PathoSEEK® Salmonella & Escherichia coli Multiplex Detection Assay with MaGiC Lysis Kit

User Guide

Real-time PCR (qPCR) assay for the detection of *Salmonella* spp. and *Escherichia coli* in food, nutraceutical, dietary supplement, and cannabis matrices.

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Introduction

Salmonella spp. and *Escherichia coli* are key indicators of microbial safety in consumable products. Absence of these organisms is a common specification for dietary supplements and nutritional ingredients (for example, under USP <2022>), and testing for them is required under many state cannabis regulatory programs.

The PathoSEEK® Salmonella and E. coli Multiplex Detection Assay v3 with MaGiC Lysis is a real-time PCR (qPCR) method for the qualitative (presence/absence) detection of *Salmonella* spp. and *Escherichia coli* following enrichment. It detects both targets in a single reaction, along with an internal control that verifies sample processing and amplification in every well. The method has been validated for the matrices listed in Table 1.

Process Overview

The PathoSEEK® Salmonella and Escherichia coli Multiplex Detection Assay with MaGiC Lysis employs a multiplexing strategy that incorporates an internal control (IC) introduced at the lysis step. This ensures that every reaction contains internal control DNA alongside target detection, providing a built-in verification of assay performance. By confirming proper reaction setup and experimental conditions in each run, this approach minimizes the risk of false negative results — a limitation of methods that lack integrated controls. The assay has been validated on the Bio Molecular Systems Mic and Bio-Rad CFX Opus 96 and CFX 96. A simplified depiction of the MaGiC lysis workflow and qPCR assay steps is provided in Figure 1 and Figure 2, respectively.



Figure 1: Overview of MaGiC Lysis

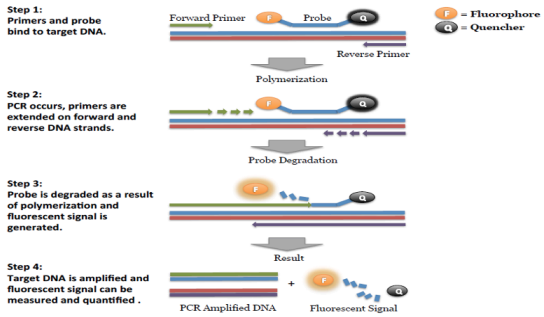


Figure 2: Overview of qPCR

Kit Components

PathoSEEK® Salmonella and E. coli Multiplex Detection Assay with MaGiC Lysis kit, **P/N 420525** (Kit contains sufficient reagents for 200 reactions).

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

Note: Actual fill volumes include overage. Some components are stored at different temperatures.

*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 Included in Kit:

Item P/N	Item Name	Qty Provided	Storage Conditions
420337	Internal Control	1 Tube (50 µL)	-15 to -20 ºC
420313	PathoSEEK Salmonella & E. coli Positive Control	1 Tube (50 µL)	-15 to -20 ºC

Item P/N	Item Name	Qty Provided	Storage Conditions
420227	Buffered Peptone Water	CS/10 x 500 mL Bottles	2-25 ºC
420184	PCR Grade Nuclease Free Water	500 mL Bottle	2-25 ºC
420205	Tryptic Soy Broth (for cannabis matrices) (TSB)	CS/10 x 500 mL Bottles	2-25 ºC

Additional **Optional** Reagents Not in Kit:

Item P/N	Item Name	Qty Provided	Storage Conditions
420145	Grim Reefer Free DNA Removal Enzyme and Buffer	~1000 Reactions	-15 to -20 ºC
	Grim Reefer Enzyme	1 Bottle (2.5 mL)	-15 to -20 ºC
	Grim Reefer Buffer	1 Bottle (12.75 mL)	-15 to -20 ºC
420150	Grim Reefer Deactivation Buffer	1 Tube (1.2 mL)	20 to 28 ºC

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:

- Bio-Molecular Systems Myra Liquid Handling System with Mic 4-Channel Real-Time PCR Instrument
    - Mic 4-Channel Real-Time PCR Instrument - **Medicinal Genomics P/N 420241**
    - Myra Liquid Handling System - **Medicinal Genomics P/N 420252**
    - Latest Version of BMS Workbench Software - Free Download from BMS
    - Lab supplied Windows PC (See vendor's requirements)
    - Myra 50 µL Robot Tips - **Medicinal Genomics P/N 420254**
    - Mic Tubes and Racked Caps - **Medicinal Genomics P/N 420244**

    - Mic Tubes and Caps (Bulk) - **Medicinal Genomics P/N 420243**
- Bio-Rad CFX-Opus
    - Software version CFX Maestro 2.3
    - Bio-Rad supplied or lab supplied Windows PC (See vendor's requirements)
- Adjustable, variable volume pipettes (single or multichannel).—P10, P20, P200, and P1000
- Adjustable, variable volume filter pipettes tips.—For P10, P20, P200, and P1000
- 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 #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, or equivalent
- 1.5 mL 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**
    - Optically clear qPCR tubes may be substituted
- Adhesive optical seals for qPCR plates — **Medicinal Genomics P/N 100177**
- Filter Bags—Whirl-Pak bags or equivalent enrichment containers of suitable dimensions. It is strongly advised to utilize enrichment bags featuring integrated filter technology.
- Crushed ice
- 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
- 25 mL 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 the pipette, as well as the 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

- 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® Salmonella & E. coli Multiplex Detection Assay with MaGiC Lysis Kit 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. Prepare the workspace. Wipe down the benchtop and all equipment with a 10% bleach solution before beginning.
2. Aliquot Buffered Peptone Water (BPW) or Tryptic Soy Broth (TSB). If not already at room temperature, remove BPW or TSB from the 2-8 °C refrigerator (it should come to room temperature, 20–28 °C, before use).

   Note: BPW and TSB are very good growth media for microbes. Therefore, it is best to pour the approximate amount of BPW or TSB into another sterile tube or container, so as not to contaminate the stock bottle.

3. 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.
4. Prepare samples according to the Table 1 below:

Table 1: Matrices with Sample Prep Conditions

Matrix	Sample Preparation	Enrichment	Dilution before qPCR (use nuclease free water)
MCT Oil (25g) Gummy Vitamins (10g) Vitamin Premix (10g)	Homogenize *n* g of sample into 9 x *n* mL of **BPW** (e.g. 25g into 225 mL of BPW)	Incubate for 20 - 24 hours at 37 ± 2°C	None required
Whey Powder (25g) Botanical Powder (10g) Energy Shot (1g)	Homogenize *n* g of sample into 9 x *n* mL of **BPW**	Incubate for 20 - 24 hours at 37 ± 2°C	1:10 dilution required
Chocolate (5g)	Homogenize *n* g of sample into 9 x *n* mL of **BPW**	Incubate for 20 - 24 hours at 37 ± 2°C	1:100 dilution required
Cannabis flower Marijuana Infused Products (MIPs) (excluding chocolate products)	Homogenize *n* g of sample into 9 x *n* mL of **TSB**	Incubate for 20 - 24 hours at 37 ± 2 °C	None required

5. 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.
6. Homogenize the sample with BPW or TSB then place it into the incubator.

Sample Lysis

Preparation of the Internal Control Dilution

Dilute Internal Control (IC) to 1:50,000 for all matrices **except chocolate**

Note: Before starting, ensure the stock IC is fully thawed, vortexed, and pulse-spun in a mini-centrifuge.

1. Step 1 — Make a 1:100 dilution: Add 2 µL of stock IC to 198 µL of nuclease-free water. Vortex thoroughly and pulse spin.

2. Step 2 — Make a 1:5,000 dilution: Add 2 µL of the 1:100 dilution to 98 µL of nuclease-free water. Vortex thoroughly and pulse spin. **This is your working IC at 1:5,000 for chocolate matrices only.**
3. Step 3 — Make the final 1:50,000 dilution: Add 10 µL of the 1:5,000 dilution to 90 µL of nuclease-free water. Vortex thoroughly and pulse spin. **This is your working IC at 1:50,000 for all other matrices.**

MaGiC Lysis

1. Remove the sample enrichments from the incubator.
2. Homogenize the samples.
3. Remove 10 µL of enriched test portion and transfer into the well of a 96 well PCR plate.

OPTIONAL Grim Reefer Free DNA removal

If performing Free DNA Removal proceed with the following steps, **if not skip to step 4.**

a. Add 10 µL of Grim Reefer Buffer to each sample being tested, using a fresh pipette tip for each transfer.
b. Add 2 µL of Grim Reefer Enzyme to each sample being tested, using a fresh pipette tip for each transfer.
c. Pipette mix thoroughly using a fresh pipette tip for each transfer. Pipette mix 15 times to ensure the sample is mixed with the Grim Reefer Buffer and Grim Reefer Enzyme.
d. If processing a full plate of samples, a "GR Working Solution" made up of Grim Reefer Buffer and Grim Reefer Enzyme can be made so that the two can be added together across the plate.
    i. For example, if testing 60 samples, make enough "GR Working Solution" for 70 samples (overage is needed when making a working solution).
    ii. Transfer 700 µL of Grim Reefer Buffer + 140 µL of Grim Reefer Enzyme to a 1.5 mL tube.
    iii. Pipette mix 15 times at 50% volume and pulse spin down in a mini centrifuge.
    iv. Pipette 12 µL of the "GR Working Solution" into each sample and pipette mix thoroughly using a fresh pipette tip for each transfer.
e. Seal plate, spin down in plate centrifuge, and incubate at 37 ºC for 10 minutes in a thermal cycler.

f. Remove the plate from the thermal cycler and spin the plate in the plate centrifuge.
g. Carefully remove the seal and add **4 µL** of Grim Reefer Deactivation Buffer to each sample. Pipette mix thoroughly using a fresh pipette tip for each transfer. Pipette mix 15 times to ensure the sample is mixed with the Grim Reefer Deactivation Buffer.

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4. Add 50 µL of MaGiC Lysis Reagent to all wells being tested, using a fresh pipette tip for each transfer. Pipette mix 15 times to ensure the sample is mixed with the lysis reagent.
5. Add 5 µL of 1:50,000 diluted IC control to each sample. If testing chocolate, add 5 µL of 1:5,000 diluted IC control.
6. Repeat for all sample wells, using a fresh tip for each transfer.
7. Seal the top of the plate and spin down in a centrifuge.
8. Place plate on a thermal cycler and run MaGiC Lysis program
    a. Incubate at 95 °C for 10 minutes, then 4 °C for 5 min (if 4 °C is not an option, set to 25 °C for 10 min).
9. Remove the plate with lysed samples 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.
10. 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 setup. See Real-Time Quantitative PCR (qPCR) Setup Protocol with PathoSEEK Amplification Mix.
11. If you are not proceeding directly to qPCR setup, seal the plate containing the sample lysates and store it in a –20 °C freezer.
    a. Sample lysates that contain MaGiC Stabilization Buffer are stable for up to 1 month when stored at –20 °C.
    b. When you are ready to use them, thaw the frozen lysates at room temperature. After thawing, mix each sample by pipetting up and down 15 times before setting up the qPCR or preparing dilutions.

Preparation of Matrix Appropriate qPCR Dilutions

12. Refer to Table 1 for matrix-specific dilution requirements prior to qPCR setup.
    a. For Whey Powder, Botanical Powder, or Energy Shot - dilute the stabilized sample 1:10 in nuclease-free water. Load 5 µL of the diluted sample per reaction.
    b. For Chocolate - dilute the stabilized sample 1:100 in nuclease-free water. Load 5 µL of the diluted sample per reaction.

If using the Myra Liquid Handler and Mic thermal cycler - Proceed to the next page.

If using the Bio-Rad CFX Opus 96 or CFX 96 - go to **Real-Time Quantitative PCR (qPCR) Setup for the Bio-Rad CFX Opus or CFX 96**

Real-Time Quantitative PCR (qPCR) Setup on the Myra Liquid Handler

1. Remove PathoSEEK Amplification Mix, PathoSEEK Salmonella & E. coli Multiplex Detection Assay, and Salmonella & E. coli Multiplex positive Control from the -20 ºC freezer.
    a. If lyophilized Amplification Mix has not been previously rehydrated, use the supplied Nuclease-Free Water to rehydrate with the volume specified on the side of the vial. Invert the bottle 5-10 times, vortex, or pipette mix to ensure complete resuspension.
        i. Following reconstitution, store the Amplification Mix at -15 to -20 °C when not in use. For improved access and storage stability, transfer the reconstituted Amplification Mix to a labeled 1.5 mL or 2.0 mL microcentrifuge tube and record the rehydration date on the tube label.
    b. Allow all frozen reagents to defrost at room temperature (20 to 28 °C).
2. Before preparing the Master Mix, invert or vortex and pulse spin down the reagents in a mini centrifuge
    a. Salmonella & E. coli Multiplex Detection Assay tube (probe mix) – vortex tube quickly followed by a pulse spin down in a minicentrifuge.
    b. Salmonella & E. coli Multiplex 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. Place reagents on ice.
3. For the positive control, use the Salmonella & E. coli Multiplex positive Control reagent. Dilute the positive control **1:10,000**
    a. Add 2 µL of Positive Control to 198 µL nuclease free water (found in the kit), vortex to mix and pulse spin down the tube in a mini centrifuge. This is a 1:100 positive control dilution.

    Note: It is best practice to add the largest volume reagent first.
    b. Add 2 µL of 1:100 Positive Control dilution to 198 µL nuclease free water, vortex to mix and pulse spin down the tube in a mini centrifuge. This is your 1:10,000 positive control dilution. This tube will be loaded into the instrument.
4. Open the Myra or Workbench software program by clicking the desktop shortcut.



Figure 3: Desktop shortcut

5. Under the “Templates” tab, double-click the appropriate run template:
    a. _MaGiC_Mic

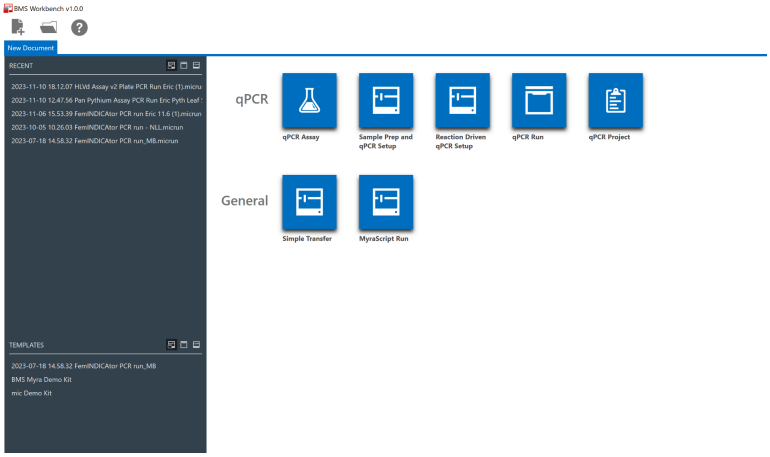


Figure 4: Template selection screen

6. Click the + Next to Assays and select the appropriate assay
    a. Salmonella & E. coli Multiplex Detection Complete Assay

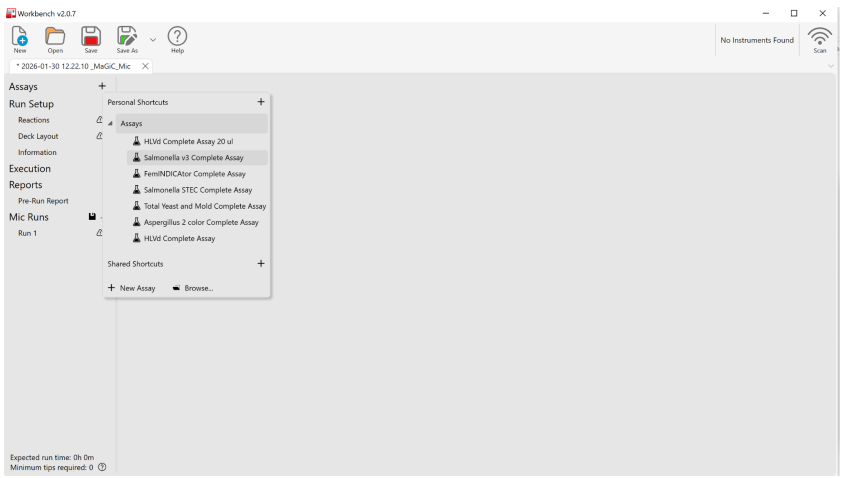


Figure 5: Example Assay selection screen

7. Connect to the Myra by using the icon in the upper right corner.



Figure 6: Connect to Myra Icon

8. Navigate to the Reactions tab and name samples, positive controls, and NTCs.
9. Use the "Type" drop down menu to identify samples as Unknown, Positive Control, or NTC.
10. Use the "Assay" drop down menu to assign Salmonella & E. coli Multiplex Detection Complete Assay to all wells being tested.

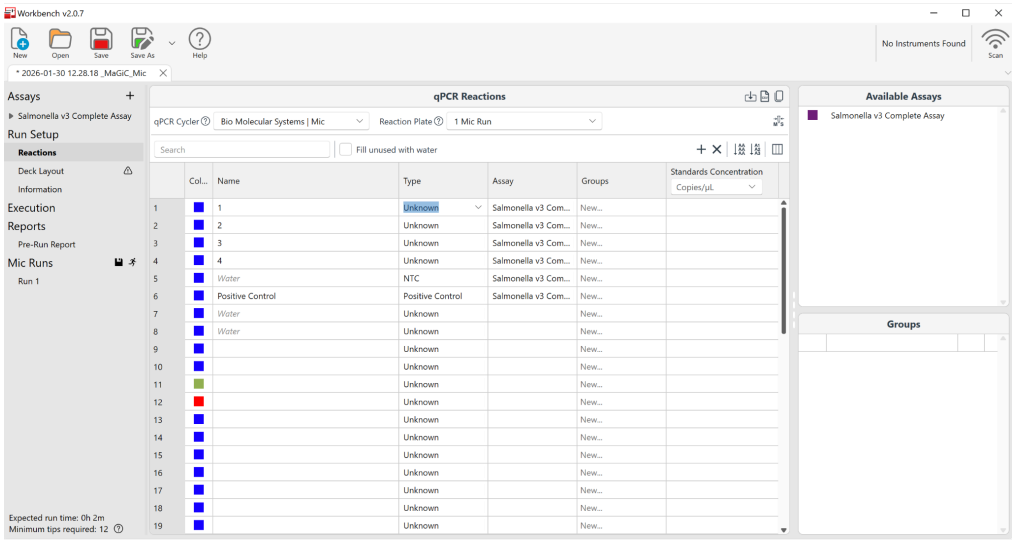


Figure 7: Example Reactions Tab

11. If stored at 4°C, remove the Myra Multipurpose Loading Block and transfer to the correct Myra deck location according to the Deck Layout screen. If the loading block is stored at 4 °C until right before setting up a run, it will keep reagents cool while on the deck.
12. Hover over the tube positions to determine the location of each reagent tube in the multipurpose loading block. Samples will be placed just to the right of the loading block according to the deck layout. The sample lysates will be placed onto the deck in a 96 well PCR plate.
13. Hovering over the reagent tubes will also indicate volumes of each reagent needed, based on the number of samples entered into the 'Samples' tab.

Note: Most master mixes will need additional volume over the volume indicated to account for viscosity of these reagents. It is best to load full tubes whenever you can.

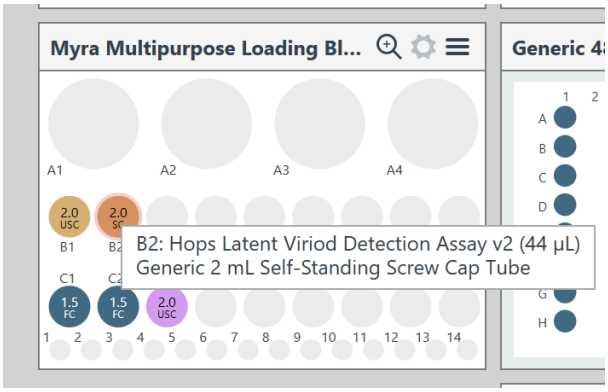


Figure 8: Example Multipurpose loading block image

14. Once the deck setup is complete, click on the Play icon to start the instrument. A "Save As" screen will allow users to save their run with a unique name.



Figure 9: Play icon

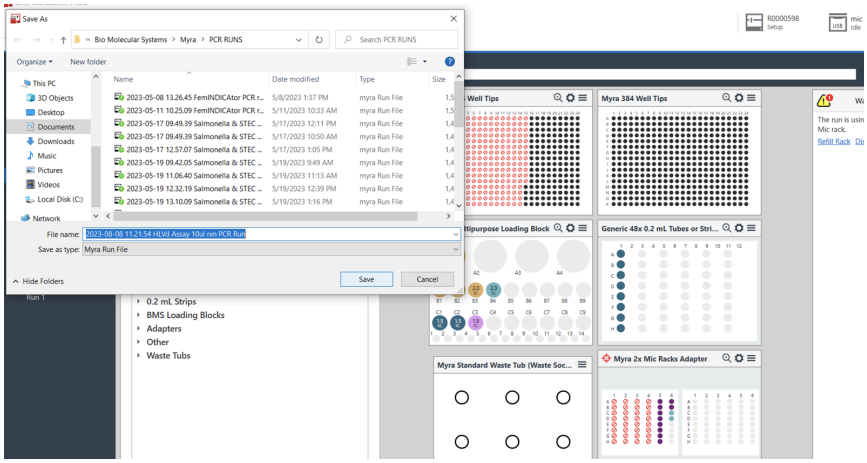


Figure 10: Example "Save As" screen

15. A pre-run checklist will appear. Check off the boxes as the steps are completed and click "Start" to initiate the run.

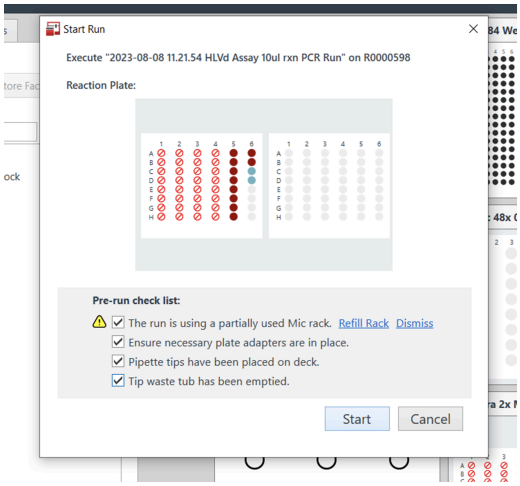


Figure 11: Example Pre-run checklist

16. Allow the instrument to run to completion.
17. Once the run has finished, your Mic tubes will contain the Master mix and samples.
18. Firmly cap the tubes using the manual capper or the Rapid Cap device.

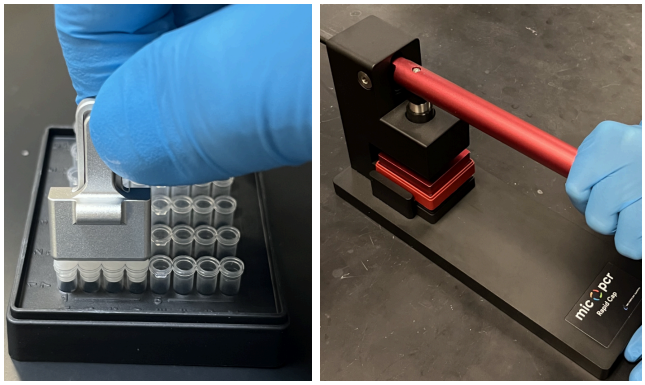


Figure 12: Capping with manual capper (left) and Rapid Cap device (right)

19. Load the Mic qPCR thermal cycler with the capped reaction tubes. Each tube is part of a strip of four, with the first tube having a small tab to ensure the strip is loaded into the instrument correctly. Start at position 1 and ensure that the small clear tab on the tubes is aiming inward towards the rotor center. If running fewer than 46 samples (48 total reactions) ensure that *water balance Mic tubes* are loaded into all unused sample locations.

    Note: Do not use old reaction tubes in the unused sample location. Not all assay reaction volumes are identical. Ensure that the proper volume of water is used in the blank tubes for each assay.
20. Replace the magnetic tube clamp and close the Mic lid when all tubes are loaded.

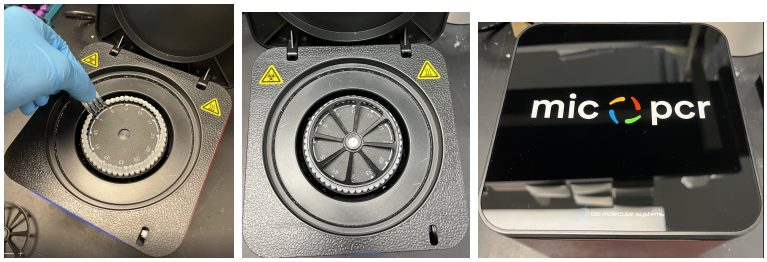


Figure 13: Loading the Mic

21. Cap the reagent tubes and sample tubes on the Myra. If another Myra run will not be performed right away, place the reagents and samples in the freezer for storage or on ice for use within the same day.
22. Click the running man figure to export the data to the Mic software.

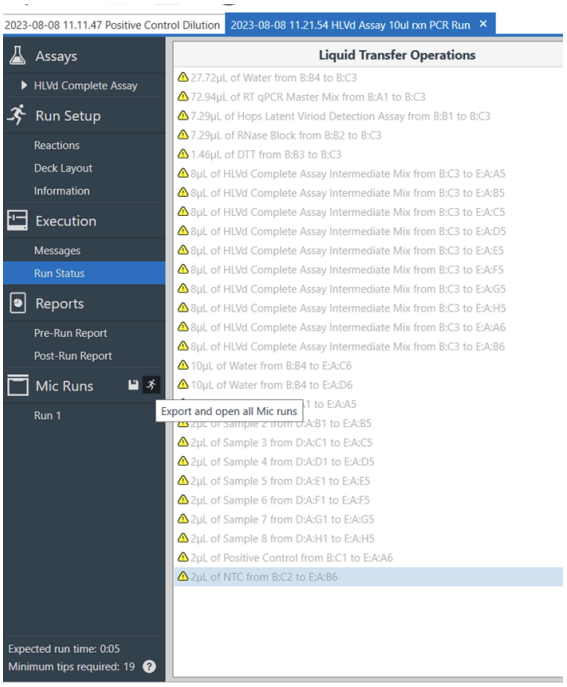


Figure 14: Example of Exporting data to the Mic

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 (data capture).

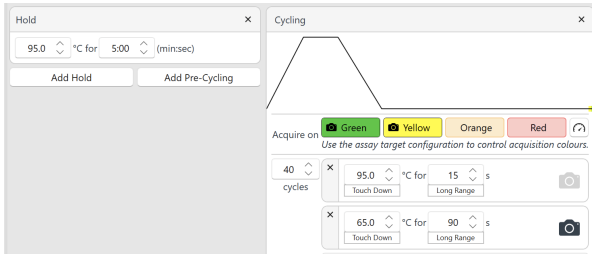


Figure 15: Thermal profile

4. Click on the Mic icon in the upper right-hand corner, and select "Start run" from the menu that appears.

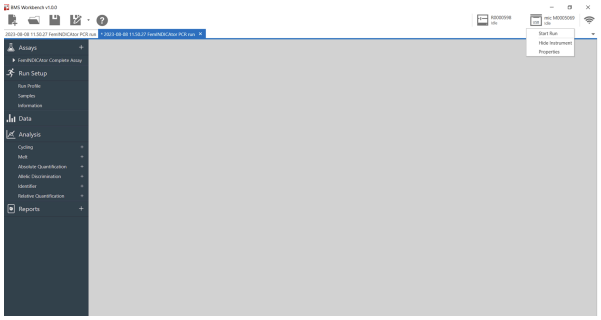


Figure 16: Example Start Run menu

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 BioMolecular System Mic

Table 2: PathoSEEK® Salmonella & E. coli Multiplex Detection Assay Data Analysis Quick Reference

PathoSEEK® Assay	Cq Value	Fluorophore	Negative Control (Cq)	CFU threshold (CFU/g)
Salmonella spp.	≤ 40	ROX	No Cq	Presence/Absence
Escherichia coli	≤ 40	FAM	No Cq	Presence/Absence
Internal Control (IC)*	≤35	HEX	*Internal control verifies the protocol worked as expected.	
Assay Positive Control	≤35	FAM, ROX		

1. Look for  next to Identifier under Analysis for the Complete Assay Analysis if it has been automatically generated. If the Complete Assay analysis is not automatically generated, click the **"+"** icon next to the **Identifier** tab and select the appropriate **Complete Assay** to display the software-generated presence/absence results.

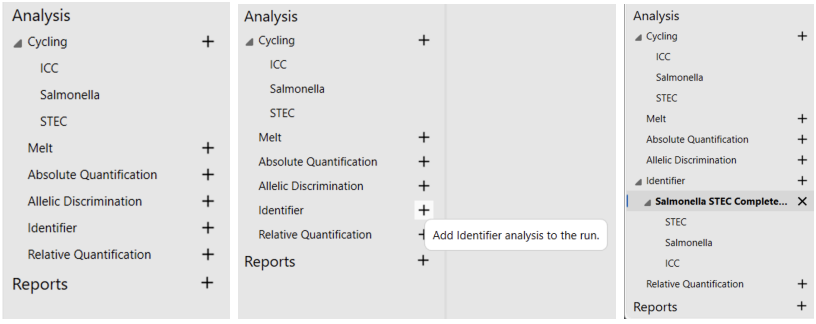


Figure 17: Add Identifier Example

2. To review a preliminary automatic report select the relevant "Complete Assay". This feature is intended to 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, see screenshot below. Various factors may affect the validity of this report and further analysis is required as outlined in the following steps.

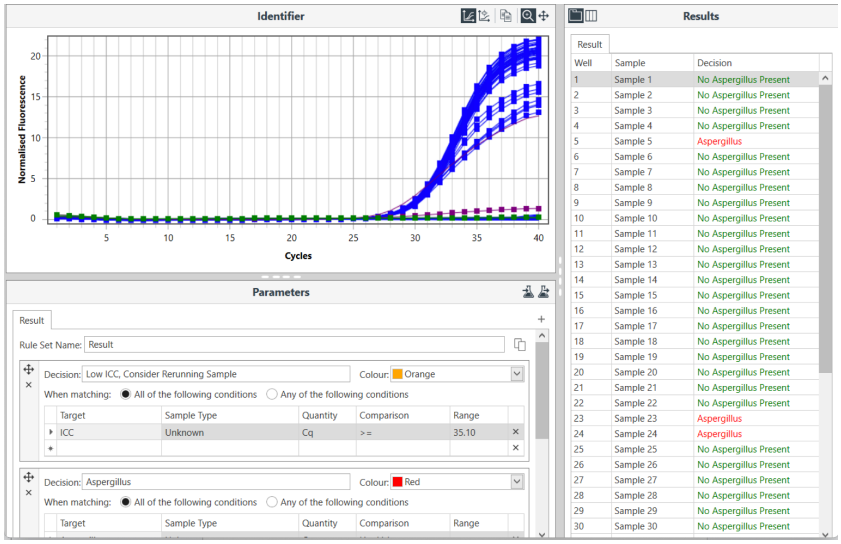


Figure 18: Example Automatic Report

3. If the automatic calls are blank, and there is amplification on all of the channels, this indicates that one of the cycles is out of threshold or the fluorescent cutoff percentage is set incorrectly. To address this, review each target under the Identifier tab.
    a. In the event that the target display lacks the requisite gray horizontal threshold indicator, the user should navigate to the "Parameters" section to adjust the *Ignore Cycles Before* or *Fluorescence Cut Off* settings.
        i. If the raw data shows an early rise at the beginning of the run followed by signal decay, increase or decrease the *Ignore Cycles Before* value one click at a time until a gray bar appears on the graph. End users may ignore up to 15 cycles.
        ii. If a weak signal overall is observed, the *Fluorescence Cutoff* percentage should be increased to a maximum of 16% or a minimum of 0%. These adjustments may have to be made with none, one, or all of the targets.

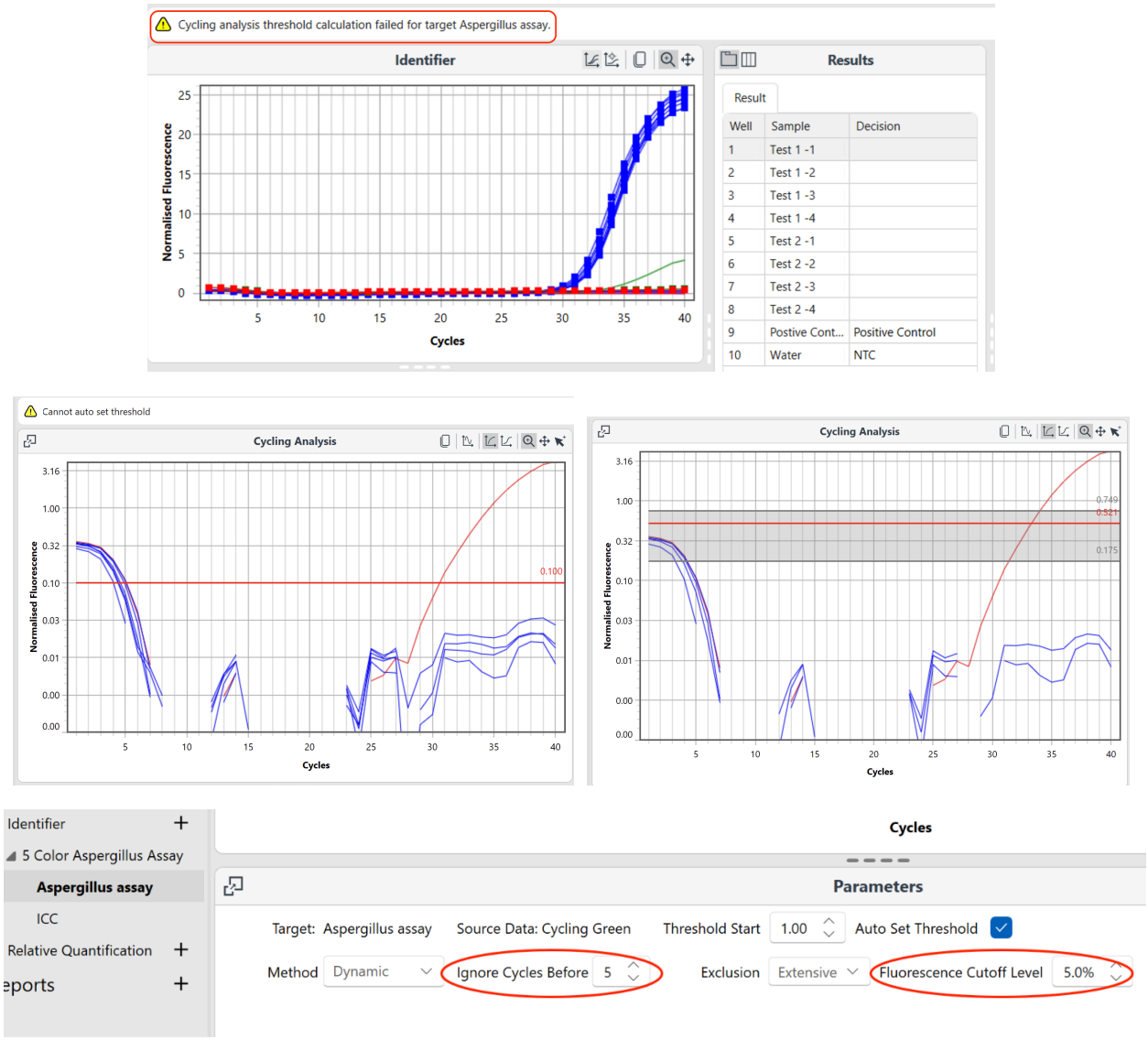


Figure 19: Example of failure to automatically set threshold

4. Conduct a manual review of the amplification curves for each sample.

   a. Click the  in the top right side of the screen next to “Samples” and select Hide All.

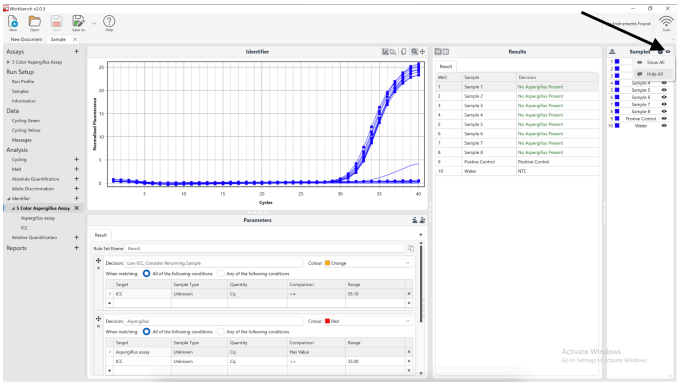


Figure 20: Example of software use

b. Next, unhide each sample, one at a time and review the curves for the Internal Control and microbial targets. Before reviewing the next sample, hide the current sample being reviewed.

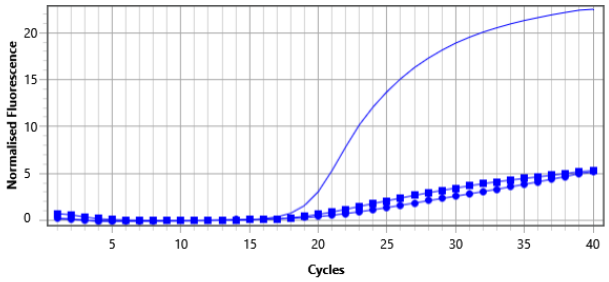


Figure 21: Example of individual sample

c. Individual targets may also be reviewed for each sample by selecting, “Display results for each target on a separate chart.”

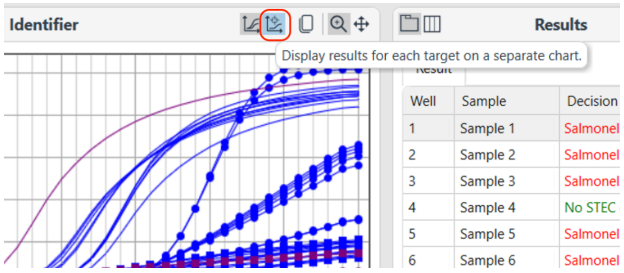


Figure 22: Example of software use

d. Confirm that the Automatic Report results and the raw amplification curves agree.

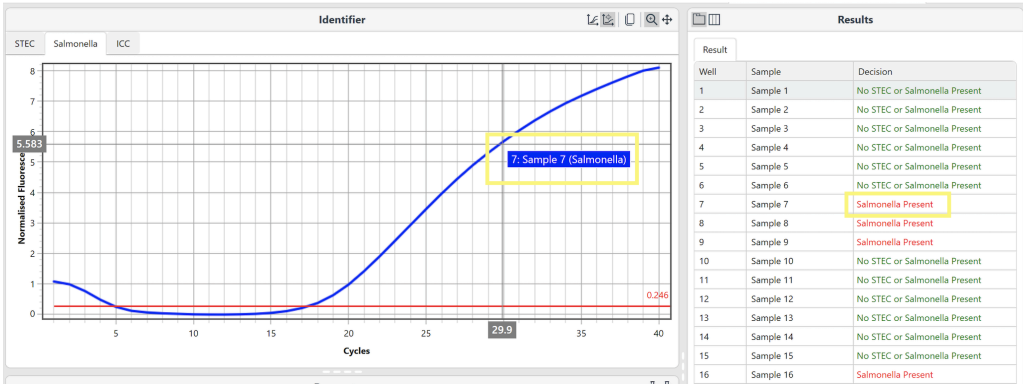


Figure 23: Example of report confirmation

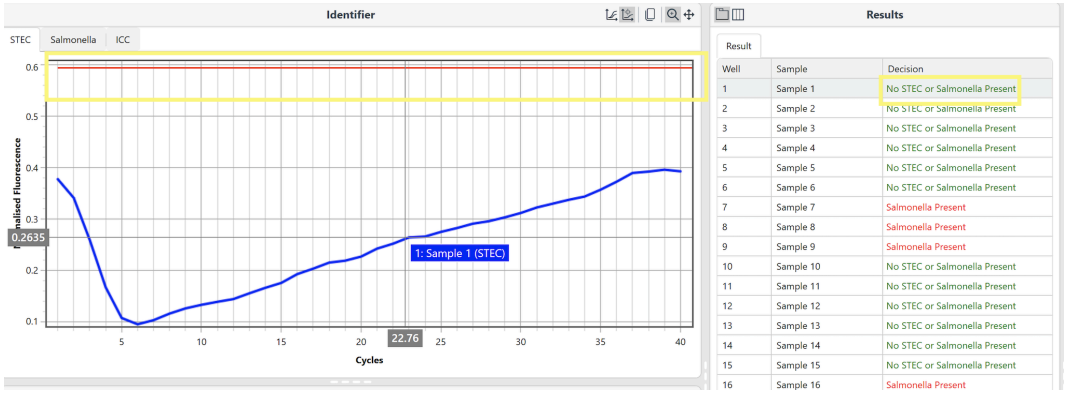


Figure 24: Example of report confirmation

e. Confirm that the Cq value generated for each target aligns with where the amplification curve crosses the threshold:

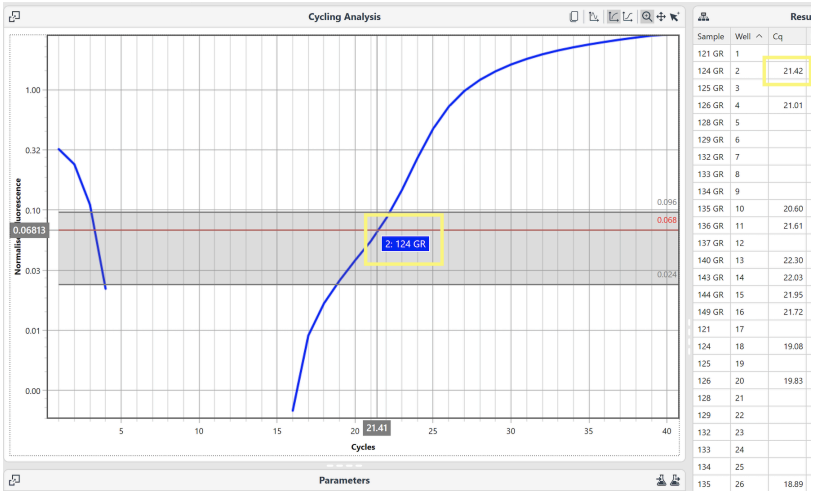


Figure 25: Example of Cq value confirmation

f. If an amplification curve is present but no Cq value is generated, the *Fluorescence Cutoff* feature or *Ignore Cycles Before* feature may require adjustment.

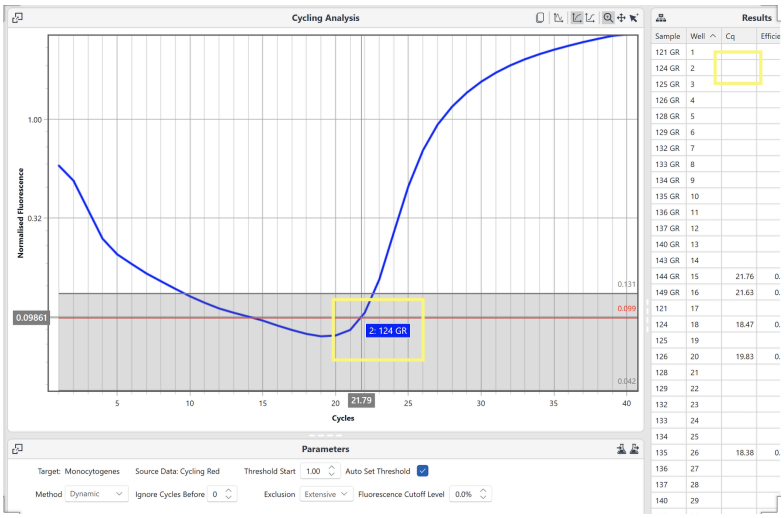


Figure 26: Example of a curve requiring adjustment of *Ignore Cycles Before* or *Fluorescence Cutoff*

g. If adjusting the *Fluorescence Cutoff* or *Ignore Cycles Before* does not allow the automatic calls to be made by the software, the already extracted DNA may be re-run on qPCR or the results may be reported manually after conducting a review of the amplification curves.

5. Controls
    a. Assay-specific Positive Control, on the FAM, ROX fluorophores, have a Cq value ≤ 35.
        i. Visually confirm with the curve on the graph.
    b. Assay-specific Negative Control in the FAM, ROX fluorophores, have no Cq value.
        i. Visually confirm with the curve on the graph.
6. Unknown *E. coli* or *Salmonella* Targets: The following fluorophore detects *E. coli* and *Salmonella* spp.:
    a. *E. coli*: FAM
    b. *Salmonella* spp.: ROX
        i. A “presence” or failing result for the unknown *E. coli*.
            1. Any Cq value for the FAM fluorophore ≤ 40.
            2. Visually confirm with the curve on the graph.
        ii. A “presence” or failing result for the unknown *Salmonella* spp.

1. Any Cq value for the ROX fluorophore ≤ 40.
2. Visually confirm with the curve on the graph.

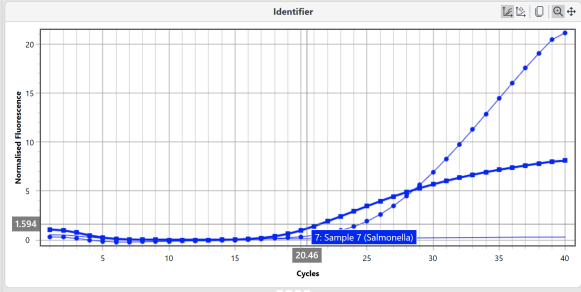


Figure 27: Example of a presence result

iii. An "absence" or passing result for the unknown targets

1. Internal Control (IC), on the HEX Fluorophore, has a Cq value ≤ 35.
2. No Cq value for the FAM or ROX fluorophores.
3. Visually confirm that there is no curve on the graph.

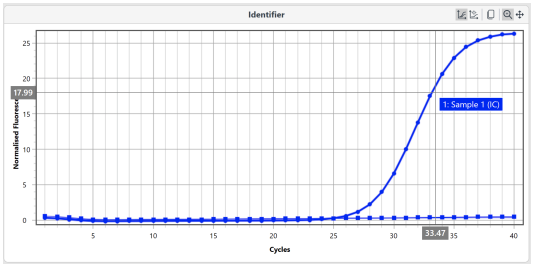


Figure 28: Example of an absence result

iv. Below is an example of acceptable amplification curves. The curves cross the threshold (horizontal red line) consistently within 40 cycles and show clear amplification consistent with typical qPCR curve shapes. The baseline remains flat until amplification occurs and a Cq is generated when the amplification curve crosses the threshold. These curves represent a positive result for their respective target.

1. Logarithmic view

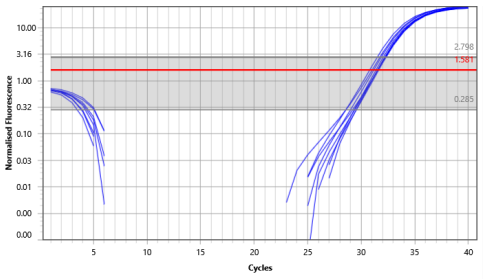


Figure 29: Example logarithmic view

2. Linear view:

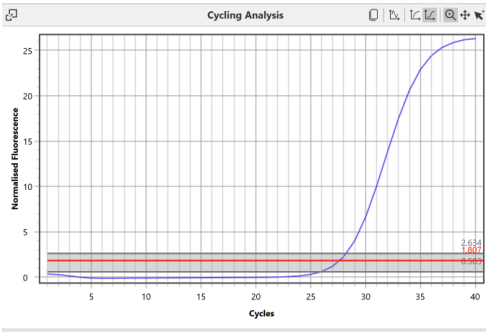


Figure 30: Example linear view

v. Below are examples where no amplification has occurred. The curves remain generally flat and do not cross the threshold or exceed the initial background fluorescence of qPCR. No Cq value is generated. The curves below represent a non-detect result for their respective target.

1. Logarithmic view

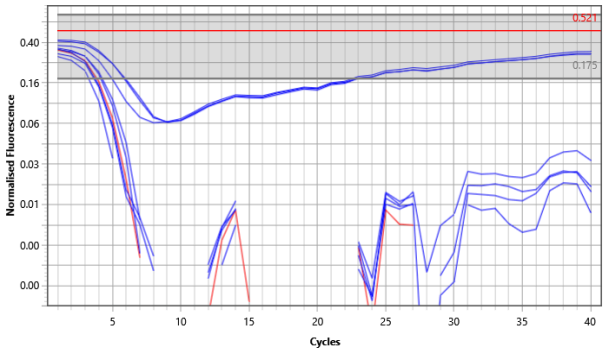


Figure 31: Example logarithmic view

2. Linear View

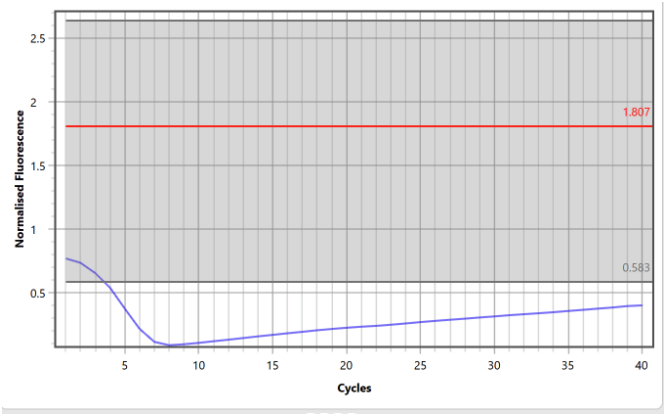


Figure 32: Example linear view

7. Raw amplification curves should be reviewed to ensure consistency with the results generated by the Identifier and that the curves are as expected. In the Data tab select Cycling Green (FAM), Cycling Yellow (HEX), or Cycling Orange (ROX), then Hide and Un-Hide samples for review. Raw data curves can be useful in determining if the "Ignore Cycles Before" or "Fluorescence Cutoff" parameters require adjusting in the "Identifier" section and whether or not amplification has occurred.

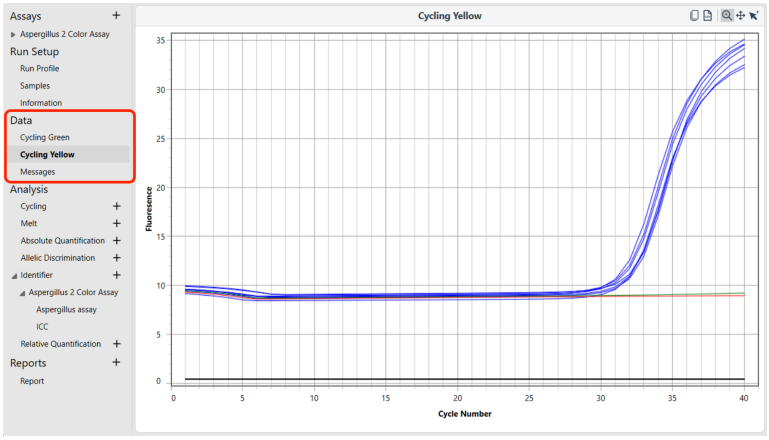


Figure 33: Example of Cycling Data

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

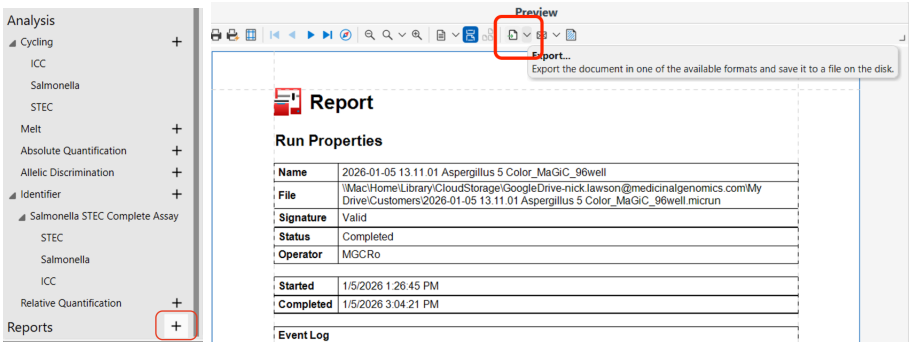


Figure 34: Example of report generation

Real-Time Quantitative PCR (qPCR) Setup for the Bio-Rad CFX Opus or CFX 96

1. Remove PathoSEEK Amplification Mix, PathoSEEK Salmonella & E. coli Multiplex Detection Assay, and Salmonella & E. coli Multiplex positive Control from the -20 ºC freezer.
    a. If lyophilized Amplification Mix has not been previously rehydrated, use the supplied Nuclease-Free Water to rehydrate with the volume specified on the side of the vial. Invert the bottle 5-10 times, vortex, or pipette mix to ensure complete resuspension.
        i. Following reconstitution, store the Amplification Mix at -15 to -20 °C when not in use. For improved access and storage stability, transfer the reconstituted Amplification Mix to a labeled 1.5 mL or 2.0 mL microcentrifuge tube and record the rehydration date on the tube label.
    b. Allow all frozen reagents to defrost at room temperature (20 to 28 °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. Salmonella & E. coli Multiplex Detection Assay tube (probe mix) – vortex tube quickly followed by a pulse spin down in a minicentrifuge.
    b. Salmonella & E. coli Multiplex 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. Place reagents on ice.
3. Prepare Master Mix in a 1.5 mL tube (the probe mix also contains the probe for the IC). Label the tube as “Master Mix”. See Table 3 (PathoSEEK Amplification Master Mix Reagent Volumes). Always prepare enough Master Mix for an additional one or two reactions to account for pipetting and dead volumes. Be sure to include 2 extra reactions for the qPCR positive and negative controls. For example, if testing 10 samples, you would need to make enough Master Mix for 13 or 14 reactions, which would account for 1 or 2 excess.

Table 3: PathoSEEK® Amplification Master Mix Reagent Volumes

Reagent	Volume for 1 qPCR Reaction
PathoSEEK® Amplification Mix	10 µL
Sal & E. coli Assay	1 µL
Nuclease Free Water	4 µL
Total	15 µL

4. Once the Master Mix is combined gently cap the tube and vortex to mix.
    a. Pulse spin down tube in minicentrifuge.
    b. 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. For the positive control, use the Salmonella & E. coli Multiplex positive Control reagent. Dilute the positive control 1:10,000
    a. Dilute the stock Salmonella & E. coli Multiplex positive Control 1:100.
        i. Ensure stock positive control is fully thawed and vortexed, and then pulse spin down in a mini centrifuge before use.
        ii. Add 2 µL of stock Salmonella & E. coli Multiplex positive Control to 198 µL of nuclease-free water. Vortex well and pulse spin down in a mini centrifuge.
    b. Serially dilute the 1:100 dilution of Salmonella & E. coli Multiplex positive Control an additional 1:100 to make a 1:10,000 dilution.
        i. Add 2 µL of 1:100 Positive Control to 198 µL nuclease free water (found in the kit), vortex to mix and pulse spin down the tube in a mini centrifuge. This is a 1:10,000 positive control dilution.

    Note: It is best practice to add the largest volume reagent first.
7. For qPCR reactions, use a 96-well optical qPCR plate or optically clear qPCR tubes.
8. Transfer lysed samples into a qPCR plate or optically clear qPCR tubes.
    a. Carefully remove the seal from the Stabilized Lysis Plate.

    Note: If lysed samples were frozen, let the DNA thaw completely and spin the plate in centrifuge to avoid cross contamination between samples. Pipette mix thawed samples wells before transferring to the qPCR plate or tubes.

b. Pipette transfer 5 µL of each sample lysate into the corresponding qPCR tube or well on the qPCR plate.
c. Add 5 µL of the 1:10,000 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

9. 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 pipette mix a few times after each addition of qPCR master mix. Be careful not to introduce bubbles during this mix. Use a fresh tip for each transfer of Master Mix to each well
10. Seal the plate with strip caps or an adhesive seal, or seal qPCR tubes with strip caps.
11. For the Bio-Rad CFX Opus 96 or CFX 96, spin down the qPCR plate or tubes for at least 1 minute in a 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.
12. Place the sealed plate or tubes onto the qPCR instrument.
13. Follow the software-specific instructions for each qPCR platform to initiate the run.

Running the Bio-Rad CFX Opus 96 or CFX 96

The following targets will be detected on the following fluorophores:

Target	Optical Channel
Escherichia coli	FAM
Salmonella spp.	ROX
Internal Control	HEX

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

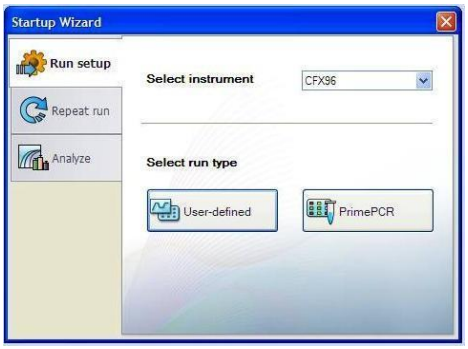


Figure 35: Startup Wizard

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 (Plate Read).
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, ROX, and HEX Fluorophores and click "OK".

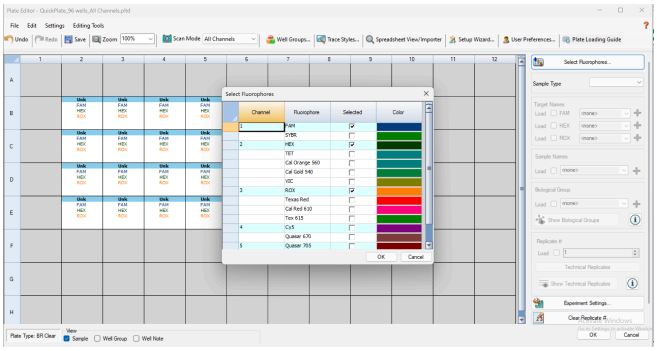


Figure 36: Plate editor window

    c. If the plate layout was previously saved, click "Edit Selected" to move to the Plate Editor Screen.

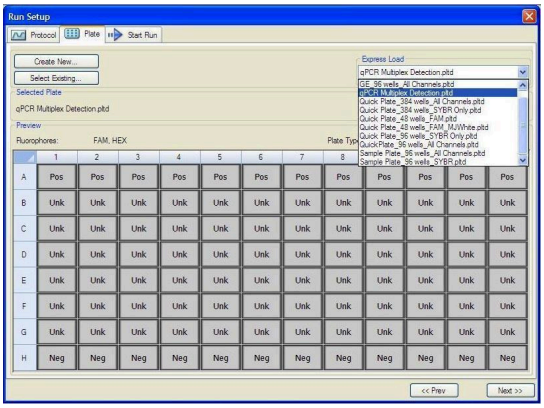


Figure 37: Plate Editor Screen

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

NTC (Negative Template Control)

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

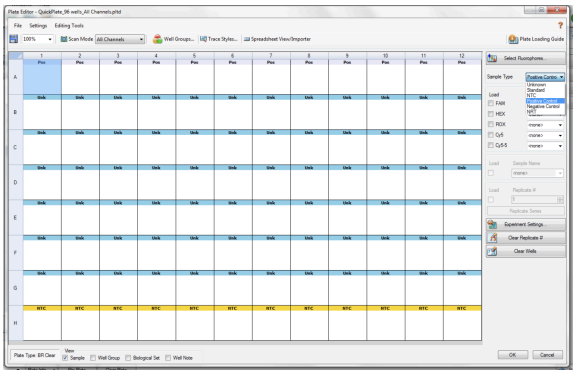


Figure 38: Channel Selection

f. Attach the fluorophores to the wells being used.

i. Highlight all the wells being used

ii. For *E. coli* & *Salmonella* spp. assay, highlight the well locations and click on FAM, ROX, and HEX.

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

4. Click “Yes” to save your plate. If creating a 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.

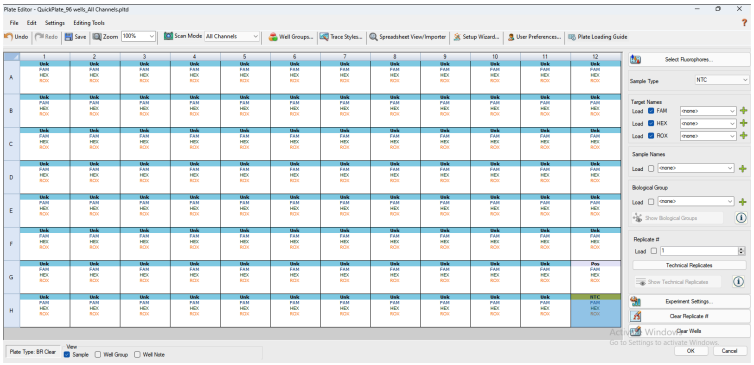


Figure 39: Plate Template

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 Bio-Rad CFX 96 Opus or CFX 96

Table 4: PathoSEEK® Salmonella & E. coli Multiplex Detection Assay Data Analysis Quick Reference Table - Biorad CFX Opus and CFX 96:

PathoSEEK® Assay	Cq Value	Fluorophore	Negative Control (Cq)	CFU threshold (CFU/g)
Salmonella spp.	≤ 40	ROX	No Cq	Presence/Absence
Escherichia coli	≤ 40	FAM	No Cq	Presence/Absence
Internal Control (IC)*	≤35	HEX	*Internal control verifies the protocol worked as expected.	
Assay Positive Control	≤35	FAM, ROX		

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

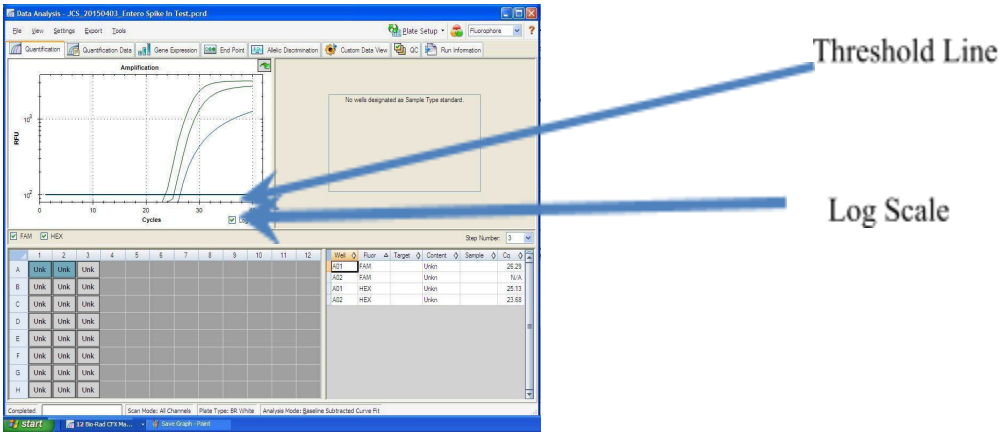


Figure 40: Example of threshold setting

4. Controls
    a. Assay-specific Positive Control on the FAM and ROX fluorophores have a Cq value ≤ 35.
        i. Visually confirm with the curve on the graph.
    b. Assay-specific Negative Control on the FAM and ROX fluorophores have no Cq value.
        i. Visually confirm with the curve on the graph.
5. Unknown Salmonella & E. coli Targets: The following fluorophore detects *Salmonella* or *E. coli*:
    a. *E. coli*: FAM
    b. *Salmonella* spp.: ROX
        i. A "presence" or failing result for the unknown *Salmonella* or *E. coli* targets.
            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 confirm with the amplification curve when a presence result occurs. Sometimes the background amplification will give a false positive reading.
                a. A Cq of ≤ 40 on the ROX channel indicates the presence of *Salmonella* spp.
                b. A Cq of ≤ 40 on the FAM channel indicates the presence of *Escherichia coli*

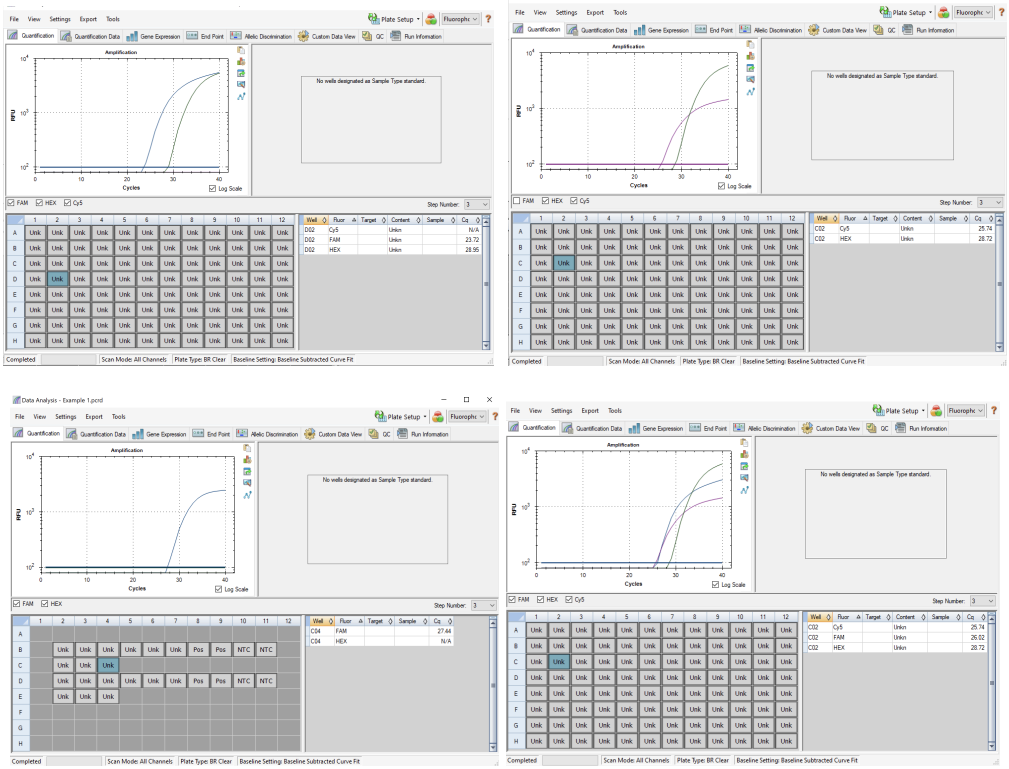


Figure 41: Examples of presence results

ii. An "absence" or passing result for the unknown *Salmonella*, or *E. coli* targets.

1. Internal Control (IC), on the HEX Fluorophore, has a Cq value ≤ 35
2. No Cq value for the FAM or ROX fluorophores.
3. Visually confirm that there is no curve on the graph.

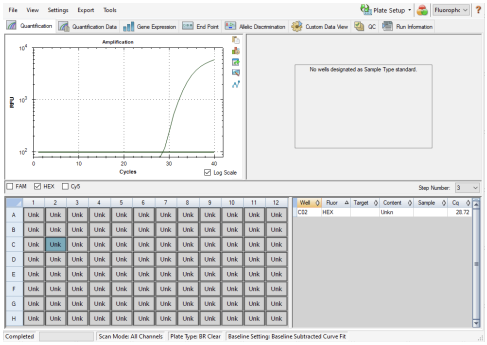


Figure 42: Example of an absence result

Confirmation of Results

Results are confirmed on an as-needed basis as determined by your laboratory. For procedures on how to confirm *Salmonella* and *E. coli* please refer to the most recent versions of the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (BAM) Chapter 4 (Enumeration of *Escherichia coli* and the Coliform Bacteria) (4), Chapter 4a (Diarrheagenic *Escherichia coli*) (4a), and Chapter 5 (*Salmonella)* (5).

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 reagents where no Cq is expected. It helps to ensure that all Assay-specific reactions are free of contaminants.

The assay-specific **Positive Controls** are the reagents 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 and ROX Fluorophores.

Amplification of the **Internal Control (**or amplification of the microbial target of interest) is expected in every reaction containing DNA isolated from a lysate. It ensures the DNA isolation procedure was successful, or the presence of target microbial contamination. The internal control targets a specific genome and uses the HEX Fluorophore.

Revision History

Version	Date	Description
v1	November 2024	Method Launch, PathoSEEK® Salmonella & E. coli Assay v3 with MaGiC Lysis
v2	January 2025	Amplification Mix packaging update
v3	October 2026	Additional matrices validated (USP equivalency); added Buffered Peptone Water enrichment for specified matrices; added Grim Reefer Free DNA Removal as an optional step

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 the factors known to affect the growth of bacterial and fungal species.

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