PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 with MaGiC Lysis

User Guide v2

Real-Time PCR (qPCR) Assay for the detection and speciation of *P. aeruginosa* and *S. aureus* in dried cannabis flower and MIP matrices

Table of Contents

Introduction

Some regulations require cannabis flower and cannabis products to be free of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (absent in 1 gram). The PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 with MaGiC Lysis method can be used to detect and speciate these species in one qPCR multiplexed assay. The method is validated for cannabis flower, concentrates, infused edibles, and infused non-edibles.

Process Overview

The PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 uses a multiplexing strategy with an internal cannabis DNA control to ensure accurate detection of *Pseudomonas aeruginosa* and *Staphylococcus aureus* as well as cannabis DNA in a single 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 setup errors or failing experimental conditions. Below is an overview of the MaGiC Lysis process as well as a simplified depiction of the qPCR assays.

Figure 1: Overview of MaGiC Lysis

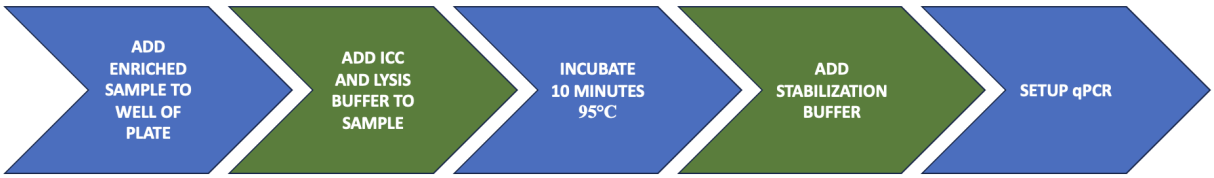
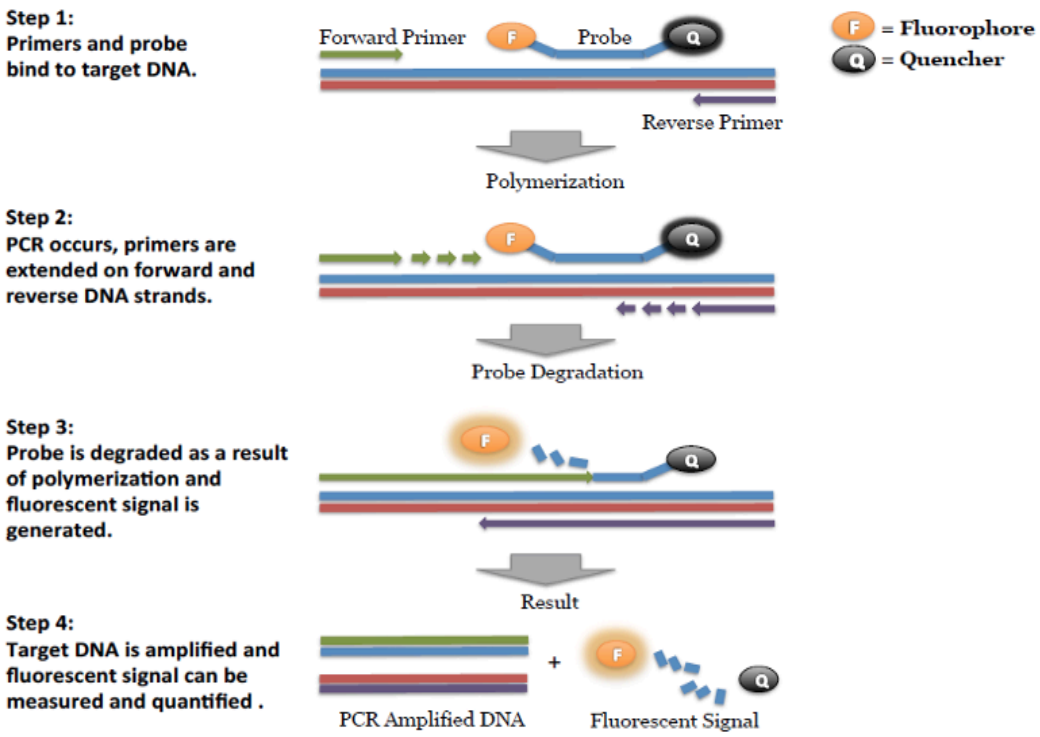


Figure 2: Overview of qPCR



The PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 can be used on the Bio Molecular Systems Mic, AriaMx Real-Time PCR Thermocycler (Agilent) or the CFX-96 (Bio-Rad). The assay uses the HEX Fluorophore for the detection of cannabis DNA, the FAM Fluorophore for the detection of *Staphylococcus aureus* and the Cy5 Fluorophore for the detection of *Pseudomonas aeruginosa*.

Limit of Detection

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

Materials and Methods

Kit Components

PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 with MaGiC Lysis Kit

P/N 420526 (contains sufficient reagents for 200 reactions)

Component Name	Qty Provided	Storage Conditions
MaGiC Lysis Reagent	1 Bottle (12 mL)	RT
MaGiC Stabilization Buffer	1 Bottle (24 mL)	RT
PathoSEEK Amplification Mix *Includes 2 tubes nuclease free water for resuspension*	4 Vials (50 rxns/each)	RT / -20 ºC*
PathoSEEK P. aeruginosa & S. aureus Detection Assay v2	1 Tube (200 µL)	-20 ºC

Note: Some actual fill volumes include overage

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

Additional Required Reagents Not in Kit:

Item P/N	Item Name	Qty Provided	Storage Conditions
420205	Tryptic Soy Broth	CS/10 x 500mL bottles	2-25 °C
420337	Internal Cannabis Control	1 Tube (50 µL)	-20 ºC
420338	PathoSEEK® P. aeruginosa & S. aureus Positive Control	1 Tube (50 µL)	-20 ºC
420184	PCR Grade Water	500 mL Bottle	2-25 °C

Additional **Optional** Reagents Not in Kit:

Item P/N	Item Name	Qty Provided	Storage Conditions
420145	Grim Reefer Free DNA Removal Enzyme and Buffer	100 Reactions	-20 ºC
	Grim Reefer Enzyme	1 Bottle (2.5 mL)	-20 ºC
	Grim Reefer Buffer	1 Bottle (12.75 mL)	-20 ºC
420150	Grim Reefer Deactivation Buffer	1 Bottle (12.75 mL)	RT

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, Cy5, 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, P50, P200, P300 and P1000
- Adjustable, variable volume filter pipettes tips.—For P10, P20, P50, P200, P300 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
- 25 mL Serological Pipette—VWR 89130-890 or 89130-900 or equivalent
- 10% bleach

Safety Precautions and Recommendations for Best Results

Safety Precautions

The PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 is a qPCR detection assay for detection of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in cannabis matrices.

1. Assay users should observe standard microbiological practices and safety precautions when performing this assay. Wear protective gloves, lab coats, eye/face protection as indicated by your quality system.
2. It is the responsibility of each laboratory to handle waste and effluents processed according to their nature and degree of hazardousness. Waste and effluents processed must be treated and disposed of in accordance with all applicable local, state, and federal regulations.

Environment

1. The quality of results depends on the strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR.
    a. Never circulate lab equipment from one workstation to another.
    b. Always use a positive and negative control for each series of amplification reactions.
    c. Periodically verify the accuracy and precision of pipettes, as well as correct functioning of the instruments.
    d. Change gloves often, especially if you suspect contamination.
    e. Clean workspaces periodically with 10% bleach and other decontaminating agents.
    f. Use powder-free gloves.
    g. If using qPCR reaction strip tubes instead of plates, avoid fingerprints and writing on caps because both can interfere with data acquisition.

Intended User

The PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 with MaGiC Lysis 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). If not already at room temperature, remove TSB from the 2-8 °C refrigerator (it should come to room temperature, 20–28 °C, before use).

   Note: TSB is a very good growth medium for microbes. It is best to pour the approximate amount of TSB into another sterile tube or container to avoid contaminating the whole bottle. Inspect stock of TSB for flocculants or signs of growth prior to aliquoting. Return it to the 2-8°C refrigerator immediately after use.

2. Wipe down the workspace with a 10% bleach solution, including the benchtop and all equipment being used.
3. Prepare consumables. Label all the filter bags with "[sample name] [date]".
4. Label lysis plate with date.
5. 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.
6. *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 ± 1°C.
7. *Infused products and concentrates,* ***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 test portion. Vortex or homogenize sample and TSB. Incubate for 16 - 24 h at 37 ± 2 °C.
8. If processing multiple samples, be sure to change gloves between each sample, to ensure there is no cross contamination between samples during the weighing process.

Figure 3: Homogenized cannabis flower and TSB.

Sample Lysis

MaGiC Lysis for Flower, Gummy Infused Products, and Extracts

1. Remove the Whirl Pak Bag, 15, or 50 mL tube from the incubator.
    a. Flower sample in Whirl Pak Bag: Mix thoroughly by hand manipulating and/or squeezing the contents for the flower bag for 1 minute.
    b. Non flower Infused Product in 15 or 50 mL tube: vortex sample thoroughly for 30 seconds.
2. Remove 10 µl of enriched sample from the bag or tube and put into the well of a 96 well PCR plate.

OPTIONAL Grim Reefer Free DNA removal

3. 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.
    b. Add 2 µl of Grim Reefer Enzyme to each sample.
    c. Pipette tip mix thoroughly 15 times.
    d. Seal plate, spin down in centrifuge, and incubate at 37 ºC for 10 minutes in a thermal cycler.
    e. Remove plate from thermal cycler and spin plate in plate centrifuge.
    f. Carefully remove the seal and add **4 µl** of Grim Reefer Deactivation Buffer to each sample. Pipette mix thoroughly 15 times.
4. Dilute Internal Cannabis Control (ICC) to 1:50,000 for all matrices **except chocolate** (see MaGiC lysis for chocolate below)

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

    a. Ensure stock ICC is fully thawed, vortexed and spun down before use.
    b. Dilute stock ICC 1:100.
        i. Add 2 µL of ICC to 198ul of Nuclease free Water. Close tube, vortex and quick spin.
    c. Dilute the 1:100 dilution of ICC to 1:10,000.

i. Add 2 µL of 1:100 ICC dilution to 198 µL of water. Close tube, vortex and quick spin.

d. Dilute 1:10,000 dilution of ICC to 1:50,000.

i. Add 20 µL of 1:10,000 ICC dilution to 80 µL of water. Close tube, vortex and quick spin.

5. Add 50 µL of MaGiC Lysis Buffer and pipette tip mix 15 times. Repeat for all wells being tested using a fresh tip for each transfer.
6. Transfer 5 µL of 1:50,000 diluted ICC control to the 60 µl of sample. Repeat for all sample wells. Be sure to use a fresh tip every time.
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. 95 °C for 10 minutes
    b. 4 °C for 10 minutes or "forever". If the thermal cycler does not go down to 4 °C set to 25 °C.

    Note: When using the Agilent AriaMX, a compression pad should be placed over the adhesive seal to prevent evaporation during thermal cycling.
9. Once the plate has been at 4 °C for 10 minutes or 25 °C for 10 minutes, remove it from the thermal cycler and spin the plate down to remove evaporation from the plate seal. Carefully remove the plate seal.
10. Add 100 µl of MaGiC Stabilization buffer to each sample well. Pipette mix 5 times.
11. Seal the plate with a plate seal and store in -20 °C freezer if not proceeding directly to qPCR setup.
    a. Samples with stabilization buffer added are stable at -20 ºC for 1 month.
    b. Extracted samples which have been frozen must be tip mixed 15 times prior to setting up qPCR or performing dilutions.
12. Proceed to qPCR

MaGiC Lysis for Chocolate Infused Product

1. Remove the 15 mL or 50 mL tube from the incubator and mix thoroughly by vortexing for 30 seconds.

2. Remove 10 µl of liquid from the bag or tube and put into the well of a 96 well PCR plate.

OPTIONAL Grim Reefer Free DNA removal

3. 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.
    b. Add 2 µl of Grim Reefer Enzyme to each sample.
    c. Pipette tip mix thoroughly 15 times.
    d. Seal plate, spin down in centrifuge, and incubate at 37 ºC for 10 minutes in a thermal cycler.
    e. Remove plate from thermal cycler and spin plate in plate centrifuge.
    f. Carefully remove the seal and add **4 µl** of Grim Reefer Deactivation Buffer to each sample. Pipette mix thoroughly 15 times.
4. Dilute ICC to 1:5,000.
    a. Dilute the stock ICC 1:50
        1. Add 2 µl stock ICC Control into 98 µl of nuclease free water, vortex well and quick spin.
    ii. Dilute the 1:50 dilution an additional 1:100.
        1. Add 2 µl of the 1:50 dilution to 198 µl of water, vortex and quick spin. This is a 1: 5,000 dilution.
5. Add 50 µL of MaGiC Lysis Buffer, and pipette tip mix 15 times.
6. Add 5 µL of 1:5,000 diluted ICC control. Do this for all wells being tested using a fresh tip every time.
7. Seal the top of the plate and spin down in the centrifuge.
8. Place plate on a thermal cycler and run MaGiC Lysis program
    a. 95 °C for 10 minutes
    b. 4 °C for 10 minutes or "forever". If the thermal cycler does not go down to 4 °C set to 25 °C.

    Note: When using the Agilent AriaMX, a compression pad should be placed over the adhesive seal to prevent evaporation during thermal cycling.

9. Once the plate has been at 4 °C for 10 minutes or 25 °C for 10 minutes, remove it from the thermal cycler and spin the plate down to remove evaporation from the plate seal. Carefully remove the plate seal.
10. Add 100 µl of MaGiC Stabilization buffer to each sample well. Pipette mix 5 times.
11. Seal the plate with a plate seal and store in -20 °C freezer if not proceeding directly to qPCR setup.
    a. Samples with stabilization buffer added are stable at -20 ºC for 1 month.
    b. Extracted samples which have been frozen must be tip mixed 15 times prior to setting up qPCR or performing dilutions.
12. Proceed to qPCR

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

1. Remove Amplification Mix, Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2, and assay positive control from the -20 ºC freezer.
    a. If Lyophilized Amplification Mix has not been rehydrated, rehydrate in 550 µl of Nuclease Free water. Swirl or Pipette mix. After resuspension, store at -15 to -20 °C when not in use.
    b. Allow all frozen reagents to defrost at room temperature. Once defrosted, place tubes on ice.
2. Before preparing the master mix, invert or vortex and spin down the reagents.
    a. Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 tube – Vortex quickly followed by a pulse spin down in a microcentrifuge.
    b. Assay Positive Control tube – Vortex quickly followed by a pulse spin down in a microcentrifuge.
    c. 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 contains the internal cannabis control probe mix). Label tube "Master Mix". Always prepare enough master mix for an additional 1 or 2 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. See Table 1 as a reference.

    Table 1: PathoSEEK Amplification Master Mix Reagent Volumes

Reagent	Volume for 1 Reaction
Amplification Mix	10 µL
S. aureus & P. aeruginosa Multiplex Detection Assay v2	1 µL
Nuclease Free Water	4 µL
Total	15 µL

    a. Once combined, cap tube and vortex to mix.
        i. Pulse spin down tube in microcentrifuge.
        ii. Place the Master Mix tube on ice until used.

4. Prepare a 1:10 dilution of the assay positive control
    a. Add 1 µL of Positive Control to 9 µL PCR Grade Water, vortex to mix well and spin down the tube.

       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.
5. For the negative control, use PCR Grade Water that was used to rehydrate your Amplification Mix.
6. Use a 96-well optical qPCR plate or optically clear qPCR tubes.
7. Transfer samples into qPCR plate or tubes
    a. Carefully remove the seal from the Lysis Plate.
        i. If lysed samples were frozen, let the DNA thaw completely and spin the plate to avoid cross contamination between samples. Tip mix thawed samples well before transferring to the qPCR plate or tubes.
    b. Transfer 5 µL of each sample 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 well or tube.
    d. Add 5 µL of water to the corresponding negative control well or tube.

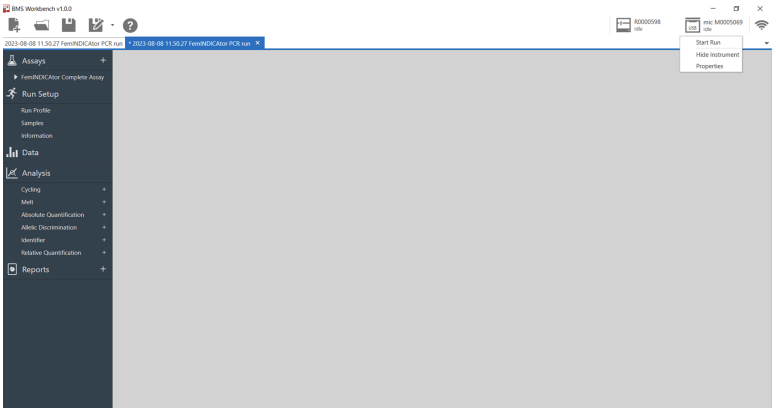
    Note: ALWAYS use a fresh tip for every liquid transfer into the qPCR plate
8. Add 15 µL of Master Mix to each corresponding sample well, positive control well, and negative control well in the qPCR plate or tubes. Gently tip mix a few times after each addition of qPCR master mix. Be careful not to introduce bubbles during this mix.
9. Seal the plate with strip caps or an adhesive seal or seal qPCR tubes with strip caps.
10. For the Agilent AriaMX or Bio-rad CFX, spin down qPCR plate or tubes for at least 1 minute in plate or tube microcentrifuge to bring well contents to the bottom of wells and help to get rid of reaction bubbles.

    Note: Check for bubbles in the wells (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the wells, *spin down* for another minute.

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 qPCR instrument.
13. Follow the software specific instructions to initiate the run.

Running the BioMolecular Systems Mic

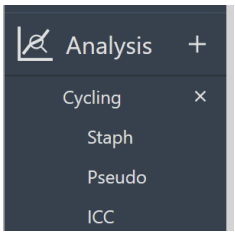
1. Open the BMS Workbench software and create a new file.
2. Select qPCR Run.
3. Select the appropriate template by clicking the "+" sign next to assays or ensure that the appropriate thermal cycling conditions are entered: Hot start at 95 ºC for 5 minutes, followed by 40 cycles of 95 ºC for 15 seconds 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.



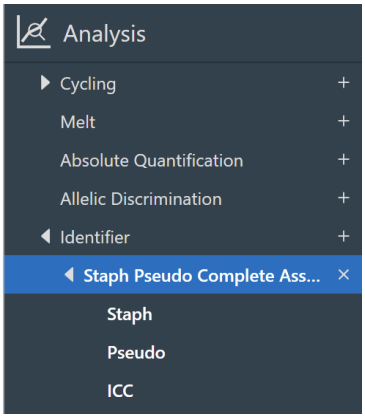
5. A pop-up will appear asking for the reaction volume, which will be auto-filled with the correct volume based on the template chosen. Close the lid and the instrument will start.
   Note: If the cover isn't closed, the program will not start.
6. Let the Mic run to completion before analyzing the data.

Data Analysis with BioMolecular System Mic

1. When the run has completed, click on the "+" sign next to the "Cycling" tab and select ICC (Internal Cannabis Control). The selection will appear under the "Cycling" tab. Next, select Staph and then Pseudo.

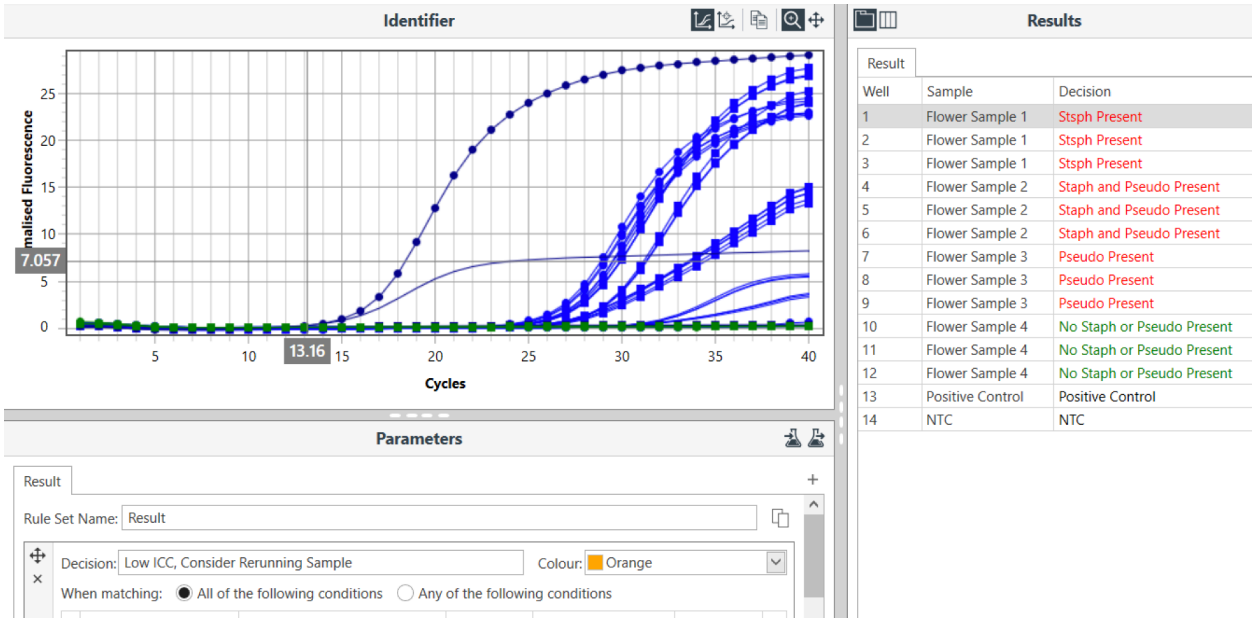


2. When these have been expanded, select the "+" sign next to the "Identifier" tab and select the available report.



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.



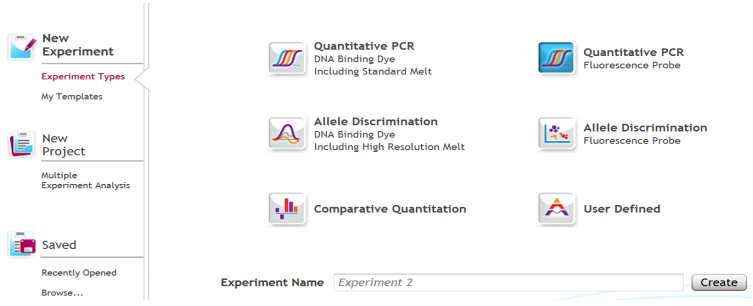
5. Data may be exported by selecting the "Report" tab, then clicking the Export icon.

Running the Agilent AriaMX

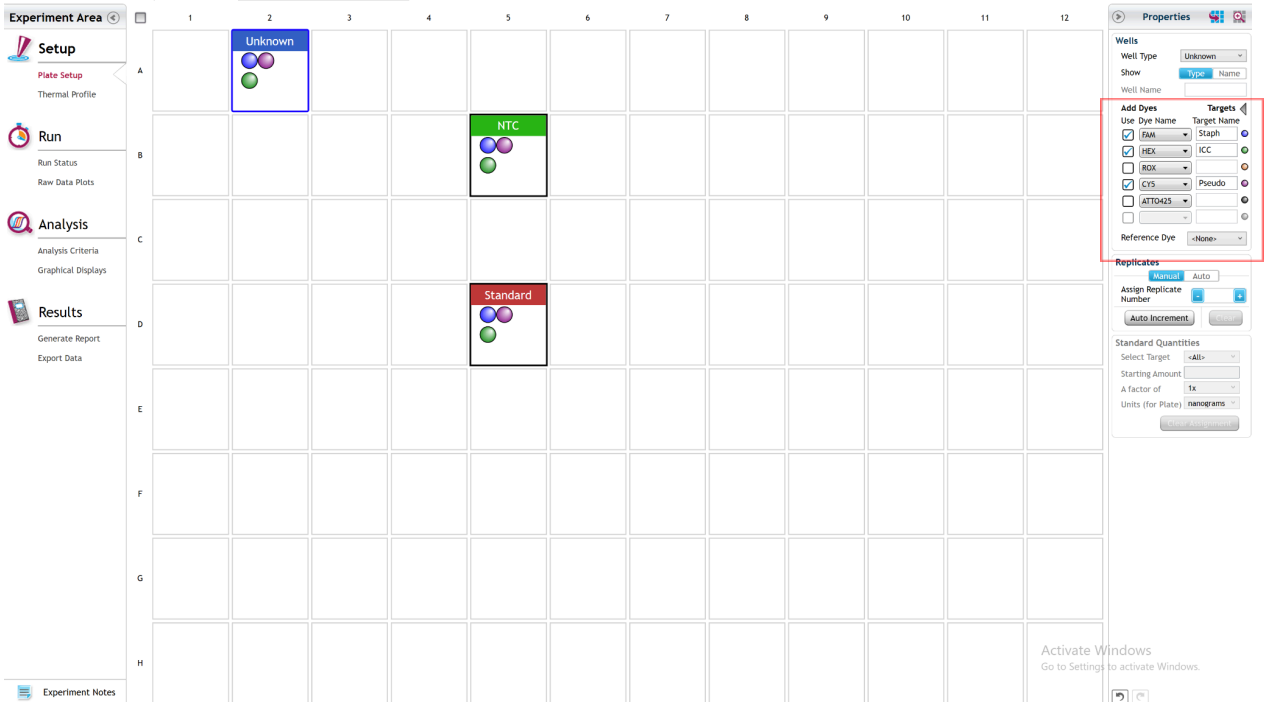
The following species will be detected on the following Fluorophores:

- S. aureus: FAM
- P. aeruginosa: Cy5
- Cannabis DNA: HEX

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



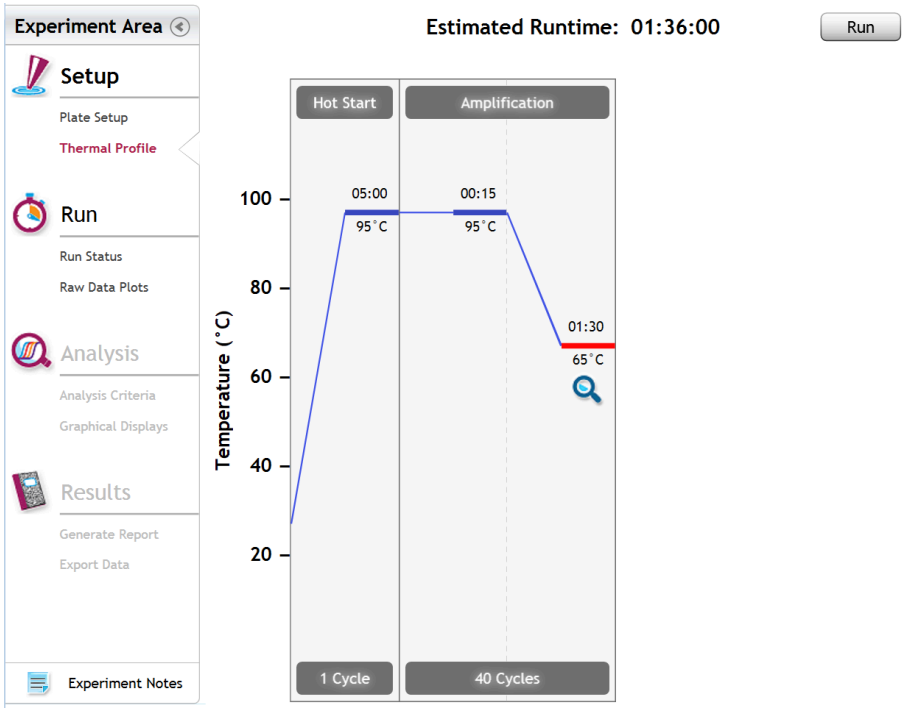
2. Under Setup > Plate Setup, highlight **only** wells that contain reactions and select FAM, Cy5, and HEX under **Add Dyes**. Having fluorescence detection turned on in empty wells can affect the calculations which the software makes to obtain the corrected data, ΔR log.



3. Change the well types to reflect your plate set up. All wells should be set to Unknown except the negative control can be set to NTC well type. Add Target names to the dyes under **Targets:**
    a. FAM: S. aureus
    b. Cy5: P. aeruginosa
    c. HEX: Internal Cannabis Control (ICC)

4. Under Setup > Thermal Profile, create the following PCR thermal profile

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



5. Close the lid and click “Start Run”

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

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

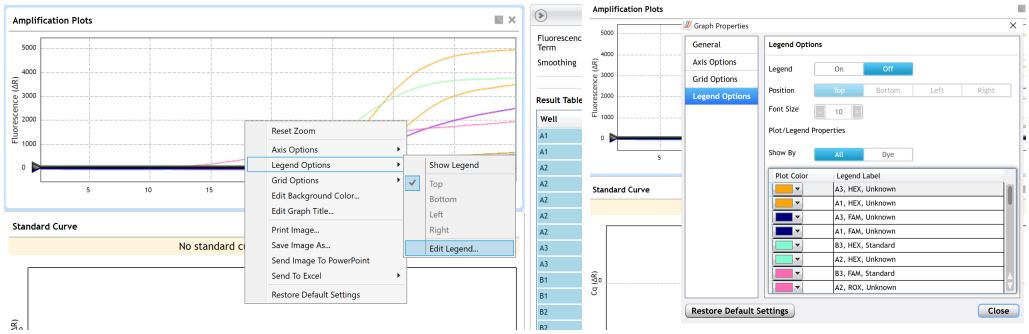
Data Analysis: Agilent AriaMX

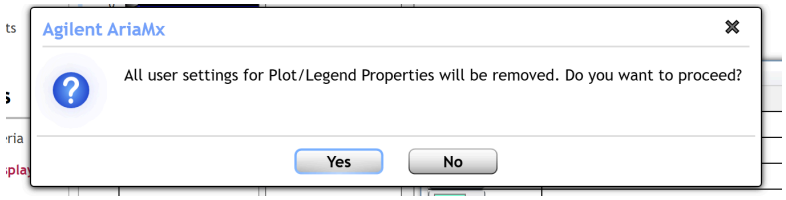
PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 - Data Analysis Quick Reference Table 3: All Matrices

PathoSEEK® Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Staphylococcus aureus	≤ 40	FAM	No Cq	Presence/Absence
Pseudomonas aeruginosa	≤ 40	Cy5	No Cq	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Control	≤35	FAM and Cy5		

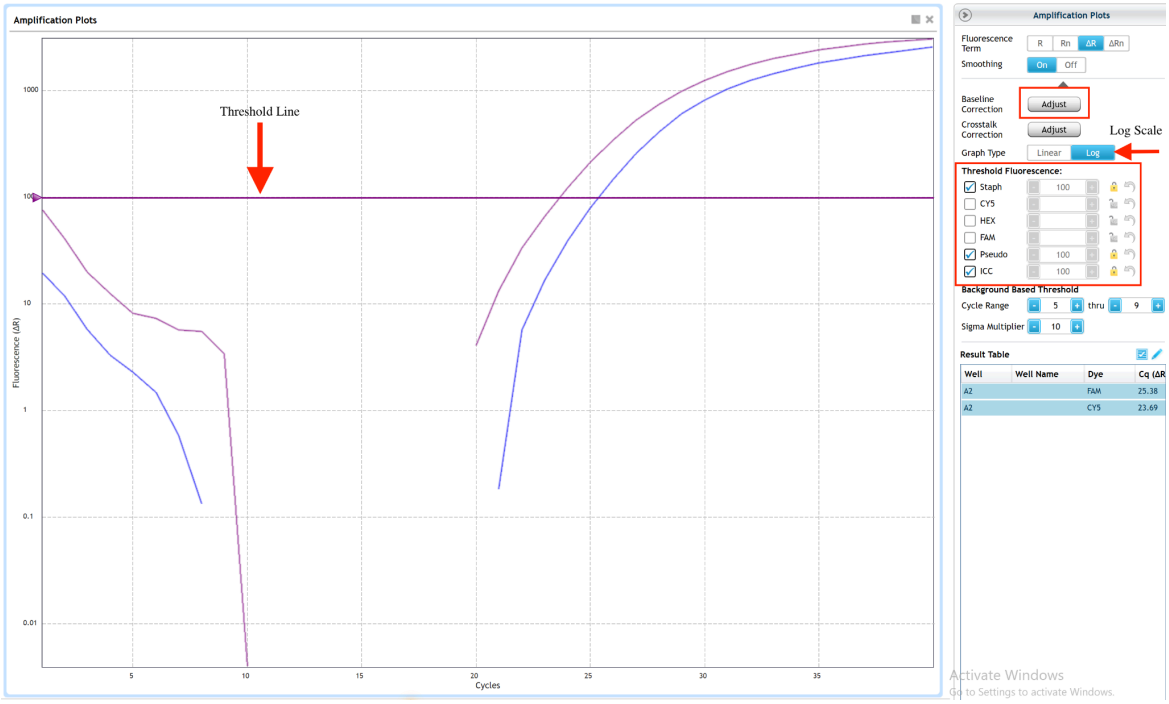
1. Open the Data Analysis window when the run is complete.

2. Highlight the wells of interest in the Analysis Criteria under Analysis, then select Graphical Display
    a. Amplification plots will be available for viewing
    b. The Cq values will appear to the right in the table
    c. Right click inside the graph, select Edit Legend under Legend Options
    d. Change “All” to “Dye”
    e. All user settings for Plot/Legend Properties will be removed. Do you want to proceed? Select “Yes”.
    f. This will assign a single color to each fluorophore.





3. To analyze the results
    a. Start by turning the graph to Log Scale 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 thresholds to 100 RFU for the HEX, FAM, and Cy5 fluorophores. (which will have the target name because you've added them in the plate setup.) Click the lock next to each target to prevent future changes.

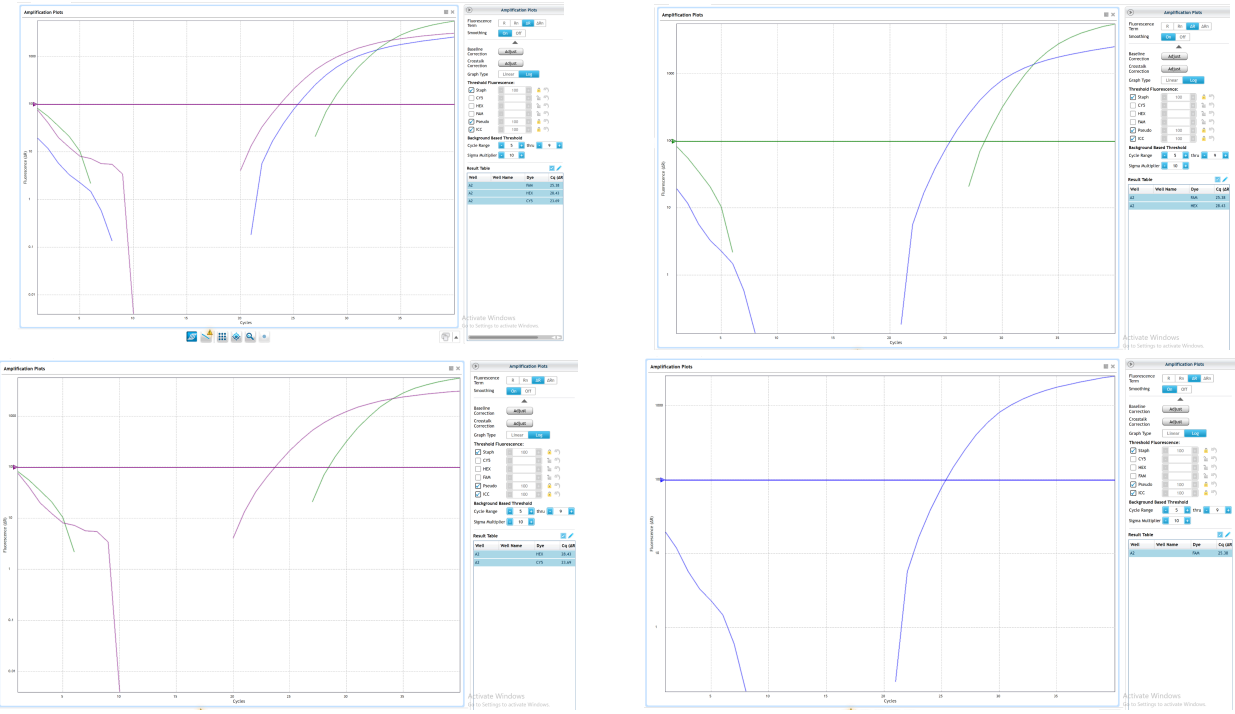


4. Controls
    a. Positive Control, on the FAM and Cy5 Fluorophores, have Cq value ≤ 35.
        i. Visually confirm with the curves on the graph.
    b. Negative Control, on the FAM and Cy5 Fluorophores, have no Cq Value.
        i. Visually confirm with the curves on the graph.

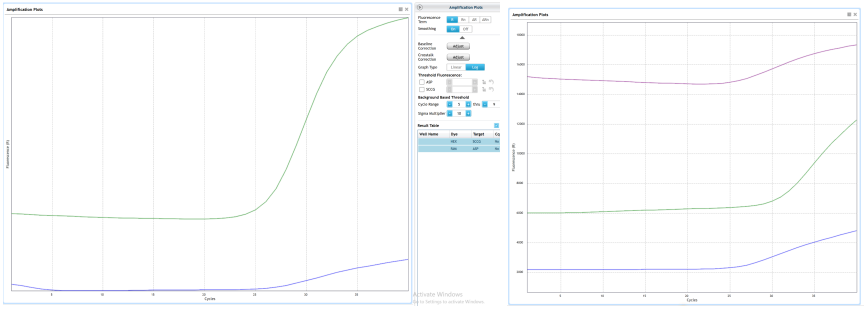
5. Unknown P. aeruginosa and/or S. aureus Targets

    a. A "presence" or failing result for the unknown P. aeruginosa and/or S. aureus target(s).

i. Any Cq value for the FAM or Cy5 Fluorophore ≤ 40.



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.



iii. 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.

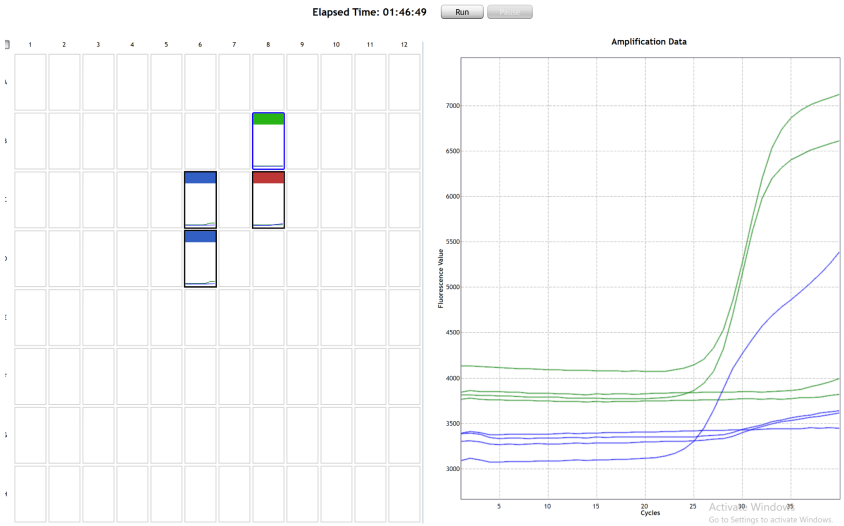


Figure 7: Raw data (R) plots for which the cycle quantification (Cq) will be generated.

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

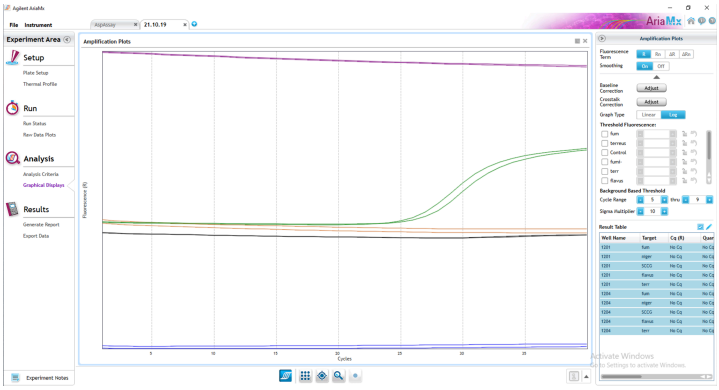
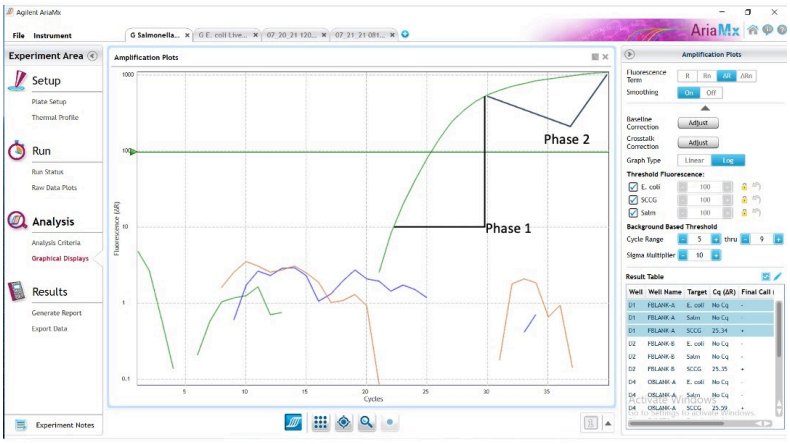


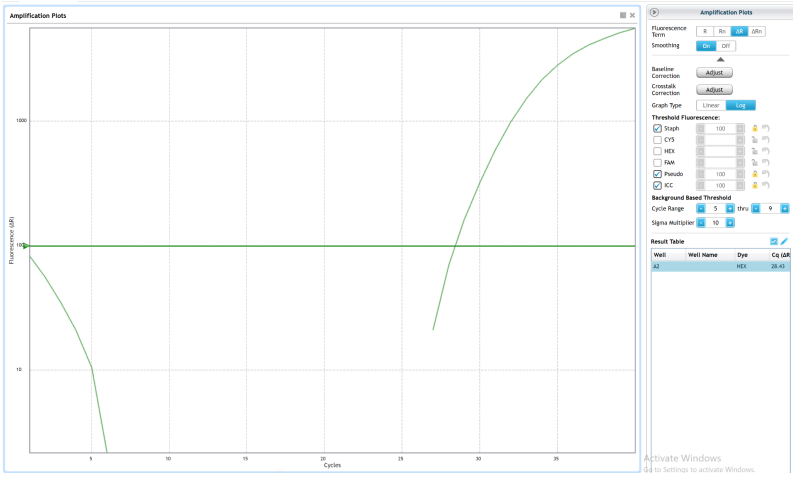
Figure: Example of good baseline with sigmoidal shaped curve increase in the internal control fluorescence.

v. Check the amplification plots for a semi-logarithmic curve with two distinct phases that crosses the threshold:

1. 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.
2. Phase 1 shows exponential growth in the fluorescence, in a span of around 5 cycles.
3. Phase 2 is a plateau where the amplification signal growth ends but remains level.



b. An “absence” or passing result for the unknown P. aeruginosa and/or S. aureus targets.
    i. No Cq value for the FAM or Cy5 Fluorophores
        1. Visually confirm that there are no curves on the graph.
        2. Cq of <35 for the internal control on the HEX fluorophore.
            a. Visually confirm the curve on the graph.



Running the BioRad CFX96

The following targets will be detected on the following fluorophores:

- S. aureus: FAM
- P. aeruginosa: CY5

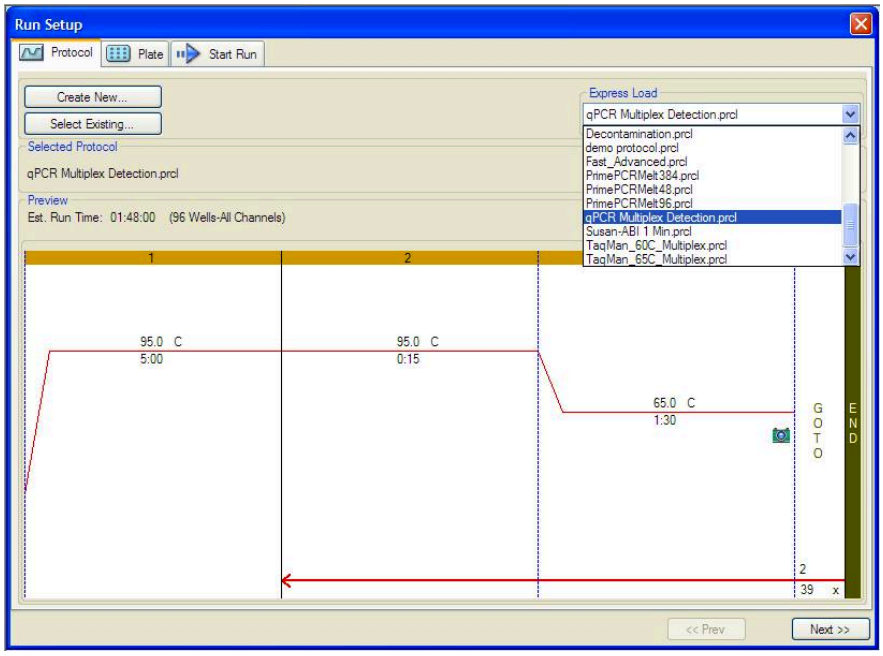
- Cannabis DNA: HEX

1. Start the qPCR Cycling program

    a. Select User-Defined in the Startup Wizard under Run setup

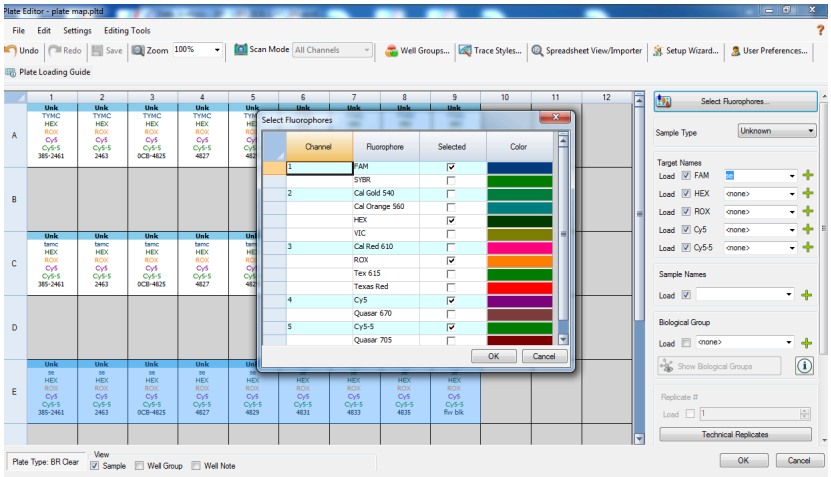
2. Use the Express Load dropdown menu to pick the qPCR Multiplex Detection Program and click "Next".
    a. If not already pre-programmed, create a cycling program with the following specifications and save as "qPCR Multiplex Detection"

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

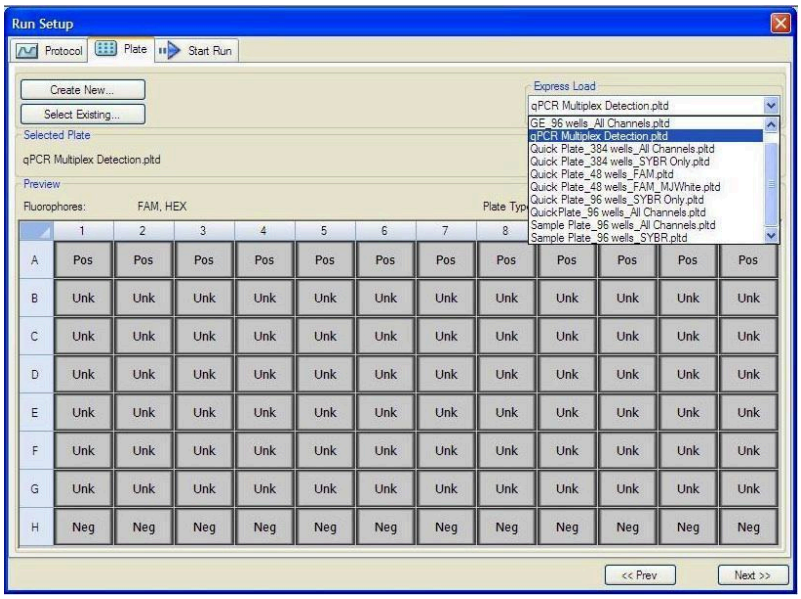


3. Design your plate under the plate tab in the Run Setup
    a. Select the qPCR Multiplex Detection from the dropdown menu. If not already present, click "Create New"

    b. Plate editor window will appear. Choose FAM, CY5, and HEX Fluorophores and click "OK".



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

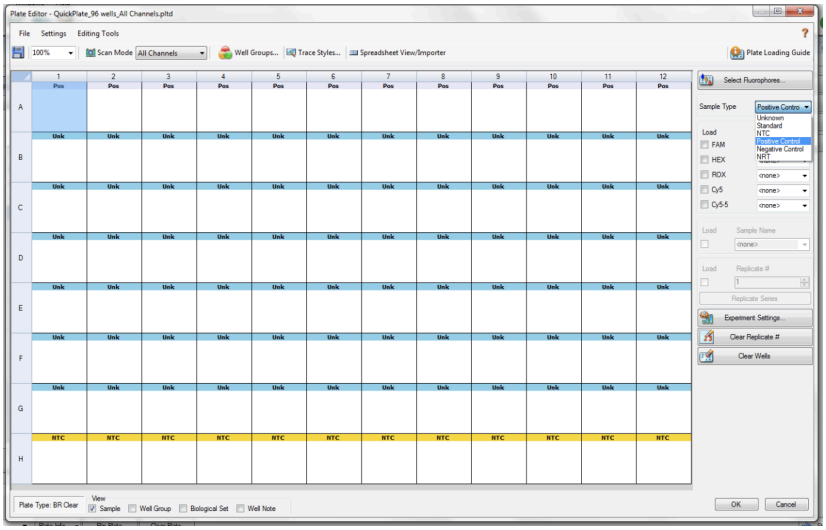


d. On the Plate Editor Screen, change the Sample Type to correlate with your specific plate setup.

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.



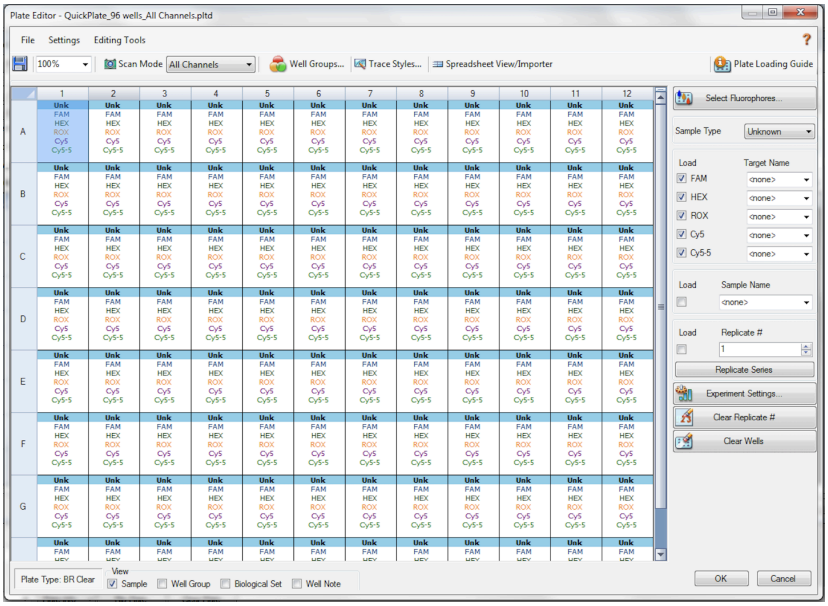
f. Attach the fluorophores to the wells being used.

i. Highlight all the wells being used

ii. For P. aeruginosa and S. aureus Assay, **Highlight the well locations and click on FAM, CY5, and HEX**

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

h. Click "yes" to save your plate. If creating plate layout for the first time, save as "qPCR Multiplex Detection". If you do not save the plate, it will return to the default plate.



Note: Saving will override the template which is acceptable..

i. Close the lid and click Start Run.

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

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

Data Analysis BioRad CFX96

PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 Quick Reference Table:

Table 4: All Matrices

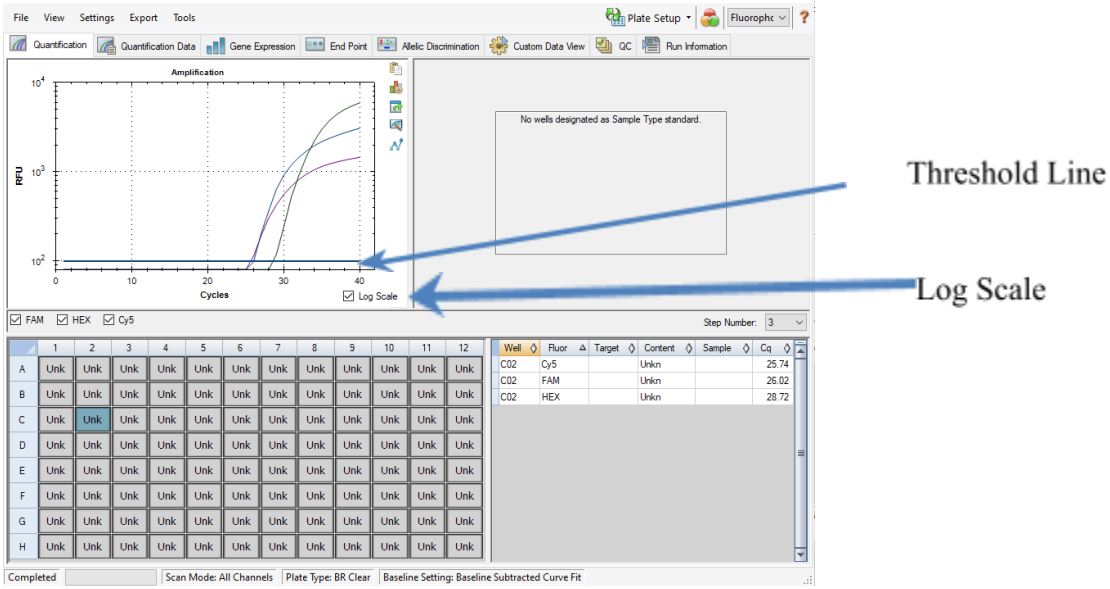
PathoSEEK® Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Staphylococcus aureus	≤ 40	FAM	No Cq	Presence/Absence
Pseudomonas aeruginosa	≤ 40	Cy5	No Cq	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Control	≤35	FAM and Cy5		

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.
    b. To turn the graph to Log Scale, click on the box at the bottom right of the graph.
    c. To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis. Alternatively, set an exact threshold value by selecting a single fluorophore at a time beneath the graph, then Settings > Baseline Threshold. In the next window select User Defined and enter 100.



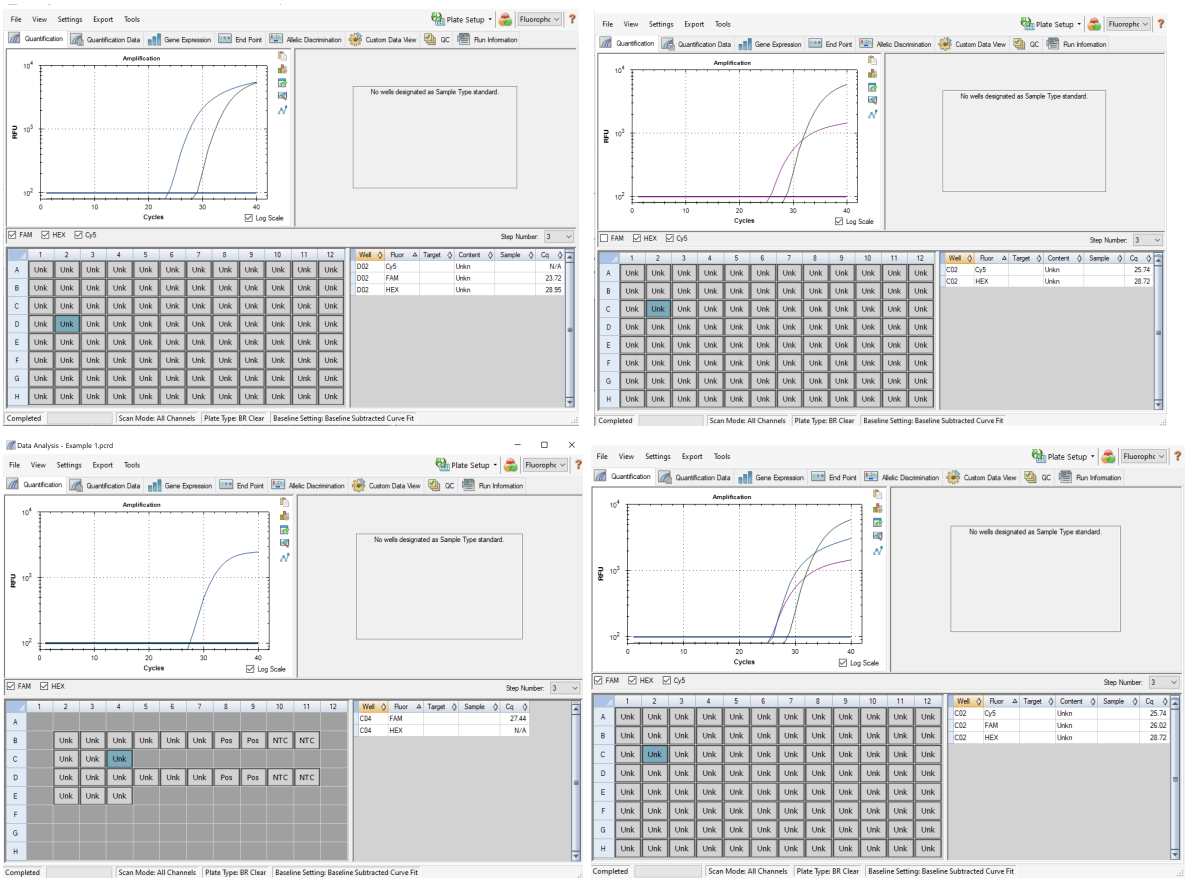
d. Controls

   i. Assay-specific Positive Control, on the FAM and Cy5 fluorophores, have a Cq value ≤ 35.
      1. Visually confirm with the curve on the graph.

   ii. Assay-specific Negative Control, on the FAM and Cy5 fluorophores, have no Cq value.
      1. Visually confirm with the curve on the graph.

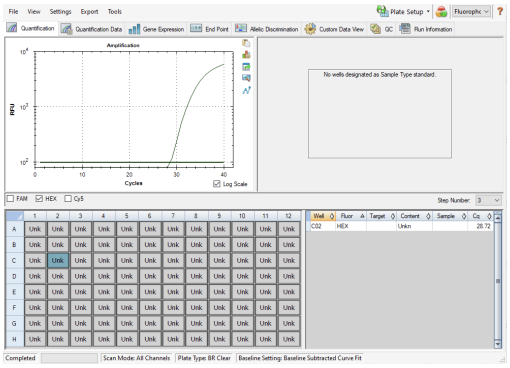
e. Unknown P. aeruginosa and S. aureus Targets: The following fluorophore detects P. aeruginosa or S. aureus:

   i. S. aureus: FAM
   ii. P. aeruginosa: Cy5
   iii. A "presence" or failing result for the unknown P. aeruginosa and/or S. aureus.
      1. Any Cq value for the FAM or Cy5 fluorophore ≤ 40.
      2. 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.



iv. An “absence” or passing result for the unknown P. aeruginosa and/or S. aureus targets.

1. No Cq value for the FAM or Cy5 fluorophores.
2. Visually confirm no curve on the graph.
3. Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, < 40 for all other matrices.
4. Visually confirm with the curve on the graph



Troubleshooting Guide

Symptom	Reason	Solution
Internal control failure	ICC 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 ICC spike not performed for flower samples for Aspergillus Assays	The ICC 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 1:10 with nuclease free water and re-perform qPCR
Internal Control Positive result on assay positive or negative control reactions or reactions containing samples that do not contain plant DNA No Cq or a Cq of more than 35 is acceptable. Any Cq lower than 35 constitutes a rerun	Cannabis DNA or ICC 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 Cannabis Control** is expected in every reaction containing cannabis DNA. It ensures the DNA isolation procedure was successful. The internal control targets cannabis 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	December 2024	Launch User Guide for PathoSEEK® Pseudomonas aeruginosa & Staphylococcus aureus Multiplex Detection Assay v2 (speciation) and MaGiC Lysis
v2	January 2025	Amplification Mix packaging update

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.

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