x *n* mL of TSB to each infused product test portion. Homogenize the test portion until thoroughly mixed in TSB. Incubate for 16 - 24 h at 37 ± 2 °C.
9. *Concentrates,* ***n*** *grams.* — Weigh concentrate into a 15 mL conical tube, 50 mL conical tube, or Whirl-Pak bag depending on the concentrate test portion. Add 9 x *n* mL of TSB to each test portion. Homogenize the test portion until thoroughly mixed in TSB. Incubate for 16 - 24 h at 37 ± 2 °C.
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. See flower example in Figure 3.

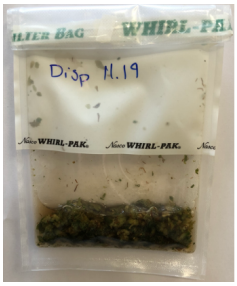



Figure 3: Homogenized cannabis flower and TSB.

**MaGiC Sample Lysis:**

Matrices: Dried Cannabis Flower, Infused Cannabis Products, and Cannabis Concentrates (for chocolate see below)

1. Dilute IC to 1:50,000

   **Note: Prepared 1:50k dilution of IC has been tested up to 24 freeze thaws with minimal effect on resulting Cq value when properly stored at -20 °C when not in use.**

   a. Dilute stock IC to 1:100.

      i. Ensure stock IC is fully thawed and vortexed, and then pulse spin down in a mini centrifuge before use.

      ii. Add 2µL of stock IC to 198ul of nuclease free water. Vortex well and pulse spin down in a mini centrifuge.

   b. Serially dilute the 1:100 dilution of IC an additional 1:100 to make a 1:10,000 dilution.

      i. Add 2µL of 1:100 IC to 198µL of nuclease free water. Vortex and pulse spin down in mini centrifuge. This is the 1:10,000 dilution IC.

   c. Dilute the 1:10,000 IC dilution an additional 1:5, which results in the final dilution of 1:50,000.

      i. Add 20µL of the 1:10,000 IC to 80µL of nuclease free water. Vortex and pulse spin down in a mini centrifuge. Final dilution is 1:50,000 IC.

2. Remove enriched samples (contained in the Whirl Pak Bag, 15, or 50 mL tube) from the incubator.

   a. **Flower sample**: Mix thoroughly by hand manipulating and/or squeezing the contents in Whirl Pak bag for the flower enrichment for 1 minute.

   b. **Infused Products:** Mix thoroughly by hand manipulating and/or squeezing the contents in Whirl Pak bag for 1 min or vortex tubes for 30 seconds.

   c. **Concentrates:** Vortex conical tube thoroughly for 30 seconds.

3. Remove 10 µl of enriched test portion and transfer into a well of a 96 well PCR plate.

4. Add 50 µL of MaGiC Lysis Buffer to all wells containing samples, using a fresh pipette tip for each transfer. Tip mix 15 times to ensure the sample is mixed with the lysis buffer.

5. Transfer 5 µL of 1:50,000 diluted IC to the 60 µl of enrichment + lysis buffer. Repeat transfer of IC for all sample wells, using a fresh pipette tip for each transfer of IC
6. Seal the top of the plate and briefly spin down in a centrifuge.
7. Place plate on the thermal cycler and run the MaGiC Lysis program
    a. Incubate 95 °C for 10 minutes, then 4 °C for 5 minutes (if 4 °C is not an option, set to 25 °C for 10 min).

        **Note: When using the Agilent AriaMX, a compression pad should be placed over the adhesive seal to prevent evaporation from the plate and seal.**
8. Remove plate with lysed samples from the thermal cycler and briefly spin the plate down in centrifuge to remove evaporation from the plate seal. Carefully remove the plate seal.
9. Add 100 µl of MaGiC Stabilization buffer to each sample well using a new pipette tip for each sample. Pipette mix 5 times. The samples are now ready for qPCR set up, see Real - Time Quantitative PCR (qPCR) Setup Protocol with PathoSEEK Amplification Mix.
10. If not proceeding directly to qPCR setup, seal the plate with sample lysates with a plate seal and store in -20°C freezer .
    a. Sample lysates with stabilization buffer added are stable at -20 ºC for 1 month.
    b. When ready to use, thaw frozen samples at room temperature. Sample lysates which have been frozen must be tip mixed 15 times prior to setting up qPCR or performing dilutions.

<u>MaGiC Lysis for Infused Chocolate Product</u>

1. Dilute IC to 1:5,000
    a. Dilute the stock IC to 1:50.
        ii. Ensure stock IC is fully thawed and vortexed, and then pulse spin down in a mini centrifuge before use.
        iii. Add 2µL of stock IC into 98ul of nuclease free water. Vortex and quick spin down in mini centrifuge.

    b. Serially dilute the 1:50 IC an additional 1:100 to make a 1:5,000 dilution.

iv. Add 2µL of the 1:50 IC dilution to 198µL of nuclease free water. Vortex and pulse spin down in a mini centrifuge. This is the final 1:5,000 dilution IC.

2. Remove the sample enrichment contained in the Whirl Pak Bag or tube from the incubator. Mix Whirl Pak Bag thoroughly by hand by manipulating and/or squeezing the contents from the enrichment for 1 min or vortex tubes for 30 seconds.
3. Remove 10 µL of enriched test portion and transfer into the well of a 96 well PCR plate.
4. Add 50 µL of MaGiC Lysis Buffer. Tip mix 15 times to ensure the sample is mixed with the lysis buffer.
5. Add 5 µL of 1:5,000 diluted IC to all wells being tested using a fresh tip each time.
6. Seal the top of the plate and spin down in the centrifuge.
7. Place plate on a thermal cycler and run the MaGiC Lysis program
    a. Incubate 95 °C for 10 minutes, then 4 °C for 5 minutes (if 4 °C is not an option, set to 25°C for 10 min.).

    **Note: When using the Agilent AriaMX, a compression pad should be placed over the adhesive seal to prevent evaporation during thermal cycling.**
8. After the plate has been at 4 °C for 5 minutes or 25 °C for 10 minutes, remove it from the thermal cycler and briefly spin the plate down in a centrifuge to remove evaporation from the plate seal. Carefully remove the plate seal.
9. Add 100 µl of MaGiC Stabilization buffer to each lysate sample well. Pipette mix 5 times.
10. If not proceeding directly to qPCR setup, seal the plate with sample lysates with a plate seal and store in -20 °C freezer.
    a. Sample lysates with stabilization buffer added are stable at -20 ºC for 1 month
    b. When ready to use, thaw frozen lysates at room temperature. Sample lysates which have been frozen must be tip mixed 15 times prior to setting up qPCR or performing dilutions.
11. On day of analysis, dilute the infused chocolate product extracts an additional 1:100 with nuclease free water before running the qPCR.
    a. Add 2 µl of lysate into 198 µl of water. To ensure thorough mixing, mix each sample 15 times with a pipette set to 100 µl. Use a fresh pipette tip for each sample.

12. Proceed to qPCR

**Real-Time Quantitative PCR (qPCR) Setup Protocol with PathoSEEK Amplification Mix:**

1. Remove PathoSEEK Amplification Mix, detection assay, and positive control from the -20 ºC freezer.
    a. If lyophilized Amplification Mix has not been previously rehydrated, rehydrate with 550 µl of Nuclease Free Water. Swirl or Pipette tip mix. After resuspension, store the remaining Amplification Mix at -15 to -20 °C when not in use.
    b. Allow all frozen reagents to defrost at room temperature (20-2 8°C). Once defrosted, place tubes on ice.
2. Before preparing the Master Mix, invert or vortex and pulse spin down the reagents in a mini centrifuge
    a. Detection Assay (probe mix) – vortex tube quickly followed by a pulse spin down in a minicentrifuge.
    b. Positive Control tube – vortex tube quickly followed by a pulse spin down in a minicentrifuge.
    c. PathoSEEK Amplification Mix – Invert the bottle 5-10 times to mix or briefly vortex.
    d. Return all reagents to the ice.
3. Prepare Master Mix in a 1.5 mL tube (the probe mix also includes the probe targeting the IC). Label the tube as “Master Mix”. See Table 1 (PathoSEEK Amplification Master Mix).
    a. Ensure adequate Master Mix is prepared by adding 1 or 2 additional reactions to accommodate pipetting and dead volumes, plus 2 more for qPCR positive and negative controls. For instance, to test 10 samples, prepare enough Master Mix for 13 or 14 reactions.

Table 1: PathoSEEK Amplification Master Mix Reagent Volumes

| Reagent | Volume for 1 qPCR Reaction |
|---|---|
| PathoSEEK Amplification Mix | 10 µL |
| Detection Assay | 1 µL |
| Nuclease Free Water | 4 µL |
| Total | 15 µL |

4. Once the Master Mix is combined then gently cap the tube and vortex to mix.
    i. Pulse spin down tube in minicentrifuge.
    ii. Place the Master Mix tube on ice until used.
5. For the negative control, use nuclease free water that was used to rehydrate your Amplification Mix.
6. Dilute the assay positive control 1:10.
    a. Add 1 µL of Positive Control to 9 µL nuclease free water (found in the kit), vortex to mix and pulse spin down the tube in mini centrifuge

    **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. Transfer lysed samples into 96-well optical qPCR plate wells or optically clear qPCR tubes
    a. Carefully remove the seal from the Extraction Plate.

    **Note: If lysed samples were frozen, let the DNA thaw completely and spin the plate in centrifuge to avoid cross contamination between samples. Tip mix thawed samples wells before transferring to the qPCR plate or tubes.**
    b. Transfer 5 µL of each sample lysate into the corresponding qPCR tube or well on the qPCR plate.
    c. Add 5 µL of the diluted Positive Control to the corresponding positive control plate well or tube.
    d. Add 5 µL of nuclease free water to the corresponding negative control plate well or tube.

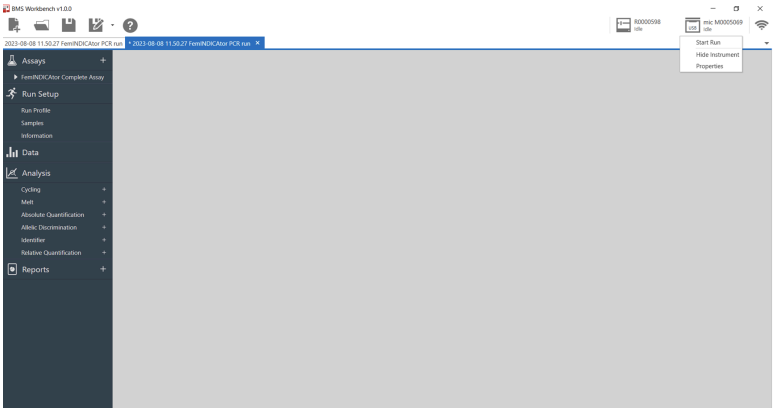
    **Note: ALWAYS use a fresh tip for every liquid transfer of sample, positive, or negative control into the qPCR plate**

8. Add 15 µL of Master Mix to each corresponding sample well, positive control well, and negative control well in the qPCR plate or tubes. Gently tip mix 5 times after each addition of Master Mix. Be careful not to introduce bubbles during this mix. Use a fresh tip for each transfer of Mater Mix to each well.
9. Seal the plate with strip caps or an adhesive seal, or seal qPCR tubes with strip caps.
10. For the Agilent AriaMX or Biorad CFX, spin down qPCR plate or tubes for at least 1 minute in plate (or tube) mini centrifuge to bring well contents to the bottom of wells (or tubes) and help to get rid of reaction bubbles.

    **Note: Check for bubbles in the wells or tubes (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the wells (or tubes), spin down for another minute in mini centrifuge**
11. For the Agilent Aria MX: 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 wells in the plate.
12. Place the sealed plate or tubes onto the PCR instrument.
13. Follow the software specific instructions for each qPCR platform to initiate the run.

Running the BioMolecular Systems MIC

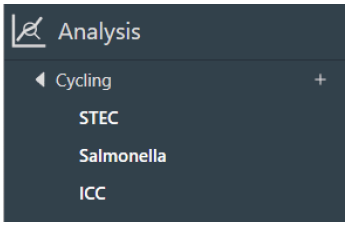
1. Open the BMS Workbench software and create a new file.
2. Select qPCR Run.
3. Select the appropriate template by clicking the "+" sign next to assays or ensure that the appropriate thermal cycling conditions are entered: Hot start at 95 ºC for 5 minutes, followed by 40 cycles of 95 ºC for 15 seconds and 65 ºC for 90 seconds.
4. Click on the Mic icon in the upper right-hand corner, and select "Start run" from the menu that appears.



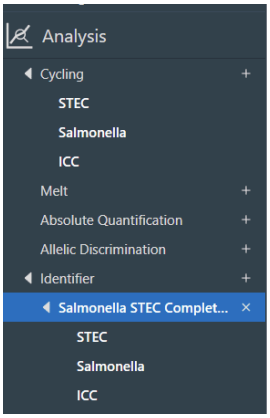

5. A pop-up will appear asking for the reaction volume, which will be auto-filled with the correct volume based on the template chosen. Close the lid and the instrument will start.
   **Note: If the cover isn't closed, the program will not start.**
6. Let the Mic run to completion before analyzing the data.

Data Analysis with BioMolecular System MIC

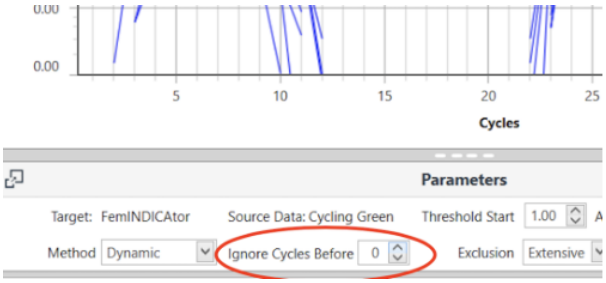
1. When the run has completed, click on the "+" sign next to the "Cycling" tab and select IC (Internal Control). The selection will appear under the "Cycling" tab. Next, select the desired target.




2. When these have been expanded, select the "+" sign next to the "Identifier" tab and select the available report.




3. If the automatic calls are blank, then one of the cycles is out of threshold. To fix this, find "Ignore Cycles Before" in the "Parameters" section, and increase its value one whole number at a time until a gray bar appears on the graph. This might have to be done with one, or all of the filters.




4. To review an automatic report, click the "+" sign next to the "Identifier" tab then select the relevant "Complete Assay". This feature will call the samples tested as detect, non-detect, or inconclusive based on the qPCR data. These results will be displayed on the right side of the screen. To interpret the results on the Bio Molecular Systems Mic, see Figure 1.

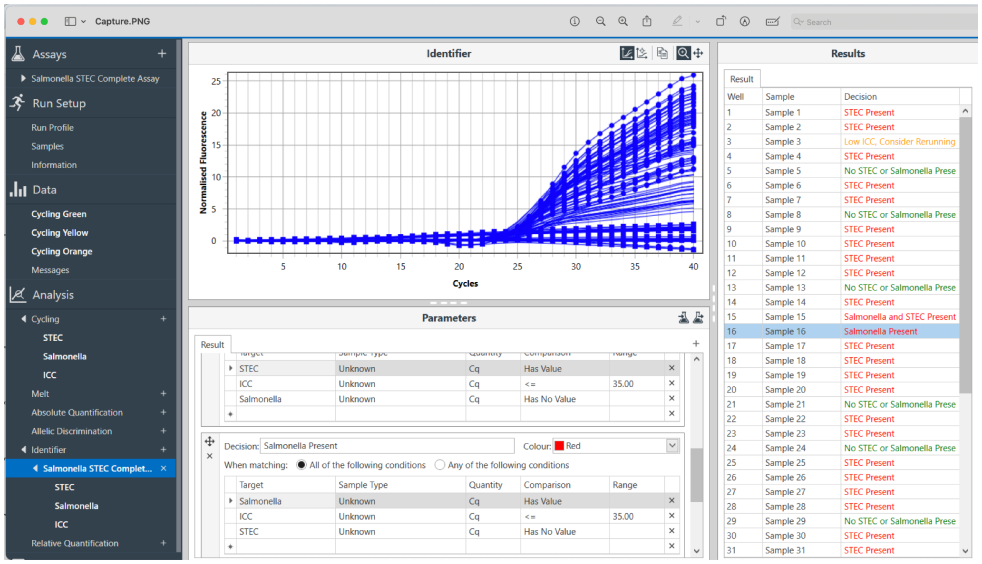



**Figure 1**. **Bio Molecular Systems MiC**: PathoSEEK Data Analysis

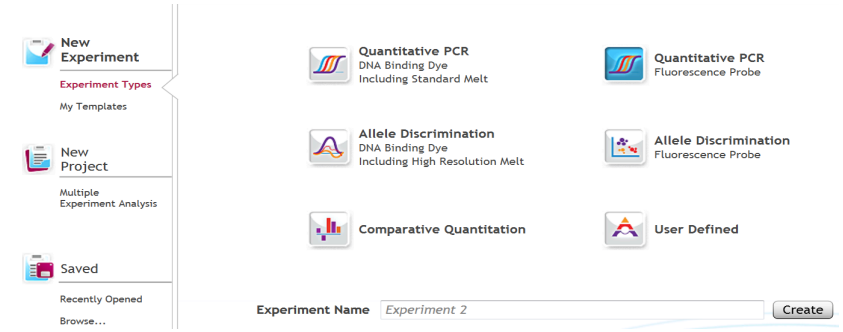
5. Data may be exported by selecting the "Report" tab, then clicking the Export icon.

## Running the Agilent AriaMX

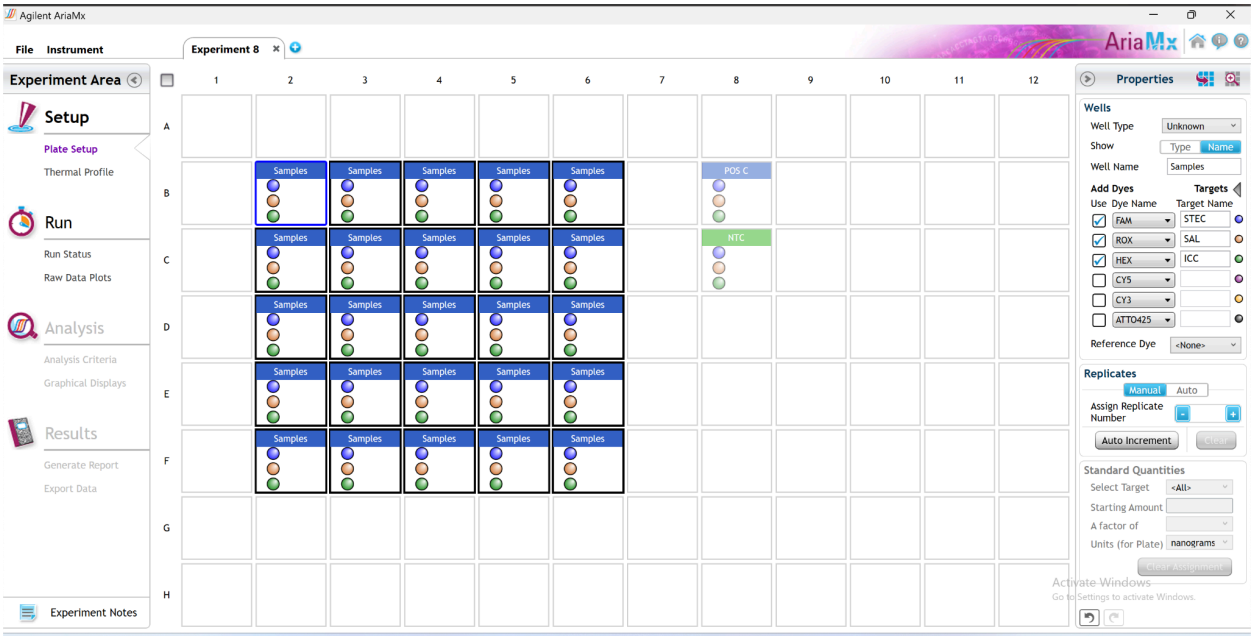
The following targets will be detected on the following fluorophores:

| Target | Optical Channel |
|---|---|
| *Salmonella sp., Total E. coli and Shiga Toxin producing E. Coli* | FAM |
| Internal Control | HEX |

1. Create a New Experiment on the Agilent qPCR instrument.
    a. Select "Quantitative PCR: Fluorescence Probe" from Experiment Types.



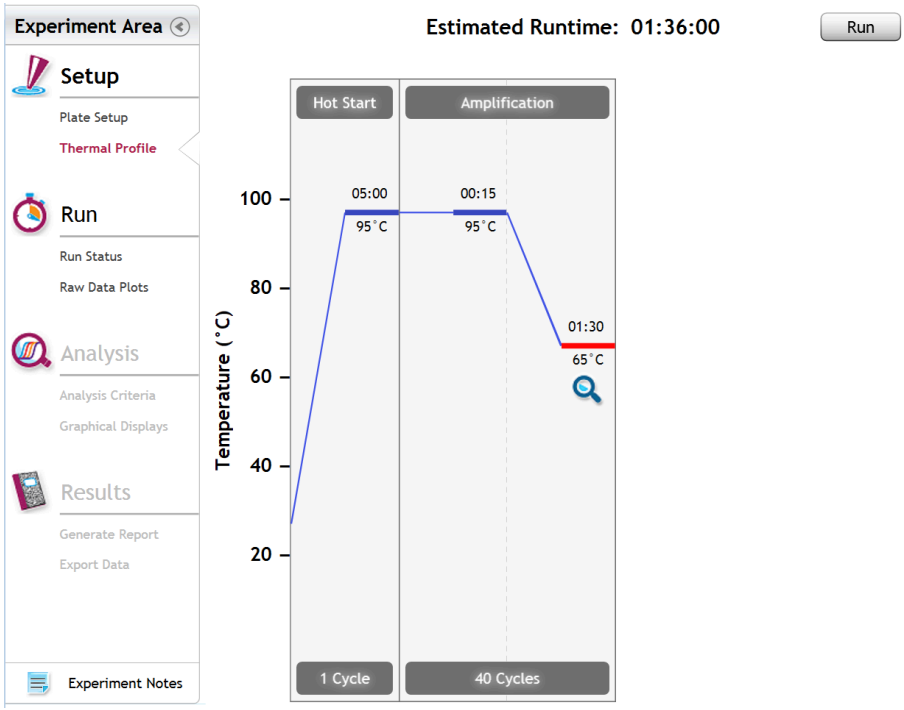

b. Under Setup > Plate Setup, highlight **only** wells that contain reactions and select FAM and HEX under **Add Dyes**. Having fluorescence detection turned on in empty wells can affect the calculations which the software makes to obtain the corrected data, ΔR log.




2. Change the well types to reflect your plate set up. All wells should be set to Unknown except the negative control may be set to NTC well type. Add Target names to the dyes under **Targets:**
    a. FAM : STEC sp., *E. coli sp, Salmonella sp.*
    b. HEX : Internal Control
3. Under Setup > Thermal Profile, create the following PCR thermal profile

a. Hot start at 95 ºC for 5 minutes, followed by 40 cycles of 95 ºC for 15 seconds and 65 ºC for 90 seconds.

**Note: A compression pad should be placed on top of the plate with an adhesive seal to prevent cross contamination.**



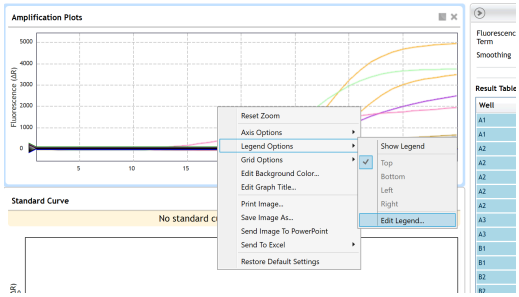

4. Close the lid and click "Start Run".
5. Save the experiment with the [User] and [date].
6. When the qPCR run is complete, immediately dispose of the plate or tubes. Do not open the plate or tubes after the run to avoid contamination in the lab.

Data Analysis: Agilent AriaMX

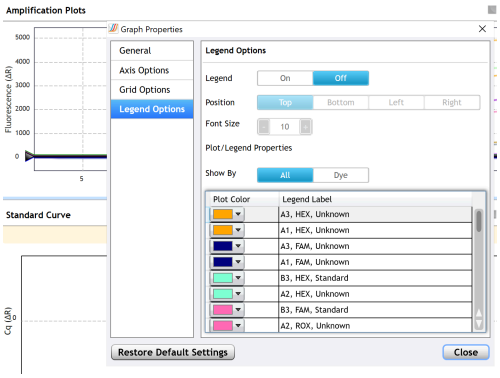
Table 2: PathoSEEK® Salmonella, *E. coli,* & STEC Detection Assays Data Analysis

| PathoSEEK Assay | Cq Value | Fluorophore | Negative Control (Cq) | CFU Threshold (CFU/g) |
|---|---|---|---|---|
| *Salmonella sp.* | ≤ 40 | FAM | No Cq | Presence/Absence |
| Total *E. coli* | ≤ 40 | FAM | No Cq | Presence/Absence |
| Shiga Toxin producing *E. Coli (STEC)* | ≤ 40 | FAM | No Cq | Presence/Absence |
| Internal Control (IC)* | ≤35 | HEX | *Internal control verifies the presence or absence of cannabis DNA | |
| Assay Positive Control | ≤35 | FAM | | |

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 Displays
    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

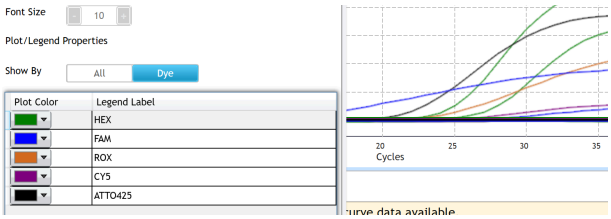



    d. Change “All” to “Dye”



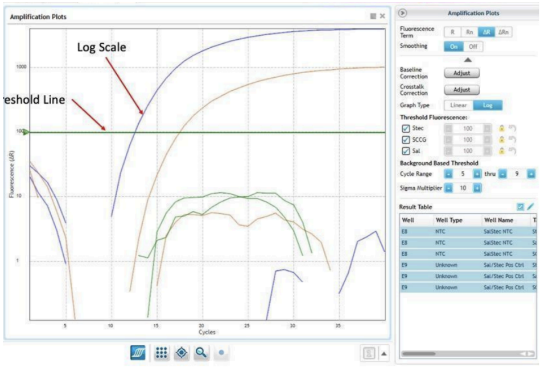

e. All user settings for Plot/Legend Properties will be removed. Do you want to proceed? Select "Yes".

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

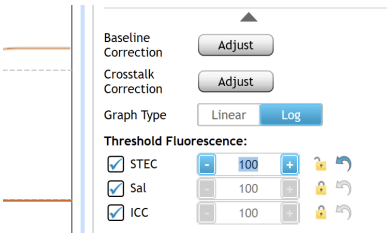



3. To analyze the results

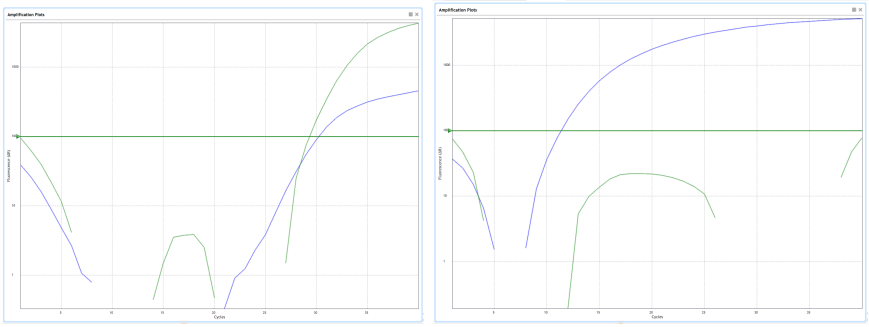
a. Start by turning the graph to Log Scale with a right click on the chart, select Axis options, enable y-axis log scale. Expand the amplification plots settings by clicking on the triangle.



b. Manually adjust and lock thresholds to 100 RFU for the HEX and FAM fluorophores. (Target names will have previously been identified.)




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.
5. Unknown *Salmonella*, *E. coli,* and STEC Targets
    a. A "presence" or failing result for the unknown *E. coli*, *Salmonella*, and STEC target(s).
        i. Any Cq value for the FAM Fluorophore ≤ 40.
            1. A Cq of ≤ 40 on the FAM channel indicates the presence of STEC when using PathoSEEK® E. coli STEC Detection Assay v2, the presence of *E. coli* when using PathoSEEK® E. coli Detection Assay v2 and the presence of *Salmonella* when using PathoSEEK® Salmonella Detection Assay v3.



ii. Visually confirm with the curve on the graph. It is very important to check the raw data view (R) to confirm with the amplification curve when a presence result occurs.

1. 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.

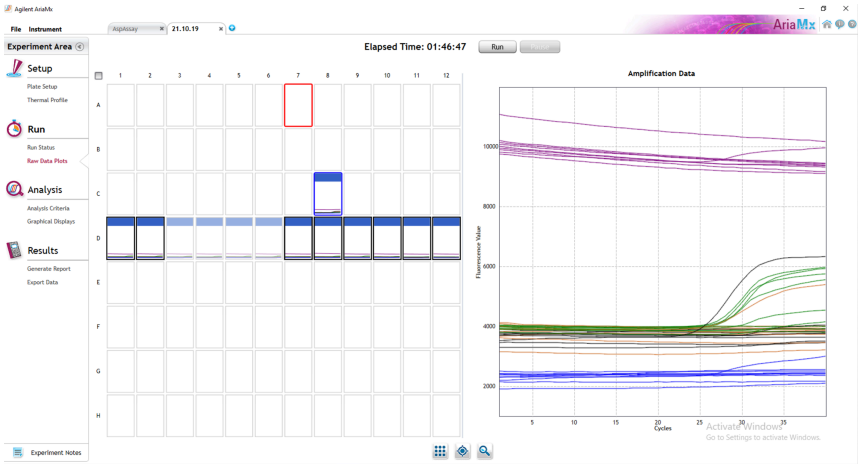



Figure 4: Raw data (R) plots for which the cycle quantification (Cq) will be generated.

2. 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.)

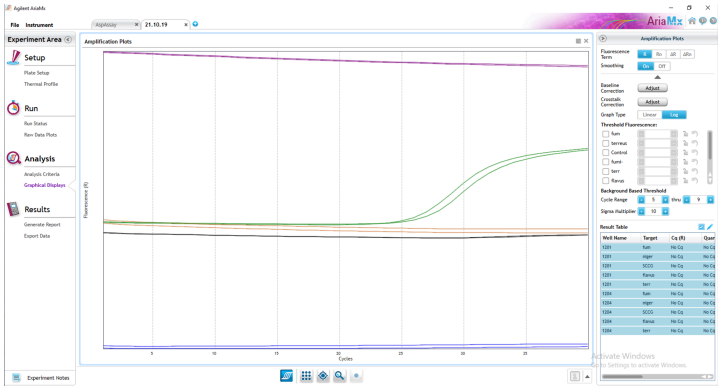
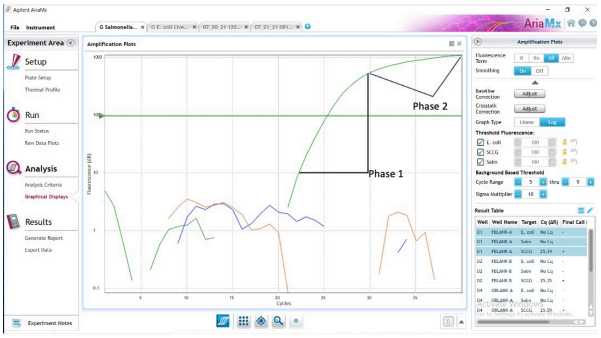


Figure 5: Example of good baseline with sigmoidal shaped curve increase in the internal control fluorescence.

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.

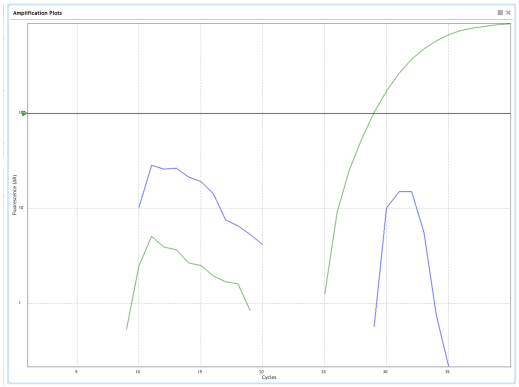



b. An "absence" or passing result for the unknown *E. coli, Salmonella*, and STEC target(s).

i. Internal Control (IC), on the HEX Fluorophore, has a Cq value ≤ 35 for flower samples, ≤40 for all other matrices.

ii. No Cq value for the FAM Fluorophore.

iii. Visually confirm that there is no curve on the graph.

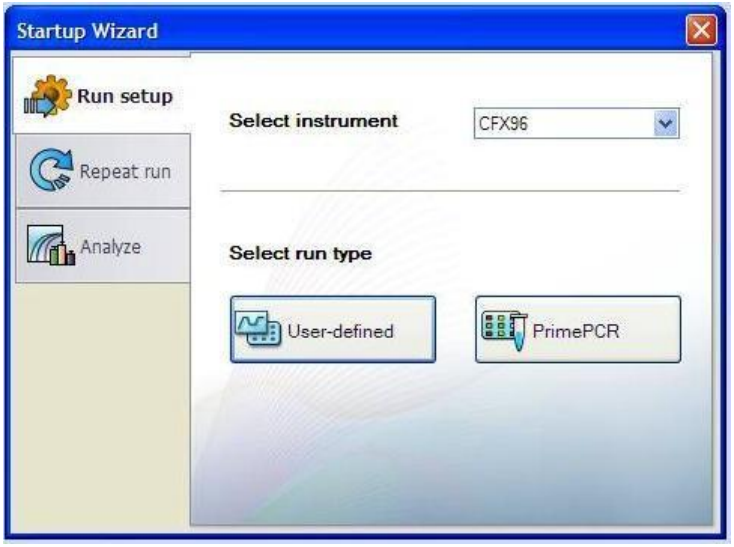



Running the BioRad CFX96

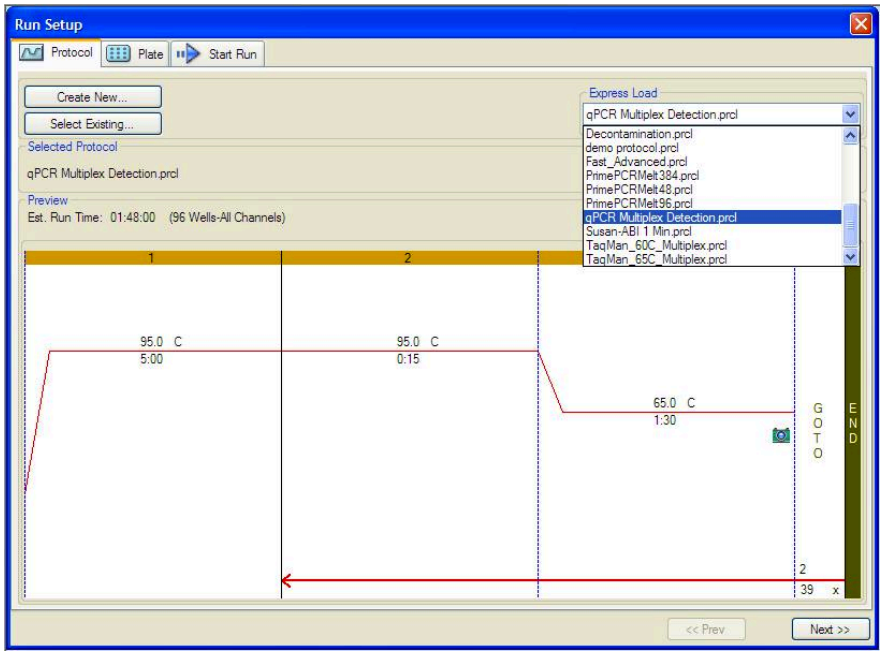
The following targets will be detected on the following fluorophores:

| Target | Optical Channel |
|---|---|
| *Salmonella sp., Total E. coli and Shiga Toxin producing E. Coli* | FAM |
| Internal Control | HEX |

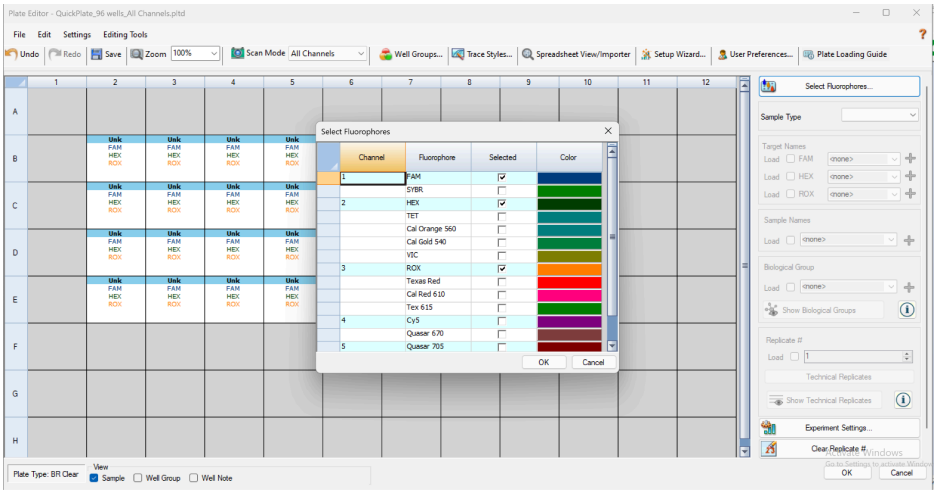
1. Start the qPCR Cycling program
    a. Select User-Defined in the Startup Wizard under Run setup



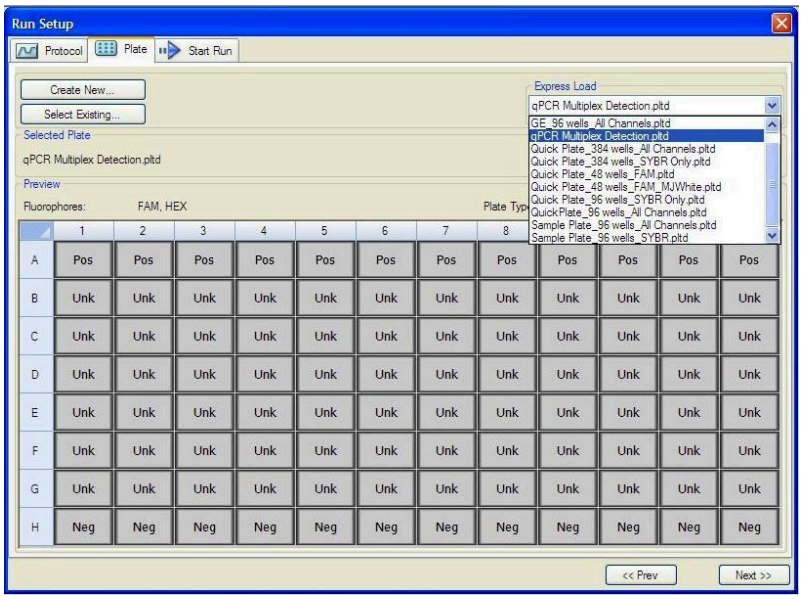

2. Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click "Next".
    a. If not already pre-programmed, create a cycling program with the following specifications and save as "qPCR Multiplex Detection"
    b. Hot start at 95 ºC for 5 minutes, followed by 40 cycles of 95 ºC for 15 seconds and 65 ºC for 90 seconds.




3. Design your plate under the plate tab in the Run Setup
    a. Select the qPCR Multiplex Detection from the dropdown menu. If not already present, click "Create New"
    b. The plate editor window will appear. Choose FAM and HEX Fluorophores and click "OK".



c. If 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.

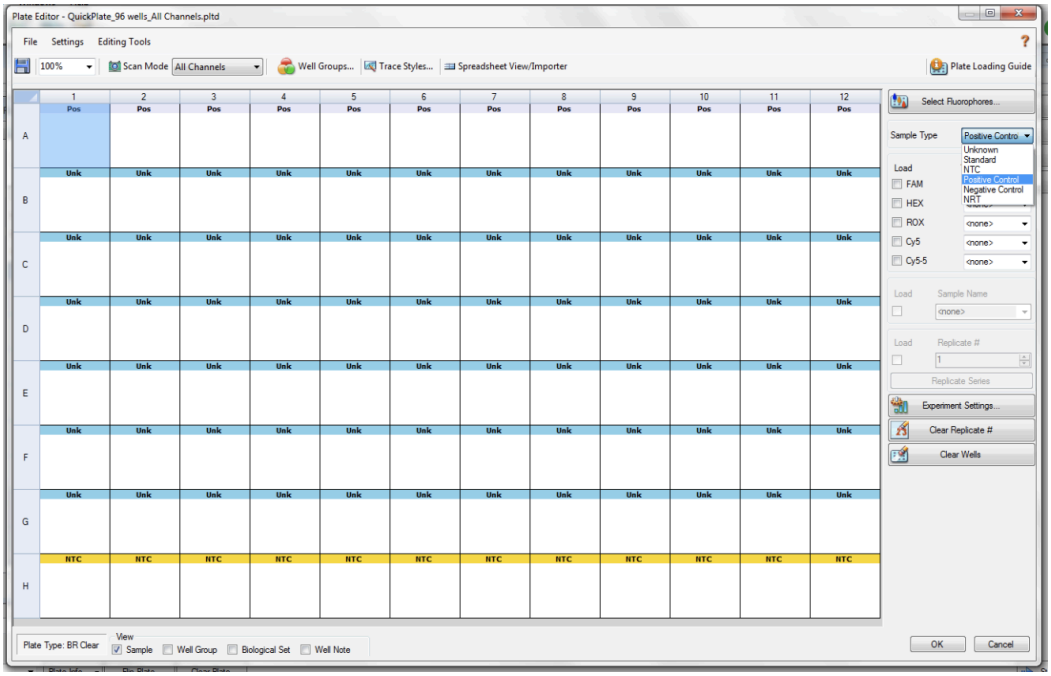
**Note: To select the Sample Type, highlight the wells you would like to define, then choose from the dropdown menu one of three types:**

*Unknown*

*Positive Control*

*Negative Control*

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



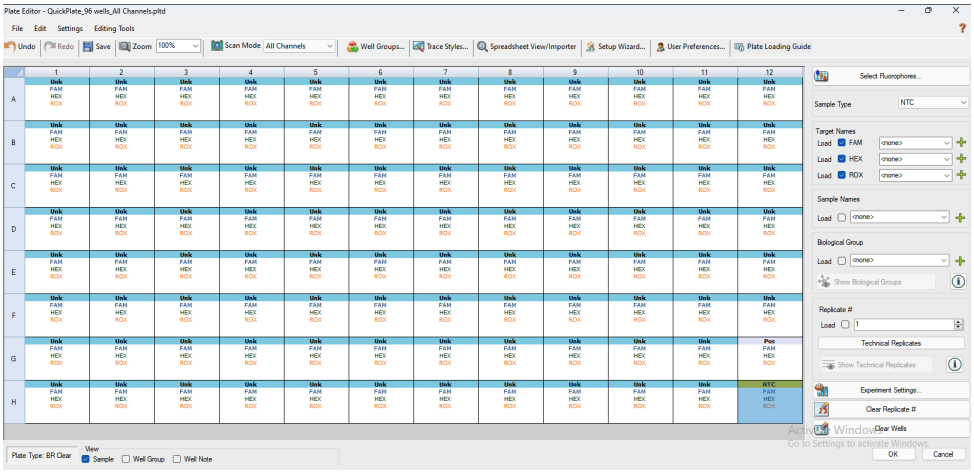

f. Attach the fluorophores to the wells being used.

   i. Highlight all the wells being used

   ii. For the Salmonella, *E. coli,* and STEC Assays, highlight the well locations and click on FAM and HEX.

g. When the plate is designed correctly, click OK.

4. 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 which is acceptable.**

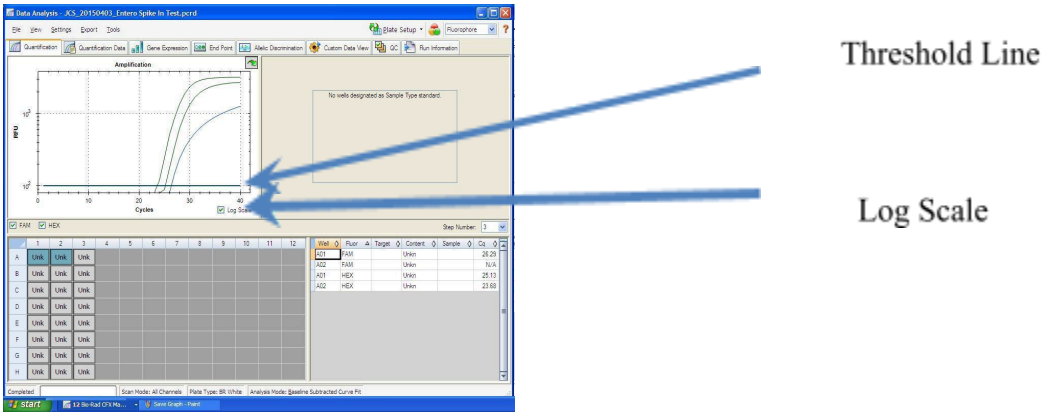
5. Close the lid and click Start Run.
6. Save the experiment with the [User] and [date].
7. When the run is complete, immediately dispose of the plate after qPCR. **Do not remove the plate seal after the run to avoid contamination in the lab.**

Data Analysis BioRad CFX96

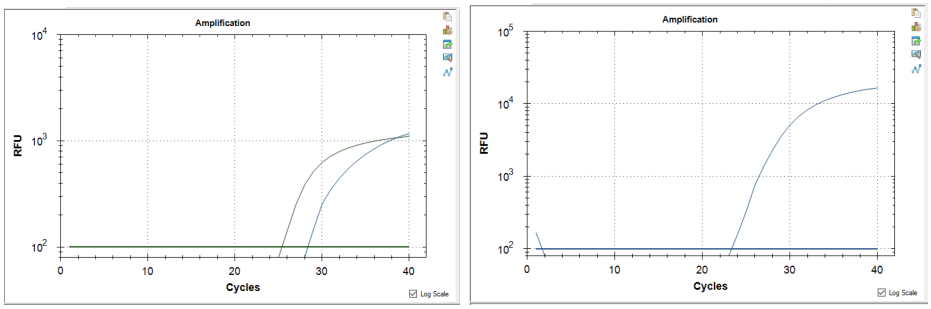
Table 3: PathoSEEK Salmonella, E. Coli and STEC Data Analysis

| PathoSEEK Assay | Cq Value | Fluorophore | Negative Control (Cq) | CFU Threshold (CFU/g) |
|---|---|---|---|---|
| *Salmonella sp.* | ≤ 40 | FAM | No Cq | Presence/Absence |
| Total *E. coli* | ≤ 40 | FAM | No Cq | Presence/Absence |
| Shiga Toxin producing *E. Coli* | ≤ 40 | FAM | No Cq | Presence/Absence |
| Internal Control (IC)* | ≤35 | HEX | *Internal control verifies the presence or absence of cannabis DNA | |
| Assay Positive Control | ≤35 | FAM | | |

1. The Data Analysis window will open automatically when the run is complete.
2. Highlight the well of interest.
    a. The graph will appear above.
    b. The Cq values will appear to the right.
3. To analyze the results
    a. Start by turning the graph to Log Scale and manually moving the threshold to $10^2$ for all fluorophores.
        i. To turn the graph to Log Scale, click on the box at the bottom right of the graph.
        ii. To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis.
        iii. Alternatively, set an exact threshold value by selecting a single fluorophore beneath the graph, then Settings > Baseline Threshold. In the next window select User Defined and enter 100.



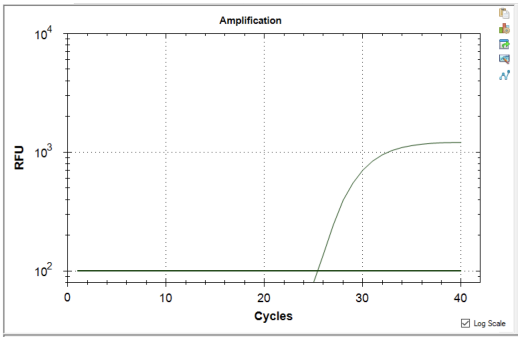

4. Controls
    a. Assay-specific Positive Control on the FAM fluorophore has a Cq value ≤ 35.
        i. Visually confirm with the curve on the graph.
    b. Assay-specific Negative Control on the FAM fluorophore has no Cq value.
        i. Visually confirm with the curve on the graph.
5. Unknown *Salmonella*, *E. coli,* and STEC Targets:
    a. A "presence" or failing result for the Unknown *Salmonella*, *E. coli,* and STEC Targets.
        i. A Cq of ≤ 40 on the FAM channel indicates the presence of STEC when using PathoSEEK® E. coli STEC Detection Assay v2, the presence of *E. coli* when using PathoSEEK® E. coli Detection Assay v2 and the presence of *Salmonella* when using PathoSEEK® Salmonella Detection Assay v3.




        a. 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.

b. An “absence” or passing result for the Unknown *Salmonella*, *E. coli,* and STEC Targets
   i. Internal Control (IC), on the HEX Fluorophore, has a Cq value ≤ 35 for flower samples, ≤40 for all other matrices.
   ii. No Cq value for the FAM fluorophore.
   iii. Visually confirm no curve on the graph.



## Troubleshooting Guide

| Symptom | Reason | Solution |
|---|---|---|
| Internal control failure | IC not spiked into sample/MaGiC lysis when applicable | Repeat MaGiC Lysis and PathoSEEK by following the protocol. |
| | Sample not added | Repeat MaGiC Lysis and PathoSEEK by following the protocol. |
| | Mix up in Reaction Setup | Repeat the qPCR by following the protocol. |
| | Missing Fluorophore on plate set up | Check plate setup in qPCR platform data file and ensure the correct fluorophores were chosen for the assay being run. |
| | Optional IC spike not performed for flower samples for Aspergillus Assays | The IC spike into flower is not mandatory when running the Aspergillus MaGiC lysis process. However if samples are low quality, it may be necessary. |
| | qPCR inhibition | Dilute MaGiC Lysates 1:2 or 1:10 with nuclease free water and re-perform qPCR. If dilution is not successful, repeat MaGiC Lysis. |
| Amplification of the Internal control is not expected in the assay positive or negative control wells. No Cq or a Cq of more than 35 is acceptable. Any Cq lower than 35 constitutes a rerun | Cannabis DNA or IC 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 MaGiC lysis area | Designate separate benches, pipettes etc. for lysis and qPCR setup. |
| Positive Negative Control | Small Cq value <15 | Visually confirm that there is a true amplification curve. If not, this is a low level background and can be ignored. |
| | Contamination | Repeat the qPCR by following the protocol. |
| | Insufficient pre-setup bleaching | Wipe down the lab workspace and all equipment with 10% Bleach. Repeat qPCR. |
| Negative Positive Control | Mix up in Reaction Setup | Ensure correct well location was chosen for assay positive control. If yes, repeat the qPCR by following the protocol. |
| 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.

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 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 |
|---|---|---|
| v1 | April 2025 | Launch of new versions of assays with MaGiC Lysis |

**DISCLAIMER**

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

This test has not been validated on remediated (irradiated, ozone treated, acid treated, hydrogen peroxide treated, etc.) samples. Samples that have undergone remediation may cause discordant results between plating methods and PathoSEEK methods. When remediated samples produce a result above the action limit on qPCR, we recommend confirming viability with an approved plating method.

Results may vary based on laboratory conditions. Altitude and humidity are among factors known to affect the growth of bacterial and fungal species.