Validation of the PathoSEEK® Salmonella and STEC E. coli Multiplex Assay using the SenSATIVAx® Extraction Kits for the Detection of *Salmonella* and/or Shiga-toxin producing *Escherichia coli* in Dried Cannabis Flower, Cannabis Concentrate and THC-Infused Chocolate

AOAC *Performance Tested Method*℠ 022202

Kevin McKernan and Yvonne Helbert

100 Cummings Center, Suite 406L, Beverly, MA 01915

Yvonne.helbert@medicinalgenomics.com

Abstract:

Background: The PathoSEEK® Salmonella and STEC E. coli Multiplex Assay with SenSATIVAx® Extraction Kits are designed to simultaneously detect *Salmonella* spp. and *Escherichia coli* carrying Shiga toxin 1 and/or 2 (*stx1* and/or *stx2*) in a single qPCR reaction.

Objective: To evaluate the candidate method according to AOAC validation requirements (*Official Methods of Analysis*℠ Appendix J) and *Standard Method Performance Requirements*℠ 2020.002 and 2020.012.

Methods: Dried cannabis flower (delta 9-tetrahydrocannabinol (THC) >0.3%; 10 g), cannabis concentrate (5 g) and THC-infused chocolate (25 g) were evaluated at three contamination levels: 20 replicates at a low-level of ~1-2 CFU/test portion, five replicates at a high-level of ~20 CFU/test portion, and five replicates at an un-inoculated control level of 0 CFU/test portion. Testing was performed on two thermal cyclers: CFX-96 and AriaMx. Two extraction protocols, SenSATIVAx for Flower/Leaf and SenSATIVAx for MIP/Extracts were used, dependent on

matrix type. Additional PTM testing requirements (inclusivity and exclusivity, robustness, and product consistency and stability) were evaluated.

Results: Data analysis using the probability of detection statistical model indicated no statistically significant difference between presumptive and confirmed results for the candidate method for each matrix with both thermocyclers. Results of robustness demonstrated that the assay was not impacted by small variations in the method. Inclusivity and exclusivity testing demonstrated the method was highly specific for *Salmonella* spp. and STEC organisms and could discriminate them from non-target organisms. Product consistency data indicated manufacturing conditions of critical reagents was consistent.

Conclusion: The PathoSEEK Salmonella and STEC E. coli Multiplex Assays with SenSATIVAx Extraction Protocol allows for a multiplex qPCR approach for the simultaneous detection of *Salmonella* spp. and STEC in select cannabis matrices.

Highlights: Using Tryptic Soy Broth, end users can now screen for the presence of *Aspergillus, Salmonella* spp. and STEC in cannabis products using a single enrichment and this assay and *Performance Tested Method*[SM] 082102.

Principle of the Method

The PathoSEEK Microbial Safety Testing Platform utilizes a novel, contamination-free, PCR-based assay and provides an internal plant DNA control for every reaction. It is a two-step protocol (DNA extraction followed by RT-PCR analysis) which is flexible and automation compatible. The PathoSEEK assay is used in combination with the SenSATIVAx extraction kit and qPCR Master Kit. The SenSATIVAx extraction kit allows for a proprietary DNA isolation process that use magnetic particles to isolate and purify both plant and microbial DNA.

The PathoSEEK Salmonella & STEC E. coli Multiplex Assay is used to detect a wide variety of species classified as *Salmonella* and STEC. The assay targets STEC using the FAM fluorophore and *Salmonella* using the ROX fluorophore. Positive controls for each target are recommended for use with each analysis.

General Information

In the cannabis industry, states establish microbial regulations which often vary. As a result, microbial testing in one state may not meet the requirements in other states [1]. In 2019, AOAC INTERNATIONAL launched the Cannabis Analytical Science Program (CASP) to standardized method validation within the cannabis industry [2]. Within CASP two *Standard Method Performance Requirements*SM (SMPR) were approved in 2020 that provide acceptance criteria for evaluating candidate methods designed to detect *Salmonella* spp.(SMPR 2020.002) and STEC (2020.012) [3,4]. Using the SMPRs, industry now has a standardized approach to validating rapid alternative methods for *Salmonella* and STEC is cannabis flower, concentrates and infused products.

Scope of Methods

(a) *Target organisms*.—*Salmonella* spp. and *Escherichia coli* carrying Shiga toxin 1 and/or 2 (*stx1* and/or *stx2*).

(b) *Matrixes*.—Dried cannabis flower (delta 9-tetrahydrocannabinol >0.3%;10 g), cannabis concentrate (5 g) and THC-infused chocolate bars (25 g).

(c) *Summary of Validated Performance Claims*.— The PathoSEEK Salmonella & STEC E.coli Multiplex Assay with SenSATIVAx® Extraction has been validated according to the

*AOAC Performance Tested Method*SM Program for the detection of Salmonella species STEC in dried cannabis flower (>0.3% THC), cannabis concentrates and THC-infused chocolate using the AriaMx and CFX-96 instruments. The validation study met the requirements as set forth in the AOAC Standard Method Performance RequirementsSM 2020.002 for the detection of Salmonella species and 2020.012 for the detection of Shiga toxin producing E.coli (STEC) in cannabis and cannabis infused products.

Definitions

(a) *Probability of Detection*.—Probability of Detection (POD) is the proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent. There are several POD measures that can be calculated, e.g., POD_{CP} (candidate method presumptive result POD) and POD_{CC} (candidate method confirmation result POD) and $dPOD_{CP}$, the difference between the two POD values.

(b) *qPCR*.—Quantitative polymerase chain reaction (qPCR) is a technology used for measuring the amplification of DNA during the PCR process.

Materials and Methods

Test Kit Name: PathoSEEK Salmonella and STEC E. coli Multiplex Assay with SenSATIVAx Extraction Protocol

Test Kit Components

(a) *PathoSEEK Salmonella and STEC E. coli Multiplex Assay*

(1) MGC P/N 420120.

i. *Assay* — 1 tube. Store kit at -15 to -20°C. Expires 2 Years from Date of Manufacture.

(b) *SenSATIVAx Flower/Leaf DNA Extraction Kit*.

(1) MGC P/N 420001.

i. *MGC Lysis Buffer*— 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture

ii. *MGC Binding Buffer*—1 bottle. Store at 2-8°C. Expires 1 Year from Date of Manufacture

iii. *MGC Elution Buffer*—1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture

(c) *SenSATIVAx MIP/Extract DNA Extraction Kit.*

(1) MGC P/N 420004.

i. *Binding Buffer* — 1 bottle. Store at 2-8°C. Expires 1 Year from Date of Manufacture

ii. *Elution Buffer* — 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture

iii. *Solution A* — 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture

iv. *Solution B* — 1 bottle. Store at 20-28°C. Expires 1 Year from Date of Manufacture

(d) *PathoSEEK Salmonella & STEC E. coli Multiplex Positive Control*—P/N 420322 (60 reactions)

(1) Control - 1 tube. Store at -15 to -20°C. Expires 2 Years from Date of Manufacture

(e) *Medicinal Genomics qPCR Master Kit v3*—P/N 420201. 1 bottle. Store at -15 to -20°C.

(1) Reaction Buffer (10x) — 1 tube

(2) Nuclease Free Water— 2 tubes

(3) qPCR Master Mix — 1 tube

Supplies, Reagents, and Equipment

(a) *Agilent AriaMx Real-Time PCR System G8830A Option 010.*—Containing the following Optical Channels: FAM, ROX, HEX, Cy5 and ATTO 425.

(1) Agilent HP Notebook PC option 650.

(2) 96 Well Optical qPCR plate.— Agilent #401490 or Fisher Scientific #AB2396

(3) Adhesive optical seal for qPCR plates.— Agilent #401492; USA Scientific TempPlate RT Optical Film #2978-2100.

(4) Optical Strip Caps.—Agilent #401425.

Note: If using adhesive seals instead of strip caps, use Applied Biosystems MicroAmp Optical Film Compression Pad, Fisher Scientific, #43-126-39 to prevent evaporation and cross contamination between wells.

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

(1) Bio-Rad Personal PC.

(2) 96 Well Optical qPCR plate.— Bio-Rad #HSP-96601 or Fisher Scientific #AB2396

(3) Adhesive optical seal for qPCR plates.— Bio-Rad #MSB-1001; USA Scientific TempPlate RT Optical Film #2978-2100.

(c) *Adjustable, variable volume pipettes (single or multichannel).*—P10, P20, P50, P200 P300 and P1000.

(d) *Adjustable, variable volume filter pipettes tips.*—For P10, P20, P50, P200, P300 and P1000.

(e) *Crushed ice.*

(f) *96 Well PCR Cryogenic Rack.*—VWR #89004-570.

(g) *1.5 µL Tube Benchtop Cryogenic Rack.*— VWR #89004-558 or equivalent.

(h) *Freezer*.—Capable of maintaining -20°C.

(i) *Table Top Mini Plate Centrifuge.*—Fisher Scientific #14-100-143 or equivalent.

(j) *Table Top Mini Centrifuge.*—VWR #10067-588, #2631-0006 or equivalent.

(k) *Vortex-Genie Pulse.*—Scientific Industries, SKU: SI-0236 or equivalent.

(l) *High Speed centrifuge.*— to accommodate 1.5mL tubes such as Eppendorf model 5414R or similar with ability to spin up to speeds of 15,000 rcf

(m)*Filter Bags.*—Whirl Pak #B01385WA

(n) *Beaker or Solo Cup.* (optional)

(o) *Tryptic Soy Broth.*—MGC P/N 420205. Store at 2-8°C.

(p) *1.5 mL Eppendorf Tubes.*

(q) *15 mL or 50 mL conical tubes.*

(r) *96 Well Plate Magnet*—MGC P/N 420202

(s) *96 Well Extraction Plate.*— Perkin Elmer P/N 6008290

(t) *Eppendorf Tube Rack.*

(u) *Scientific Scale*—Capable of measuring to milligram.

(v) *Refrigerator*—Capable of maintaining 2–8°C.

(w)*Incubator*.—Capable of maintaining 37 ± 2°C, VWR #97025-630 or equivalent.

(x) *25mL Serological Pipette*—VWR 89130-890 or 89130-900 or equivalent.

(y) *10% bleach*.

(z) *70% Ethanol*.— MGC P/N 420030

Safety Precautions

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

(b) Assay users should observe standard microbiological practices and safety precautions when performing this assay. Wear protective gloves, lab coats, eye/face protection as indicated by your quality system.

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

(d) Hazard Statement: Chloroform

(1) Harmful if inhaled or swallowed.

(2) Do not breathe vapor or mist. Do not ingest. Avoid contact with eyes, skin and clothing. Use only with adequate ventilation, which may require a chemical fume hood.

(3) Keep container tightly closed and sealed until ready for use. Wash thoroughly after handling.

(4) Please refer to the Safety Data Sheet (SDS) for more information and proper disposal

(e) Hazard Statement: 70% Ethanol

(1) Highly flammable liquid and vapor May cause respiratory irritation

(2) May cause drowsiness or dizziness Causes damage to organs

(3) May cause damage to organs through prolonged or repeated exposure

(4) Please refer to the Safety Data Sheet (SDS) for more information and proper disposal

Sample Preparation

(a) Aliquot Tryptic Soy Broth (TSB).

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

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

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

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

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

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

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

(h) *Dried cannabis flower (>0.3% THC),* ***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. For the PTM validation, 10 g of matrix was enriched with 90 mL TSB and tested at both 16 and 24 h.

(i) *Cannabis concentrate and THC-infused chocolate,* ***n*** *grams.* — Weigh concentrate into a 15 mL conical tube, 50 mL conical tube or Whirl-Pak bag depending on matrix volume. Add 2.4 x *n* mL of TSB to each test portion. Vortex or homogenize sample and TSB. Incubate for 16 - 24 h at 37 ± 1°C. For the PTM validation, 5 g of cannabis concentrate and 25 g of THC-infused chocolate were enriched with 12 mL TSB and 60 mL TSB, respectively, and tested at both 16 and 24 h..

(j) If processing multiple samples, be sure to change gloves between each, to ensure there is no cross contamination of samples during the weighing process.

DNA Extraction – SenSATIVAx Flower/Leaf DNA Extraction

Note* This protocol was used for the analysis of dried cannabis flower (>0.3% THC) in the PTM matrix study.

(a) Aspirate **1 mL** from side of the filter bag, free of plant debris, and dispense into the 1.5mL tube.

(b) Add 50 µL of MGC Lysis buffer and vortex for 10 seconds then let incubate on the bench for 2 minutes.

(c) After 2 minute incubation, spin for at least 1-3 minutes in a bench top mini centrifuge.

Note: The supernatant should be translucent at this point. If the sample is still opaque (cloudy) spin for longer. This is important for removing cellular debris.

(d) Remove the 200 µL of supernatant from the 1.5ml tube containing the centrifuged sample, being careful not to disturb the pellet at the bottom of the tube. Place the 200 µL in a labeled 96 well extraction plate labeled with Extraction Plate Day1 [date]"

Note: Pellet size will vary depending on trichome density.

(e) Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer at least 30 seconds.

(f) Add 200 µL of MGC Binding Buffer to each sample, and pipette tip mix 15 times.

Note: Be careful to avoid adding too many bubbles by pipetting gently when tip mixing. This is extremely important as to not contaminate the wells in proximity.

(g) Incubate the plate on the bench for 5 - 10 minutes.

(h) Place the extraction plate onto the 96 well plate magnet plate for 5 - 10 minutes.

(i) After the incubation on the magnet, remove as much of the 400 µL of the supernatant as possible. Be careful not to disturb or aspirate the beads.

(1) Add 400 µL of 70% ethanol (EtOH) with the extraction plate still on the magnet plate.

(2) Wait at least 30 seconds and remove all the EtOH.

Note: Place the pipet tip at the bottom center of the well to remove all liquid.

(j) Repeat 400 µL 70% EtOH wash with the extraction plate still on the magnet plate. Wait at least 30 seconds and remove all the EtOH.

Note: If EtOH still remains in the wells, go back in with a smaller pipet tip to remove the excess. Leftover EtOH can inhibit qPCR efficiency.

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

Note: It is important to NOT allow the beads to dry for an extended period of time. Over-drying can cause a reduction in DNA yield.

(l) Remove the extraction plate from the magnet plate and add 50 µL of MGC Elution Buffer.

(1) Tip mix approximately 15 times or until the beads are completely re-suspended.

Note: The re-suspensions may appear varied in their appearance, but the result will be the same.

(2) Incubate the plate for at least 1 minute on the bench, before returning the plate to the magnetic plate.

(3) Let the plate sit on the magnet for at least 1 minute before transferring the eluant to a new extraction plate labeled with "Final Extract [date]".

(m) Seal the plate with the adhesive seal, making sure to completely seal the plate wells using a pen or flat object to slide back and forth along the seal. Store at -20°C until ready to perform qPCR protocol.

DNA Extraction – SenSATIVAx MIP/Extracts

Note* This protocol was used for the analysis of cannabis concentrate and THC-infused chocolate in the PTM matrix study.

a) Prepare a SCCG positive control dilution of 1:5,000 (internal control)

(1) Label a new 1.5 mL Eppendorf tube (SCCG 1:50), add 1 µL of SCCG positive control into 49 µl of dH2O. Vortex to mix thoroughly and quick spin tube. Label another 1.5 mL Eppendorf tube (SCCG 1:5,000), add 99 µl of dH2O, then add 1 µl of the SCCG 1:50 dilution. Vortex to mix thoroughly and quick spin tube. This will result in a 1:5,000 dilution of SCCG.

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

(2) Place on ice until use.

Note: The 100 µL dilution prepared in step 6a is enough diluted SCCG for approximately 20 extractions. If more extractions are going to be prepared at the same time, the initial 1:50 dilution can be used to make multiple 1:5,000 dilutions of SCCG.

b) Add initial sample weight (*n*) x 4.6 mL SenSATIVAx Solution A to conical tube with enriched sample. Vortex the sample vigorously until homogenized.

c) Transfer 1 mL of the homogenized sample into a 1.5 mL tube.

d) Add 10 µL of the SCCG internal control (1:5,000) to 1.5 mL tube and vortex to mix well.

e) Centrifuge for 10 minutes at 15,000 rcf using a high-speed bench top centrifuge.

(1) If no bench top centrifuge is available, centrifuge for 15 minutes using a mini centrifuge.

Note: Some matrices will require the use of a high-speed centrifuge due to the presence of certain substances such as gelatin that hinder phase separation.

f) Transfer 600 µl of the solution to a new tube. Push pipet tip through the top solid layer (if one exists), without disturbing the pellet on the bottom to aspirate the sample.

g) Add 600 µL chloroform and vortex vigorously until solution turns a milky white color throughout

Note: This may require longer vortexing for some matrices

Caution: ALWAYS WEAR GLOVES WHEN HANDLING CHLOROFORM

h) Centrifuge for 5 minutes at 15,000 rcf using a bench top centrifuge

i) If no bench top centrifuge is available, centrifuge for 15 minutes using a mini centrifuge.

NOTE: If there is still any color in your aqueous layer (top layer) after centrifugation another chloroform wash may help give you a strong internal control signal (HEX) for every assay. Transfer 300 µL of the top layer to a new 1.5 mL tube and add 300 µL chloroform, vortex and centrifuge again.

j) Transfer 100 µL of aqueous layer (TOP LAYER) from Step 7 to a well of the labeled 96 well extraction plate. Be careful not to disturb the lower chloroform layer.

k) Add 100 µL of SenSATIVAx Solution B to the 100ul sample in the 96 well extraction plate.

l) Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in buffer for at least 30 seconds.

m) Add 200 µL of MGC Binding Buffer to each sample, and pipette tip mix 15 times.

Note: Be careful to avoid adding too many bubbles by pipetting gently when tip mixing. This is extremely important as to not contaminate the wells in proximity.

n) Incubate the plate on the bench for 5 - 10 minutes.

o) Place the extraction plate onto the 96 well plate magnet plate for5 - 10 minutes.

p) After the 5 min incubation, remove as much of the 400 µL of the supernatant as possible. Be careful not to disturb or aspirate the beads.

(1) Add 400 µL of 70% ethanol (EtOH) with the extraction plate still on the magnet plate.

(2) Wait at least 30 seconds and remove all the EtOH.

Note: Place the pipet tip at the bottom center of the well to remove all liquid.

q) Repeat 400 µL 70% EtOH wash with the extraction plate still on the magnet plate. Wait at least 30 seconds and remove all the EtOH.

Note: If EtOH still remains in the wells, go back in with a smaller pipet tip to remove the excess. Leftover EtOH can inhibit qPCR efficiency.

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

Note: It is important to NOT allow the beads to dry for an extended period of time. Over-drying can cause a reduction in DNA yield.

s) Remove the extraction plate from the magnet plate and add 50 µL of MGC Elution Buffer.

(1) Tip mix approximately 15 times or until the beads are completely re-suspended.

Note: The re-suspensions may appear varied in their appearance, but the result will be the same.

(2) Incubate the plate for at least 1 minute on the bench, before returning the plate to the magnetic plate.

(3) Let the plate sit on the magnet for at least 1 minute before transferring the

t) Seal the plate with the adhesive seal, making sure to completely seal the plate wells using a pen or flat object to slide back and forth along the seal. Store at -20°C until ready to perform qPCR protocol.

Real-Time Quantitative PCR (qPCR) Setup Protocol

a) Remove qPCR reagents including qPCR Master Mix, water, reaction buffer and assay probe mixes to be used from the -20ºC freezer. Place qPCR master mix on ice or leave at -20°C until ready to use. Allow remaining tubes to thaw at room temperature. Once thawed, immediately place tubes on ice.

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

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

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

(3) Return all reagents to the ice.

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

c) Make a separate master mix in a 1.5mL tube for each assay type being run. All probe mixes contain the internal plant control, SCCG probe mix, and the probe for the microbial targets. Label each tube with [Assay Name] MM. Always prepare enough master mix for 1

or 2 additional reactions over the total number of tests to account for pipetting and dead volumes.

Note: *It is best to add the largest volume reagent first, in this case water.*

Table 1: PCR Reagent Volumes

Reagents	1 Reaction	24 Reactions (Plus 1 excess rxn)	48 Reactions (Plus 2 excess rxn)
qPCR Master Mix	3.75 µL	93.75 µL	187.5 µL
Assay Probe Mix	1 µL	25 µL	50 µL
Reaction Buffer	0.8 µL	20 µL	40 µL
Water	8.2 µL	205 µL	410 µL
Total Assay Probe MM	13.75 µL	343.75 µL	687.5 µL

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

(1) Pulse spin-down tube in microcentrifuge.

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

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

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

ii. For the negative control, use water (found in the kit)

Note: It is best to add the largest volume reagent first, in this case the 9 µL water then the 1 µL of positive control, pipette mix or vortex control dilution to ensure control DNA is in solution.

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

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

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

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

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

h) Add 13.75 µL of specific Assay Probe MM to each corresponding sample well, positive control well, and negative control well in the qPCR plate. Gently tip mix a few times after each addition of qPCR master mix. Be careful not to introduce bubbles during this mix.

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

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

j) Spin-down for at least 1 minute in plate microcentrifuge to bring well contents to the bottom of wells and help to rid of reaction bubbles.

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

k) For the Agilent Aria: If using an adhesive seal; place the reusable compression pad (gray side down) on the plate directly lining up the holes in the pad with the holes in the plate.

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

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

n) Upon completion of the run save your results and proceed to confirmation if necessary.

Table 2: Summary of Results

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

Confirmation of Positive Results

All positive results should be confirmed according to the following protocol.

Salmonella spp.

(a) Mix enriched sample thoroughly by hand. Transfer 0.1 mL of each test portion to 10 mL Rappaport Vassiliadis (RV) medium and 1 mL to 10 mL Tetrathionate (TT) broth. Incubate RV medium at 42 ± 0.2°C for 22–26 h in a circulating water bath. Incubate TT broth at 37 ± 1°C for 22–26 h.

(b) From the secondary enrichments, streak a loopful to Xylose Lysine Deoxycholate (XLD) and CHROMagar *Salmonella*. Incubate at 37 ± 2°C for 22–26 h.

(c) Pick up to 2 colonies from each agar plate and stab/streak to triple sugar iron (TSI) and lysine iron agar (LIA). Incubate at 37 ± 2°C for 22–26 h.

(d) Refer to US FDA BAM Chapter 5 [6] or USDA FSIS MLG 4.11 [7] for guidance on

reading TSI and LIA slants. If further confirmation is required based on reactions in TSI and LIA, streak growth from TSI onto a tryptic soy agar (TSA) plate. Incubate for 18–24 h at 35 ± 2°C.

(e) Pick one well isolated colony and perform a spot polyvalent O and polyvalent H serology test.

(f) Biochemically analyzing using an AOAC PTM or OMA approved method or alternatively confirm via sequencing.

STEC

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

(b) Screen typical colonies with antigen specific latex agglutination test. Pick presumptive positive colony and streak to tryptic soy agar with yeast (TSAYE). Place a ColiComplete (CC) disc into the heaviest streak area. Incubate at 37 ± 2°C for 18–24 h.

(c) STEC will produce a blue color (galactopyranosidase; X-gal positive) and not fluorescence under ultraviolet (UV) light (glucuronidase, 4-methylumbelliferyl-β-D-glucuronide (MUG) negative).

(d) Wet filter paper with Kovac's reagent and perform a spot indole test using growth from the TSAYE plate.

(e) Confirm the presence of *Stx* in the isolate by reprocessing an isolated colony with the PathoSEEK Salmonella and STEC E. coli assay.

(f) Biochemically analyzing using an AOAC PTM or OMA approved method or alternatively confirm via sequencing.

Method Developer Studies

Study Overview

This validation study was conducted under the AOAC Research Institute *Performance Tested Method*SM (PTM) program and the AOAC INTERNATIONAL Appendix J: Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces. The PathoSEEK Salmonella and STEC E. coli Multiplex Assay with SenSATIVAx Extraction Protocols was evaluated for three matrices (dried cannabis flower (>0.3% THC), cannabis concentrates and THC-infused chocolate). Matrix studies, inclusivity and exclusivity, robustness, instrument variation, and product consistency and stability were conducted by the method developer (Beverly, MA). The independent laboratory study involved a matrix study for dried cannabis flower (>0.3% THC) and was performed by TEQ Analytical Laboratories (Aurora, CO).

Inclusivity/Exclusivity

Methodology.— For inclusivity evaluation, 100 strains of *Salmonella* and 51 strains of STEC were tested. Strains were grown for 16 hours at 37 ± 1°C in TSB. The cultures were then diluted to 100 x the Limit of Detection (LOD) of the method. For the exclusivity evaluation, a total of 45 non-targeted strains were tested. Exclusivity organisms were cultured under optimal condition to achieve growth at the stationary phase. Inclusivity and exclusivity strains were randomized and blind coded prior to analysis.

Results.—Detailed results for the inclusivity and exclusivity evaluations are presented in Tables 3-5. For the inclusivity evaluation, all 100 strains of *Salmonella* and all 51 strains of STEC were detected by the assay using both extraction protocols (Flower/Leaf; MIP) on both thermal cyclers (CFX-96; AriaMx). For the exclusivity, 45/45 of the organisms were correctly excluded on the *Salmonella* channel and 44/45 of the organisms were correctly excluded on the STEC channel. One organism, *Shigella dysenteriae,* contains the shiga-toxin genes *stx* and is expected to be detected by the assay on the STEC channel.

Table 3. Inclusivity Results for the PathoSEEK Salmonella and STEC E.coli Multiplex Assay - *Salmonella*

#	Organism	Species/subspecies	Serotype	Source	Origin	Results[a, b]	
						STEC	Salmonella
1	*S. enterica*	*arizonae*	Not Available	ATCC[c] 33952	Not Available	-	+
2	*S. enterica*	*arizonae*	Not Available	ATCC 29933	Not Available	-	+
3	*S. enterica*	*arizonae*	Not Available	ATCC BAA-731	Tissue of corn snake	-	+
4	*S. enterica*	*diarizonae*	IIIb 35:i:z	ATCC BAA-216	Blood	-	+
5	*S. enterica*	*diarizonae*	47:i:z53:z57	ATCC 12325	Not Available	-	+
6	*S. enterica*	*diarizonae*	Not Available	ATCC 29934	Not Available	-	+
7	*S. enterica*	*enterica*	Aberdeen	NCTC[d] 5791	Not Available	-	+
8	*S. enterica*	*enterica*	Abortusequi	NCTC 5727	Not Available	-	+
9	*S. enterica*	*enterica*	Abortusovis	ATCC 31684	Not Available	-	+
10	*S. enterica*	*enterica*	Adelaide	NCTC 6586	Not Available	-	+
11	*S. enterica*	*enterica*	Agona	ATCC 51957	Not Available	-	+
12	*S. enterica*	*enterica*	Anatum	ATCC 9270	Pork liver	-	+
13	*S. enterica*	*enterica*	Bareilly	ATCC 9115	Not Available	-	+
14	*S. enterica*	*enterica*	Berta	ATCC 8392	Not Available	-	+
15	*S. enterica*	*enterica*	Bispebjerg	ATCC 9842	Not Available	-	+
16	*S. enterica*	*enterica*	Braenderup	ATCC 700136	Not Available	-	+
17	*S. enterica*	*enterica*	Bredeney	ATCC 10728	Not Available	-	+
18	*S. enterica*	*enterica*	Breukelen	ATCC 15782	Cuscus	-	+
19	*S. enterica*	*enterica*	Bristol	ATCC 700136	Not Available	-	+
20	*S. enterica*	*enterica*	Caracas	NCTC 9937	Not Available	-	+

21	*S. enterica*	*enterica*	Cerro	NCC 5801	Not Available	-	+
22	*S. enterica*	*enterica*	Champaign	NCTC 6851	Not Available	-	+
23	*S. enterica*	*enterica*	Chester	ATCC 11997	Not Available	-	+
24	*S. enterica*	*enterica*	Choleraesuis	ATCC 10708	Not Available	-	+
25	*S. enterica*	*enterica*	Crossness	NCTC 11059	Not Available	-	+
26	*S. enterica*	*enterica*	Cubana	ATCC 12007	Not Available	-	+
27	*S. enterica*	*enterica*	Dahlem	NCTC 9949	Not Available	-	+
28	*S. enterica*	*enterica*	Derby	ATCC 6960	Tank water and pork pies	-	+
29	*S. enterica*	*enterica*	Deversoir	NCTC 9792	Not Available	-	+
30	*S. enterica*	*enterica*	Dublin	ATCC 15480	Not Available	-	+
31	*S. enterica*	*enterica*	Enteritidis	ATCC 13076	Not Available	-	+
32	*S. enterica*	*enterica*	Essen	ATCC 6961	Not Available	-	+
33	*S. enterica*	*enterica*	Gallinarum	NCTC 10532	Not Available	-	+
34	*S. enterica*	*enterica*	Gaminara	ATCC 8324	Not Available	-	+
35	*S. enterica*	*enterica*	Give	ATCC 9268	Not Available	-	+
36	*S. enterica*	*enterica*	Hadar	NCTC 9877	Not Available	-	+
37	*S. enterica*	*enterica*	Hartford	NCTC 6802	Not Available	-	+
38	*S. enterica*	*enterica*	Havana	NCTC 6086	Not Available	-	+
39	*S. enterica*	*enterica*	Heidelberg	ATCC 8326	Not Available	-	+
40	*S. enterica*	*enterica*	Hillingdon	ATCC 9184	Not Available	-	+
41	*S. enterica*	*enterica*	Indiana	NCTC 11304	Not Available	-	+
42	*S. enterica*	*enterica*	Infantis	NCTC 10679	Not Available	-	+
43	*S. enterica*	*enterica*	Inverness	NCTC 6591	Not Available	-	+
44	*S. enterica*	*enterica*	Javiana	NCTC 6495	Not Available	-	+
45	*S. enterica*	*enterica*	Jerusalem	ATCC 700137	Not Available	-	+
46	*S. enterica*	*enterica*	Johannesburg	NCTC 8272	Not Available	-	+
47	*S. enterica*	*enterica*	Kentucky	NCTC 5799	Not Available	-	+
48	*S. enterica*	*enterica*	Krefeld	NCTC 9884	Not Available	-	+
49	*S. enterica*	*enterica*	Lille	NCTC 9885	Not Available	-	+
50	*S. enterica*	*enterica*	London	ATCC 8389	Not Available	-	+
51	*S. enterica*	*enterica*	Matadi	NCTC 9887	Not Available	-	+
52	*S. enterica*	*enterica*	Mbandaka	ATCC 51958	Not Available	-	+
53	*S. enterica*	*enterica*	Meleagridids	NCTC 6023	Not Available	-	+
54	*S. enterica*	*enterica*	Menden	ATCC 15992	Feces	-	+
55	*S. enterica*	*enterica*	Mgulani	NCTC 8492	Not Available	-	+
56	*S. enterica*	*enterica*	Minnesota	NTCT 5800	Not Available	-	+
57	*S. enterica*	*enterica*	Montevideo	ATCC 8387	Not Available	-	+

58	*S. enterica*	*enterica*	Muenchen	NCTC 6246	Not Available	-	+
59	*S. enterica*	*enterica*	Newport	ATCC 6962	Food poisoning	-	+
60	*S. enterica*	*enterica*	Nottingham	NCTC 7832	Not Available	-	+
61	*S. enterica*	*enterica*	Oranienburg	ATCC 9239	Illinois	-	+
62	*S. enterica*	*enterica*	Panama	ATCC 7378	Baby	-	+
63	*S. enterica*	*enterica*	Paratyphi A	ATCC 9150	Not Available	-	+
64	*S. enterica*	*enterica*	Paratyphi B	ATCC 8759	Gall bladder	-	+
65	*S. enterica*	*enterica*	Pomona	NCTC 6589	Turkey intestine	-	+
66	*S. enterica*	*enterica*	Poona	NCTC 5792	Not Available	-	+
67	*S. enterica*	*enterica*	Potsdam	ATCC 25957	Child	-	+
68	*S. enterica*	*enterica*	Pullorum	ATCC 9120	Clinical isolate	-	+
69	*S. enterica*	*enterica*	Reading	ATCC 6967	Guinea pig	-	+
70	*S. enterica*	*enterica*	Saintpaul	ATCC 9712	Cystitis	-	+
71	*S. enterica*	*enterica*	Sandiego	ATCC 231999	Urinary bladder, turtle	-	+
72	*S. enterica*	*enterica*	Schwarzengrund	NCTC 6756	Not Available	-	+
73	*S. enterica*	*enterica*	Senftenberg	ATCC 8400	Not Available	-	+
74	*S. enterica*	*enterica*	Sloterdijk	ATCC 15791	Family outbreak	-	+
75	*S. enterica*	*enterica*	Stanley	ATCC 7308	Not Available	-	+
76	*S. enterica*	*enterica*	Sundsvall	NCTC 6758	Not Available	-	+
77	*S. enterica*	*enterica*	Tennessee	ATCC 10722	Not Available	-	+
78	*S. enterica*	*enterica*	Thompson	ATCC 8391	Not Available	-	+
79	*S. enterica*	*enterica*	Typhi	ATCC 19430	Not Available	-	+
80	*S. enterica*	*enterica*	Typhimurium	ATCC 13311	Feces	-	+
81	*S. enterica*	*enterica*	Typhisuis	ATCC 8321	Not Available	-	+
82	*S. enterica*	*enterica*	Urbana	NCTC 6248	Not Available	-	+
83	*S. enterica*	*enterica*	Utrecht	NCTC 10077	Not Available	-	+
84	*S. enterica*	*enterica*	Vellore	ATCC 15611	Rectal swab	-	+
85	*S. enterica*	*enterica*	Virchow	ATCC 51955	Not Available	-	+
86	*S. enterica*	*enterica*	Waycross	NCTC 7401	Not Available	-	+
87	*S. enterica*	*enterica*	Weltervreden	NCTC 6534	Not Available	-	+
88	*S. enterica*	*enterica*	Zwickau	ATCC 15804	Not Available	-	+
89	*S. enterica*	*houtenae*	45:g,z51:-	ATCC 43974	Not Available	-	+
90	*S. enterica*	*houtenae*	Not Available	ATCC BAA-1580	Not Available	-	+
91	*S. enterica*	*houtenae*	11:z4,z23: -	ATCC 15788	Not Available	-	+

92	*S. enterica*	*indica*	45:a:e,n,x	ATCC BAA-1578	Not Available	-	+
93	*S. enterica*	*indica*	1,6,14,25:a:e,n,x	ATC 43976	Not Available	-	+
94	*S. enterica*	*indica*	6, 14, 25:a:e,n,x	NCTC 10458	coconut	-	+
95	*S. enterica*	*salamae*	56:b:-	ATCC 700149	Not Available	-	+
96	*S. enterica*	*salamae*	1,9,12:l,w:e,n	ATCC 6959	Urine	-	+
97	*S. enterica*	*salamae*	55:k:z39	ATCC 700148	Not Available	-	+
98	*S. enterica*	*salamae*	1,9,12:l,w:e,n,x	ATCC 43972	Not Available	-	+
99	*S. bongori*		66:z41:-	ATCC 43975	Not Available	-	+
100	*S. bongori*		48:z35	NCTC 14392	Not Available	-	+

[a] + = Positive Result; - = Negative Result; [b] Results identical between extraction protocols (flower or marijuana infused products) and between thermal cyclers (CFX-96, AriaMx); [c]ATCC – American Type Culture Collection, Manassas, VA; [d] NCTC – National Type Culture Collection, London, UK.

Table 4: Inclusivity Results for the PathoSEEK Salmonella and STEC E.coli Multiplex Assay - STEC

#	Organism	Source	Origin	Results[a, b]	
				STEC	Salmonella
1	*Escherichia coli* O3	TW01413[c]	Germany	+	-
2	*Escherichia coli* O3	TW01414	Germany	+	-
3	*Escherichia coli* O5	TW00021	Cow (MI)	+	-
4	*Escherichia coli* O5	TW05097	Cow (CA)	+	-
5	*Escherichia coli* O26:H11	ATCC[d] BAA-2205	Feces	+	-
6	*Escherichia coli* O26:H11	ATCC BAA-2196	Feces	+	-
7	*Escherichia coli* O26:H11	ATCC BAA-2204	Feces	+	-
8	*Escherichia coli* O26:H11	ATCC BAA-2181	Not Available	+	-
9	*Escherichia coli* O26:H11	ATCC BAA-2188	Feces	+	-
10	*Escherichia coli* O45:H2	ATCC BAA-2193	Feces	+	-
11	*Escherichia coli* O45:H2	ATCC BAA-2189	Feces	+	-
12	*Escherichia coli* O45:H2	ATCC BAA-2185	Not Available	+	-
13	*Escherichia coli* O45:H2	ATCC BAA-2202	Feces	+	-
14	*Escherichia coli* O45:H2	ATCC BAA-2198	Feces	+	-
15	*Escherichia coli* O91:H21	ATCC 51434	Not Available	+	-
16	*Escherichia coli* O91:H21	ATCC 51435	Clinical Isolate	+	-
17	*Escherichia coli* O103:H11	ATCC BAA-2215	Not Available	+	-
18	*Escherichia coli* O103:H11	ATCC BAA-2200	Not Available	+	-
19	*Escherichia coli* O103:H11	NJDPH[e] 151297-1	Not Available	+	-
20	*Escherichia coli* O103:H11	NJDPH 130928-3	Not Available	+	-

21	*Escherichia coli* O103:H11	TW08869	Human Isolate	+	-
22	*Escherichia coli* O103:H11	TW08872	Human Isolate	+	-
23	*Escherichia coli* O111:H8	ATCC BAA-2201	Feces	+	-
24	*Escherichia coli* O111:H8	ATCC BAA-2180	Feces	+	-
25	*Escherichia coli* O111:H8	ATCC BAA-184	Feces	+	-
26	*Escherichia coli* O111:H8	ATCC BAA-180	Feces	+	-
27	*Escherichia coli* O111:H8	ATCC BAA-181	Feces	+	-
28	*Escherichia coli* O113	ATCC BAA-177	Feces	+	-
29	*Escherichia coli* O113	ATCC BAA-176	Feces	+	-
30	*Escherichia coli* O113	ATCC BAA-183	Urine	+	-
31	*Escherichia coli* O118	TW08644	Feces	+	-
32	*Escherichia coli* O118	TW08134	Cow Feces	+	-
33	*Escherichia coli* O121:H19	ATCC BAA-2219	Feces	+	-
34	*Escherichia coli* O121:H19	ATCC BAA-2203	Feces	+	-
35	*Escherichia coli* O121:H19	ATCC BAA-2187	Not Available	+	-
36	*Escherichia coli* O121:H19	ATCC BAA-2220	Feces	+	-
37	*Escherichia coli* O121:H19	TW08868	Feces	+	-
38	*Escherichia coli* O145:NM	TW09356	Human Isolate	+	-
39	*Escherichia coli* O145:NM	TW07596	Feces	+	-
40	*Escherichia coli* O145:NM	ATCC BAA-2223	Feces	+	-
41	*Escherichia coli* O145:NM	ATCC BAA-2192	Feces	+	-
42	*Escherichia coli* O145:NM	NJDPH 17257-1	Not Available	+	-
43	*Escherichia coli* O145:NM	NJDPH 161296-1	Not Available	+	-
44	*Escherichia coli* O157:H7	ATCC 35150	Feces	+	-
45	*Escherichia coli* O157:H7	ATCC 43889	Feces	+	-
46	*Escherichia coli* O157:H7	ATCC 43894	Feces	+	-
47	*Escherichia coli* O157:H7	ATCC 700599	Salami	+	-
48	*Escherichia coli* O157:H7	TW00116	Feces	+	-
49	*Escherichia coli* O157:H7	TW00975	Human Isolate	+	-
50	*Escherichia coli* O157:H7	TW02302	Human Isolate	+	-
51	*Escherichia coli* O157:H7	TW04863	Feces	+	-

[a] + = Positive Result; - = Negative Result; [b] Results identical between extraction protocols (flower or marijuana infused products) and between thermal cyclers (CFX-96, AriaMx); [c]TW – Michigan St. STEC Center, Lansing, MI; [d]ATCC – American Type Culture Collection, Manassas, VA; [e] NJDPH – New Jersey Department of Public Health, Trenton, New Jersey.

Table 5. Exclusivity Results for the PathoSEEK Salmonella and STEC E.coli Multiplex Assay -

#	**Organism**	**Source**	Origin	Results[a, b]	
				STEC	Salmonella

1	*Aeromonas bestiarum*	ATCC[c] BAA-231	Cake	-	-
2	*Aeromonas hydrophila*	ATCC 7966	Milk	-	-
3	*Burkholderia multivorans*	ATCC 17616	Soil	-	-
4	*Bacillus subtilis*	ATCC 11774	Not Available	-	-
5	*Campylobacter jejuni*	ATCC 29428	Feces	-	-
6	*Candida tropicalis*	ATCC 13803	Not Available	-	-
7	*Citrobacter braakii*	ATCC 3037	Urine	-	-
8	*Citrobacter farmerii*	ATCC 51112	Feces	-	-
9	*Citrobacter freundii*	ATCC 8090	Not Available	-	-
10	*Citrobacter koseri*	ATCC 25408	Throat	-	-
11	*Citrobacter murliae*	ATCC 51118	United States; Illinois	-	-
12	*Citrobacter youngae*	ATCC 29935	Meat scraps	-	-
13	*Edwardsiella tarda*	ATCC 23672	Not Available	-	-
14	*Enterobacter aerogenes*	ATCC 13048	Sputum	-	-
15	*Enterobacter cloacae*	ATCC 13047	Spinal fluid	-	-
16	*Enterobacter gergoviae*	ATCC 33028	Urine	-	-
17	*Enterobacter sakazakii*	ATCC BAA-894	Clinical specimen	-	-
18	*Enterobacter amnigenus*	ATCC 51818	Milk	-	-
19	*Enterobacter cancerogenus*	ATCC 35318	Cerebrospinal fluid	-	-
20	*Erwinia rhapontici*	ATCC 29290	English pink wheat grains	-	-
21	*Escherichia coli*	ATCC 25922	Clinical isolate	-	-
22	*Escherichia fergusonii*	ATCC 35469	Feces	-	-
23	*Escherichia hermannii*	ATCC 700368	Not Available	-	-
24	*Escherichia vulneris*	ATCC 33821	Wound	-	-
25	*Hafnia alvei*	ATCC 51873	Feces	-	-
26	*Klebsiella oxytoca*	ATCC 51983	Blood	-	-
27	*Klebsiella pneumonia*	ATCC BAA-2146	Urine	-	-
28	*Listeria monocytogenes*	ATCC 7647	Bovine	-	-
29	*Morganella morganii*	ATCC 25829	Stool	-	-
30	*Pantoea agglomerans*	ATCC 43348	Gypsophila paniculata galls	-	-
31	*Proteus hauseri*	ATCC 13315	Not Available	-	-
32	*Proteus mirabilis*	ATCC 43071	Rectum	-	-
33	*Proteus vulgaris*	ATCC 8427	Inner ear infection	-	-
34	*Pseudomonas aeruginosa*	ATCC 15442	Water bottle in animal room	-	-
35	*Pseudomonas fluorescens*	ATCC 13525	Pre-filter tanks	-	-

36	*Pseudomonas putida*	ATCC 47054	Not Available	-	-
37	*Rahnella aquatilis*	ATCC 33991	Soil	-	-
38	*Ralstonia insidiosa*	ATCC 49129	Clinical isolate	-	-
39	*Serratia marcescens*	ATCC	Not Available	-	-
40	*Shigella dysenteeriae*	ATCC 13313	Foreign seaman	+	-
41	*Shigella flexneri*	ATCC 12022	Not Available	-	-
42	*Shigella sonnei*	ATCC 9290	Not Available	-	-
43	*Trichoderma harzianum*	ATCC 60850	Soil	-	-
44	*Vibrio vulnificus*	ATCC 29307	Blood	-	-
45	*Yersinia ruckeri*	ATCC 29473	Rainbow trout	-	-

[a] + = Positive Result; - = Negative Result; [b] Results identical between extraction protocols (flower or marijuana infused products) and between thermal cyclers (CFX-96, AriaMx); [c]ATCC – American Type Culture Collection, Manassas, VA;

Matrix Studies

Methodology.— The PathoSEEK Salmonella and STEC E.coli Multiplex Assay with SenSATIVAx Extraction protocols were validated for three matrices, dried cannabis flower (10 g, >0.3% THC), cannabis concentrates (5 g) and THC-infused chocolate (25 g) at the method developer (Medicinal Genomics, Beverly, MA). Dried cannabis flower (10 g, >0.3% THC) was also evaluated at the independent laboratory (TEQ Analytical Laboratories, Aurora, CO). The study was conducted following guidance defined in AOAC SMPR 2020.002, SMPR 2020.012 and AOAC Appendix J. Each matrix was evaluated at 16 h and 24 h of enrichment by the candidate method. Regardless of the presumptive results, all matrix enrichments were culturally confirmed after 24 h of enrichment.

Matrices were obtained following local state regulations and prescreened for natural contamination of *Salmonella* and STEC with the candidate method and by cultural procedures as described in this report. No natural contamination was found with either the candidate method or the culture method, so each matrix were artificially contaminated. Total aerobic plate count was determined following the FDA BAM Chapter 3: Aerobic Plate Count (8). Dried cannabis flower (>0.3% THC) was inoculated using a dry inoculum. Cannabis concentrates and THC-infused chocolate were evaluated with a heat stressed liquid culture.

For dried cannabis flower (>0.3% THC), lyophilized pellets of *Salmonella* and STEC cultures were crushed and mixed together with the flower matrix. Using non-inoculated matrix as the diluent, contaminated matrix was diluted to a low-level expected to yield fractional positive results (5-15 positive results) and a high-level expected to yield all positive results. The matrix was mixed with sterile mixing utensils and allowed to equilibrate for two weeks at room temperature (20-25°C) prior to testing. Inoculation protocols were consistent between the method developer and independent laboratory.

For heat stressed matrices, *Salmonella* and STEC cultures were grown in TSB for 18-24 h at 37 ± 1°C. After incubation, cultures were heat stressed for 10-12 min at 50-55°C to achieve injury. The level of injury was determined by plating the culture onto selective (Xylose lysine deoxycholate (XLD) – *Salmonella*) or Levine's Eosin-Methylene Blue (L-EMB) – STEC) and non-selective agar (Tryptic soy agar (TSA)). The percent injury was determined using the following formula:

$$(1-\frac{n_{select}}{n_{nonselect}})x100$$

Where n_{select} = number of colonies on selective agar
And $n_{nonselect}$ = number of colonies on nonselective agar

Using TSB as the diluent, the heat stressed cultures were diluted to a low-level expected to yield fractional positive results (5-15 positive results) and a high-level expected to yield all positive results. For the chocolate matrix, the inoculum were added dropwise to melted, tempered chocolate and mixed by hand. After inoculation, the chocolate was separated into test portions and allowed to harden. Cannabis concentrate and THC-infused chocolate were held for 2 weeks at 20-25ºC prior to analysis to allow time for the organism to equilibrate within the sample.

Each matrix was analyzed at three target levels of artificial contamination: non-inoculated (0 CFU/test portion), low level (1-2 CFU/test portion), and high level (~2-20 CFU/test portion). For the 10 g test portions, an MPN for the low level was performed on the day of testing by analyzing 20 x 10 g (test portions from matrix study), 3 x 5 g, and 3 x 1 g test portions. For the

high levels, 5 replicates were used instead of 20 for the largest test portion size. For the 25 g test portions, an MPN for the low level was performed on the day of testing by analyzing 20 x 25 g (test portions from matrix study), 3 x 10 g, and 3 x 5 g test portions. For the high levels, 5 replicates were used instead of 20 for the largest test portion size. For the 5 g test portions, an MPN for the low level was performed on the day of testing by analyzing 20 x 5 g (test portions from matrix study), 3 x 2.5 g, and 3 x 1 g test portions. For the high levels, 5 replicates were used instead of 20 for the largest test portion size. Each test portion for MPN determination was enriched following the candidate method protocol and confirmed following cultural procedures described in this report. The number of positives from the 3 test levels was used to calculate the MPN using the LCF MPN calculator (version 1.6) provided by AOAC RI (9).

PathoSEEK Salmonella and STEC E. coli Multiplex Assays with SenSATIVAx Extraction

All matrices were enriched and incubated according to the protocol as described previously in "*Sample Preparation*". After incubation all test portions were processed by the qPCR assay. All samples, regardless of presumptive result, were culturally confirmed.

Salmonella Confirmation.— To confirm the presence of the target analyte, 0.1 mL of primary enrichment was transferred into 10 mL of RV medium and 1.0 mL into 10 mL of TT broth. RV was incubated at 42 ± 0.2°C for 24 ± 2 h in a circulating water bath. TT was incubated at 37 ± 1°C for 24 ± 2 h. Following incubation, a loopful of the secondary enrichments were streaked to XLD and CHROMagar *Salmonella* and incubated at 37 ± 2°C for 24 ± 2 h. A minimum of two suspect colonies from each selective agar were transferred to TSI and LIA slants and incubated at 37 ± 2°C for 24 ± 2 h. Following incubation, TSI and LIA slants were examined for typical and atypical reactions. Slants producing reactions requiring further confirmation (as outlined in

BAM Chapter 5 and MLG 4.11) were streaked to TSA and incubated for 35 ± 2°C for 18–24 h. Following incubation, isolates were serologically tested for both somatic O and flagellar H agglutination. Additionally, purified TSA isolates were identified using API 20E AOAC Official Method **978.24** (independent laboratory) or sequencing (method developer).

STEC Confirmation.— To confirm the presence of the target analyte, an isolation streak of the primary enrichment was performed onto selective agars. For *E. coli* O157:H7 test portions, TC SMAC and CHROMagar STEC are used. For non-O157 STEC test portions, L-EMB and CHROMagar STEC were used. All plates were incubated at 37 ± 1.0°C for 18–24 h.

For confirmation of test portions, typical isolates were screened by antigen specific latex agglutination. Presumptive positive isolates were struck to TSAYE, and a CC disc was placed into the heaviest streaked area. Plates were incubated at 37 ± 1.0°C for 18–24 h. Isolates were considered STEC if they produced a blue color (X-gal positive) and did not fluorescence under UV light (MUG negative). Presumptive positive isolates were screened for production of indole using Kovac's reagent and biochemically confirmed using API 20E AOAC Official Method **978.24** (independent laboratory) or sequencing (method developer). A final confirmation for the presence of *stx* genes in the isolates was performed by reprocessing an isolated colony with PTM #121806 and the candidate method.

Results.— Aerobic plate count results for the matrices are as follows: dried cannabis flower (>0.3% THC) – 2.8 x 10^3 CFU/g; cannabis concentrate - 1.6 x 10^3 CFU/g; THC-infused chocolate -2.4 x 10^3 CFU/g. As per criteria outlined in Appendix J, fractional positive results were obtained at the low level of inoculation for all matrices. Method comparison results and statistical analyses are presented in Table 7 (AriaMx) and Table 8 (CFX-96). The POD comparisons (10) between the candidate method presumptive and confirmed results using either

extraction protocols (Flower/Leaf and MIP/Extracts) indicated there was no significant difference at the 5% level for all matrices and on either thermocycler.

Table 7: PathoSEEK Salmonella and STEC E. coli Multiplex Assays with SenSATIVAx Extraction Presumptive vs Confirmed Results (Paired) for AriaMx – POD Results

Matrix and Inoculum	Enrichment Time Point	MPN_a / Test Portion	N[b]	x[c]	Presumptive		x	Confirmed		$dPOD_{cp}$[f]	95% CI[g]
					POD_{cp}[d]	95% CI		POD_{cc}[e]	95% CI		
Dried cannabis flower (>0.3% THC)[h]; 10g *Salmonella* Typhimurium ATCC 13311	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.08 (0.60, 1.86)	20	13	0.65	0.43, 0.82	13	0.65	0.43, 0.82	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.08 (0.60, 1.86)	20	13	0.65	0.43, 0.82	13	0.65	0.43, 0.82	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Dried cannabis flower (>0.3% THC) [h]; 10g *E. coli* O26 ATCC BAA-2188	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.09 (0.52, 1.67)	20	11	0.55	0.34, 0.74	12	0.60	0.39, 0.78	-0.05	-0.21, 0.11
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.09 (0.52, 1.67)	20	12	0.60	0.39, 0.78	12	0.60	0.39, 0.78	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Cannabis concentrates [h]; 5 g *Salmonella* Newport ATCC 6962	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.41 (0.83, 2.47)	20	14	0.70	0.48, 0.86	14	0.70	0.48, 0.86	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.41 (0.83, 2.47)	20	14	0.70	0.48, 0.86	14	0.70	0.48, 0.86	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Cannabis concentrates [h]; 5 g *E. coli* O103 ATCC BAA-2215	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.59 (0.95, 2.79)	20	15	0.75	0.53, 0.89	15	0.75	0.53, 0.89	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.59 (0.95, 2.79)	20	15	0.75	0.53, 0.89	15	0.75	0.53, 0.89	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47

THC-Infused Chocolate [h]; 25 g *Salmonella* Heidelberg ATCC 8326	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.08 (0.60, 1.85)	20	14	0.70	0.48, 0.86	13	0.65	0.43, 0.82	0.05	-0.11, 0.21
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.08 (0.60, 1.85)	20	13	0.65	0.43, 0.82	13	0.65	0.43, 0.82	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
THC-Infused Chocolate [h]; 25 g *E. coli* O145 ATCC BAA-2192	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.85 (0.45, 1.49)	20	11	0.55	0.34, 0.74	11	0.55	0.34, 0.74	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.85 (0.45, 1.49)	20	11	0.55	0.34, 0.74	11	0.55	0.34, 0.74	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Dried cannabis flower [h] (>0.3% THC); 10g *Salmonella* Typhimurium ATCC 16888 (Independent Laboratory)	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.44 (0.85, 2.57)	20	13	0.65	0.43, 0.82	14	0.70	0.48, 0.86	-0.05	-0.21, 0.11
		11.4 (2.97, 43.3)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.44 (0.85, 2.57)	20	14	0.70	0.48, 0.86	14	0.70	0.48, 0.86	0.00	-0.13, 0.13
		11.4 (2.97, 43.3)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Dried cannabis flower (>0.3% THC) [h]; 10g *E. coli* O26 ATCC 16888 (Independent Laboratory)	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.87 (0.46, 1.46)	20	10	0.50	0.30, 0.70	11	0.55	0.34, 0.74	-0.05	-0.21, 0.11
		6.16 (1.91, 19.9)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.87 (0.46, 1.46)	20	11	0.55	0.34, 0.74	11	0.55	0.34, 0.74	0.00	-0.13, 0.13
		6.16 (1.91, 19.9)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47

[a]MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval.
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]$dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h] Dried cannabis flower evaluated using SenSATIVAx Flower/Leaf extraction kit, all other matrixes used SenSATIVAx MIP/Extract

Table 8: PathoSEEK Salmonella and STEC E. coli Multiplex Assays with SenSATIVAx Extraction Presumptive vs Confirmed Results (Paired) for CFX-96 – POD Results

Matrix and Inoculum	Enrichment Time Point	MPN_a / Test Portion	N[b]	x[c]	Presumptive		x	Confirmed		$dPOD_{cp}$[f]	95% CI[g]
					POD_{cp}[d]	95% CI		POD_{cc}[e]	95% CI		
Dried cannabis flower (>0.3% THC)[h]; 10g *Salmonella* Typhimurium ATCC 13311	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.08 (0.60, 1.86)	20	13	0.65	0.43, 0.82	13	0.65	0.43, 0.82	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.08 (0.60, 1.86)	20	13	0.65	0.43, 0.82	13	0.65	0.43, 0.82	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Dried cannabis flower (>0.3% THC)[h]; 10g *E. coli* O26 ATCC BAA-2188	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.09 (0.52, 1.67)	20	11	0.55	0.34, 0.74	12	0.60	0.39, 0.78	-0.05	-0.21, 0.11
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA 0.09 (0.52, 1.67) >10 (8.00, >30.0)	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
			20	11	0.55	0.34, 0.74	12	0.60	0.39, 0.78	-0.05	-0.21, 0.11
			5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Cannabis concentrates[h]; 5 g	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.41 (0.83, 2.47)	20	14	0.70	0.48, 0.86	14	0.70	0.48, 0.86	0.00	-0.13, 0.13

Salmonella Newport ATCC 6962		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.41 (0.83, 2.47)	20	14	0.70	0.48, 0.86	14	0.70	0.48, 0.86	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Cannabis concentrates [h]; 5 g *E. coli* O103 ATCC BAA-2215	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.59 (0.95, 2.79)	20	15	0.75	0.53, 0.89	15	0.75	0.53, 0.89	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.59 (0.95, 2.79)	20	15	0.75	0.53, 0.89	15	0.75	0.53, 0.89	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
THC-Infused Chocolate [h]; 25 g *Salmonella* Heidelberg ATCC 8326	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.08 (0.60, 1.85)	20	14	0.70	0.48, 0.86	13	0.65	0.43, 0.82	0.05	-0.11, 0.21
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.08 (0.60, 1.85)	20	13	0.65	0.43, 0.82	13	0.65	0.43, 0.82	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
THC-Infused Chocolate [h]; 25 g *E. coli* O145	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.85 (0.45, 1.49)	20	11	0.55	0.34, 0.74	11	0.55	0.34, 0.74	0.00	-0.13, 0.13

ATCC BAA-2192		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.85 (0.45, 1.49)	20	11	0.55	0.34, 0.74	11	0.55	0.34, 0.74	0.00	-0.13, 0.13
		>10 (8.00, >30.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Dried cannabis flower (>0.3% THC) [h]; 10g *Salmonella* Typhimurium ATCC 16888 (Independent Laboratory)	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.44 (0.85, 2.57)	20	13	0.65	0.43, 0.82	14	0.70	0.48, 0.86	-0.05	-0.21, 0.11
		11.4 (2.97, 43.3)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.44 (0.85, 2.57)	20	14	0.70	0.48, 0.86	14	0.70	0.48, 0.86	0.00	-0.13, 0.13
		11.4 (2.97, 43.3)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Dried cannabis flower (>0.3% THC) [h]; 10g *E. coli* O26 ATCC 16888 (Independent Laboratory)	16 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.87 (0.46, 1.46)	20	4	0.20	0.08, 0.42	11	0.55	0.34, 0.74	-0.35	-0.59, -0.11
		6.16 (1.91, 19.9)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	24 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.87 (0.46, 1.46)	20	9	0.45	0.26, 0.66	11	0.55	0.34, 0.74	-0.10	-0.28, 0.83
		6.16 (1.91, 19.9)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47

[a]MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval.
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]$dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h] Dried cannabis flower evaluated using SenSATIVAx Flower/Leaf extraction kit, all other matrixes used SenSATIVAx MIP/Extract

Robustness Study

Methodology.—All the assays were carried out by the method developer. SenSATIVAx extraction kits had previously been validated (PathoSEEK 5 – Color Aspergillus Multiplex Assay, PTM #082102) and were not included in the study. Parameters varied for the PathoSEEK assay included: extraction aliquot volume (4.5 µL, 5.5 µL), assay probe mix volume (0.8 µL, 1.2 µL) and master mix volume (3.5 µL, 4.0 µL), along with one combination of the nominal values (5.0 µL, 2.0 µL, 3.75 µL).

Each parameter was evaluated in dried cannabis flower (>0.3% THC) using both thermal cyclers by analyzing the candidate method with ten replicates of a target strains (*Salmonella* Enteritidis ATCC 13076 and *E. coli* O111:H8 ATCC BAA-2201) diluted to achieve fractional positive results and ten replicates of non-target organism (*Enterobacter cloacae* ATCC 13047). POD values and confidence intervals were calculated, and the data analyzed for potential variable detection due to changes in parameter settings.

Results.—Results were decoded with POD values and confidence intervals calculated for combinations 1–8 when compared to the nominal combination 9. Data was analyzed for variable detection due to changes in parameters.

For both *Salmonella* and STEC targets, no statistically significant results were observed in the dPOD analysis with 95% confidence intervals between combinations 1-8 and the nominal combination (9). For the non-target organism, there were 0 presumptive positives out of 10 replicates for all test combinations 1-9. Detailed results are presented in table 9-10).

Table 9: Robustness study of the PathoSEEK Salmonella and STEC E. coli Multiplex Assay - *Salmonella*, POD comparison

	Parameters													
Parameter test combination[a]	Extraction Aliquot Volume (µL)	Assay Probe Mix volume (µL)	Master Mix Volume (µL)	N[b]	x[c]	POD_E[d]	95% CI	Nominal condition[e]	N	x	POD_N[f]	95% CI	$dPOD_{EN}$[g]	95% CI[h]
Target analyte: dried hemp flower inoculated with *Salmonella* Enteritidis ATCC 13076														
1	4.5 µL	0.8 µL	3.50 µL	10	6	0.60	0.31, 0.83	9	10	5	0.50	0.24, 0.77	0.10	-0.29, 0.45
2	4.5 µL	0.8 µL	4.00 µL	10	4	0.40	0.17, 0.69	9	10	5	0.50	0.24, 0.77	-0.10	-0.45, 0.29
3	4.5 µL	1.2 µL	3.50 µL	10	4	0.40	0.17, 0.69	9	10	5	0.50	0.24, 0.77	-0.10	-0.45, 0.29
4	4.5 µL	1.2 µL	4.00 µL	10	5	0.50	0.24, 0.76	9	10	5	0.50	0.24, 0.77	0.00	-0.37, 0.37
5	5.5 µL	0.8 µL	3.50 µL	10	2	0.20	0.06, 0.51	9	10	5	0.50	0.24, 0.77	-0.30	-0.60, 0.11
6	5.5 µL	0.8 µL	4.00 µL	10	5	0.50	0.24, 0.76	9	10	5	0.50	0.24, 0.77	0.00	-0.37, 0.37
7	5.5 µL	1.2 µL	3.50 µL	10	4	0.40	0.17, 0.69	9	10	5	0.50	0.24, 0.77	-0.10	-0.45, 0.29
8	5.5 µL	1.2 µL	4.00 µL	10	4	0.40	0.17, 0.69	9	10	5	0.50	0.24, 0.77	-0.10	-0.45, 0.29
9	5.0 µL	1.0 µL	3.75 µL	10	5	0.50	0.24, 0.76	9	10	5	0.50	0.24, 0.77	0.00	-0.37, 0.37
Non-target analyte: dried hemp flower inoculated with *Enterobacter cloacae* ATCC #13047														
1	4.5 µL	0.8 µL	3.50 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
2	4.5 µL	0.8 µL	4.00 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
3	4.5 µL	1.2 µL	3.50 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
4	4.5 µL	1.2 µL	4.00 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
5	5.5 µL	0.8 µL	3.50 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
6	5.5 µL	0.8 µL	4.00 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
7	5.5 µL	1.2 µL	3.50 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
8	5.5 µL	1.2 µL	4.00 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
9	5.0 µL	1.0 µL	3.75 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28

[a]Each parameter test combination is being compared to the nominal test condition.
[b]N = Number of test portions experimental combination.
[c]x = Number of positive test portions experimental combination.
[d]POD_E = Positive outcomes divided by the total number of trials experimental combination.
[e]Nominal condition = parameter test combination No. 9.
[f]POD_N = Positive outcomes divided by the total number of trials nominal condition.
[g]$dPOD_{EN}$ = Difference in POD between the nominal condition and experimental combinations.

[h]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Table 10: Robustness study of the PathoSEEK Salmonella and STEC E. coli Multiplex Assay - STEC, POD comparison

	Parameters													
Parameter test combination[a]	Extraction Aliquot Volume (µL)	Assay Probe Mix volume (µL)	Master Mix Volume (µL)	N[b]	x[c]	POD_E[d]	95% CI	Nominal condition[e]	N	x	POD_N[f]	95% CI	$dPOD_{EN}$[g]	95% CI[h]
Target analyte: dried hemp flower inoculated with *E. coli* O111:H8 ATCC BAA-2201														
1	4.5 µL	0.8 µL	3.50 µL	10	5	0.50	0.24, 0.76	9	10	5	0.50	0.24, 0.77	0.00	-0.37, 0.37
2	4.5 µL	0.8 µL	4.00 µL	10	7	0.70	0.40, 0.89	9	10	5	0.50	0.24, 0.77	-0.20	-0.20, 0.53
3	4.5 µL	1.2 µL	3.50 µL	10	6	0.60	0.31, 0.83	9	10	5	0.50	0.24, 0.77	0.10	-0.29, 0.45
4	4.5 µL	1.2 µL	4.00 µL	10	5	0.50	0.24, 0.76	9	10	5	0.50	0.24, 0.77	0.00	-0.37, 0.37
5	5.5 µL	0.8 µL	3.50 µL	10	6	0.60	0.31, 0.83	9	10	5	0.50	0.24, 0.77	0.10	-0.29, 0.45
6	5.5 µL	0.8 µL	4.00 µL	10	6	0.60	0.31, 0.83	9	10	5	0.50	0.24, 0.77	0.10	-0.29, 0.45
7	5.5 µL	1.2 µL	3.50 µL	10	4	0.40	0.17, 0.69	9	10	5	0.50	0.24, 0.77	-0.10	-0.45, 0.29
8	5.5 µL	1.2 µL	4.00 µL	10	8	0.80	0.49, 0.94	9	10	5	0.50	0.24, 0.77	0.40	-0.22, 0.60
9	5.0 µL	1.0 µL	3.75 µL	10	5	0.50	0.24, 0.76	9	10	5	0.50	0.24, 0.77	0.00	-0.37, 0.37
Non-target analyte: dried hemp flower inoculated with *Enterobacter cloacae* ATCC #13047														
1	4.5 µL	0.8 µL	3.50 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
2	4.5 µL	0.8 µL	4.00 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
3	4.5 µL	1.2 µL	3.50 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
4	4.5 µL	1.2 µL	4.00 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
5	5.5 µL	0.8 µL	3.50 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
6	5.5 µL	0.8 µL	4.00 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
7	5.5 µL	1.2 µL	3.50 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
8	5.5 µL	1.2 µL	4.00 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
9	5.0 µL	1.0 µL	3.75 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28

[a]Each parameter test combination is being compared to the nominal test condition.
[b]N = Number of test portions experimental combination.
[c]x = Number of positive test portions experimental combination.
[d]POD_E = Positive outcomes divided by the total number of trials experimental combination.
[e]Nominal condition = parameter test combination No. 9.

[f]POD_N = Positive outcomes divided by the total number of trials nominal condition.
[g]$dPOD_{EN}$ = Difference in POD between the nominal condition and experimental combinations.
[h]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Product Consistency (lot-to-lot)

Methodology.— Three lots of PathoSEEK Salmonella and STEC E. coli Multiplex Assay, consisting of assays newly manufactured, mid-shelf life and end of shelf life, were evaluated for lot-to-lot consistency (SenSATIVAx Extraction assays were previously evaluated for lot-to-lot consistency). One strain for each target, *Salmonella* Typhimurium ATCC 13311 and *E. coli* O157:H7 ATCC 35150, and 1 non-target strain, *E. coli* ATCC 25922, were incubated in TSB for 48 h at 37 ± 1°C. The target strain was diluted to a level that yields fractional recovery and the non-target strain was tested without dilution. Each lot of PCR assay was tested with 10 replicates each diluted target strain and 10 replicates of undiluted non-target strain in a randomized blind coded fashion. POD values and confidence intervals were calculated, and data analyzed for potential variable detection in lots.

Results.—For the 10 non-target strain, there were 0 presumptive positives out of 10 replicates for each lot evaluated. For the evaluation of the target strains, there were no observed statistical differences obtained by POD analysis with 95% confidence intervals for all lots evaluated (Table 11) indicating the claimed shelf life as appropriate for the assay.

Table 11: Product consistency (lot-to-lot) and stability study of the PathoSEEK Salmonella and STEC E. coli Multiplex Assay, Paired Lot POD comparison

PCR Kit	Lot[f,g,h]	N[a]	x[b]	POD[c]	95% CI	Lots	N	x	POD	95% CI	dPOD[d]	95% CI[e]
Target analyte: *Salmonella* Typhimurium ATCC 13311												
Salmonella and STEC E. coli Multiplex	A	10	8	0.80	0.49, 0.94	B	10	7	0.70	0.40, 0.89	0.10	-0.27, 0.44
	B	10	7	0.70	0.40, 0.89	C	10	7	0.70	0.40, 0.89	0.00	-0.36, 0.36
	C	10	7	0.70	0.40, 0.89	A	10	8	0.80	0.49, 0.94	-0.10	-0.44, 0.27
Target analyte: *E. coli* O157:H7 ATCC 35150												
Salmonella and STEC E. coli Multiplex	A	10	7	0.70	0.40, 0.89	B	10	6	0.60	0.31, 0.83	0.10	-0.28, 0.45
	B	10	6	0.60	0.31, 0.83	C	10	8	0.80	0.49, 0.94	-0.20	-0.52, 0.18
	C	10	8	0.80	0.49, 0.94	A	10	7	0.70	0.40, 0.89	0.10	-0.27, 0.44
Non-target analyte: *E. coli ATCC# 25922*												
Salmonella and STEC E. coli Multiplex	A	10	0	0.00	0.00, 0.28	B	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
	B	10	0	0.00	0.00, 0.28	C	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
	C	10	0	0.00	0.00, 0.28	A	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28

[a]N = Number of test portions.
[b]x = Number of positive test portions.
[c]POD = Positive outcomes divided by the total number of trials.
[d]$dPOD_{AB}$ = Difference in POD between the paired comparison.
[e]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[f]Lot A – 3770003, newly manufactured; [g]Lot B – 3757347, mid-shelf life; [h]Lot C – 4219995, end of shelf life.

Discussion

The PathoSEEK Salmonella and STEC E. coli Multiplex Assay with SenSATIVAx Extraction Protocols successfully detected *Salmonella* species and STEC from dried cannabis flower (>0.3% THC), cannabis concentrates and THC-infused chocolate. POD statistical analysis indicated no difference between the candidate presumptive and confirmed results for the matrices evaluated. Minimal discrepant results were observed during the method developer matrix studies. One false negative for STEC and *Salmonella* were observed at 16 h which were detected at 24 h indicating that the concentration of the organism is just at the LOD of the method in these samples at the earlier time point. The independent laboratory did have more discrepant results than the method developer and did not recover STEC at 16 h on the CFX-96 as well as on the AriaMx. This may be a result of the performance of the instrument being impacted by the cannabinoid profile with this particular strain of cannabis flower. Based on the results from the independent laboratory and the variability in cannabinoid profile of flower matrix and the impact it has on the growth rates of the target organisms, it may benefit end users to extend the incubation time beyond the minimum to achieve optimal results. Overall, the assay produced 725 out of 730 accurate results for the method developer and 228 out of 240 accurate results for the independent laboratory, with 7 of these results coming for one matrix at one time point for one analyte.

In the inclusivity and exclusivity evaluations, all inclusivity organisms were correctly identified for both *Salmonella* and STEC. One exclusivity organism (*Shigella dysenteriae*) was detected in the STEC channel by the candidate method; however, this strain contains the *stx* genes targeted by the assay and would be expected to be detected. For the lot-to-lot consistency and stability study, results indicated no statistical significant differences observed between lots

and that results support the claimed shelf life is appropriate. Using POD analysis, the robustness study indicated no impact of minor variations on the performance of the assay.

Conclusion

The data from these studies, within their statistical uncertainty, support the product claims of the PathoSEEK Salmonella and STEC E. coli Multiplex Assay with SenSATIVAx Extraction Protocols (Flower/Leaf and MIP/Extracts) for dried cannabis flower (10 g, >0.3% THC), cannabis concentrate (5 g) and THC-infused chocolate (25 g). The results obtained by the POD analysis of the method comparison study demonstrated that there were no statistically significant differences between the number of positive samples detected by the candidate method and the culturally confirmed results. The validation study met the requirements as set forth in the AOAC Standard Method Performance RequirementsSM 2020.002 for the detection of Salmonella species and 2020.012 for the detection of Shiga toxin producing E.coli (STEC) in cannabis and cannabis infused products.

Method Authors

Kevin McKernan and Yvonne Helbert

Medicinal Genomics

100 Cummings Center, Suite 406L

Beverly, MA 01915

Submitting Company

Medicinal Genomics

100 Cummings Center, Suite 406L

Beverly, MA 01915

Independent Laboratory

TEQ Analytical Laboratories

12635 E. Montview Blvd., Suite 175

Aurora, CO 80045

Reviewers

Yvonne Salfinger,

Independent Consultant, Colorado, USA 80517

Jim Agin

Ohio Department of Agriculture (Retired), Columbus OH 45214

Wayne Ziemer

USDA FERN (Retired) 1301 Kristen Ln., Loganville, GA 30052

References

1. Bird et. al (2021). *Laboratory Safety and Compliance Testing for Microorganisms Contamination in Marijuana.* Cannabis Laboratory Fundamentals. Pp. 281-318
2. AOAC INTERNATIONAL**.** 2019. *Cannabis Analytical Science Program Prospectus*

3. AOAC INTERNATIONAL**.** 2020. *Standard Methods Performance Requirements (SMPR) 2020.002 Detection of Salmonella species in Cannabis and Cannabis Products*. https://www.aoac.org/wp-content/uploads/2020/07/SMPR-2020_002.pdf [Accessed October 2021]
4. AOAC INTERNATIONAL**.** 2020. *Standard Methods Performance Requirements (SMPR) 2020.012 Detection of Shiga-Toxin Producing E. coli in Cannabis and Cannabis Products*. https://www.aoac.org/wp-content/uploads/2020/07/SMPR-2020_002.pdf [Accessed October 2021]
5. Official Methods of Analysis (2019) 21st Ed., Appendix J: AOAC INTERNATIONAL, Rockville, MD, http://www.eoma.aoac.org/app_j.pdf [Accessed October 2021]
6. U.S. Food and Drug Administration (2019) *Bacteriological Analytical Manual*, Chapter 5 Salmonella. https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam-chapter-5-salmonella [Accessed October 2021]
7. USDA/FSIS MLG 4.11: Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg, and Siluriformes (Fish) Products and Carcass and Environmental Sponges. Updated: August 2021. https://www.fsis.usda.gov/sites/default/files/media_file/2021-08/MLG-4.11.pdf [Accessed October 2021]
8. U. S. Food and Drug Administration *Bacteriological Analytical Manual*, Chapter 3 *Aerobic Plate Count* (January 2001) (Accessed October 2021) https://www.fda.gov/food/laboratory-methods-food/bam-aerobic-plate-count
9. Least Cost Formulations, Ltd., MPN Calculator Version 1.6

10. Wehling, P., LaBudde, R., Brunelle, S., & Nelson, M. (2011) Probability of detection (POD) as a statistical model for the validation of qualitative methods. *J. AOAC Int.*, **94**, 335-347.