Validation of the PathoSEEK® 5-Color Aspergillus Multiplex Assays with SenSATIVAx® Extraction for the Detection of *Aspergillus* in Dried Cannabis Flower and THC-infused Chocolate

AOAC *Performance Tested Method*^{SM} 082102

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Abstract:

Background: States currently require cannabis flower and cannabis products to be free of select species of *Aspergillus.* The PathoSEEK® 5-Color Aspergillus Multiplex Assays with SenSATIVAx® Extraction Protocol are designed to detect *Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus,* and *Aspergillus terreus* in a single qPCR reaction.

Objective: To evaluate the PathoSEEK 5-Color Aspergillus Multiplex Assays with SenSATIVAx Extraction Protocol according to AOAC validation requirements (Appendix J) : and *Standard Method Performance Requirements*^{SM} 2019.001.

Methods: Dried cannabis flower (delta 9-tetrahydrocannabinol (THC) >0.3%; 10 g sample size) and THC-infused chocolate 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 thermocyclers: CFX-96 and AriaMx.

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 most variations in the method. Inclusivity and exclusivity testing demonstrated the method was highly specific for the target organisms with minimal cross-reaction to other *Aspergillus* species.

Conclusion: The PathoSEEK 5-Color Aspergillus Multiplex Assays with SenSATIVAx Extraction Protocol provides the cannabis industry a validated multiplex qPCR assay for the detection of *A. fumigatus, A. niger, A. flavus,* and *A. terreus* in dried cannabis flower (>0.3% THC) and THC-infused chocolate.

Highlights: Using the PathoSEEK 5-Color Aspergillus Multiplex with SenSATIVAx Extraction Protocols allows an end user to simultaneously detect 4 species of *Aspergillus* from a 24 - 48 h enrichment.

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 simple two-step protocol (DNA extraction followed by RT-PCR analysis) which is flexible and automation compatible.

The PathoSEEK 5 – Color Aspergillus Multiplex microbial detection assays use a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of 4 species of *Aspergillus* as well as cannabis DNA in every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting pathogens, resulting in the minimization of false negative results due to reaction set-up errors or failing experimental conditions.

The PathoSEEK process includes real-time quantitative PCR assays using a multiplex system of primers to detect potential pathogens within the plant, extract or MIP (Marijuana Infused Product) sample. Below is a simplified depiction of the qPCR assays. The forward and reverse primers have universal primer tails to enable potential Next Generation Sequencing of resulting products.

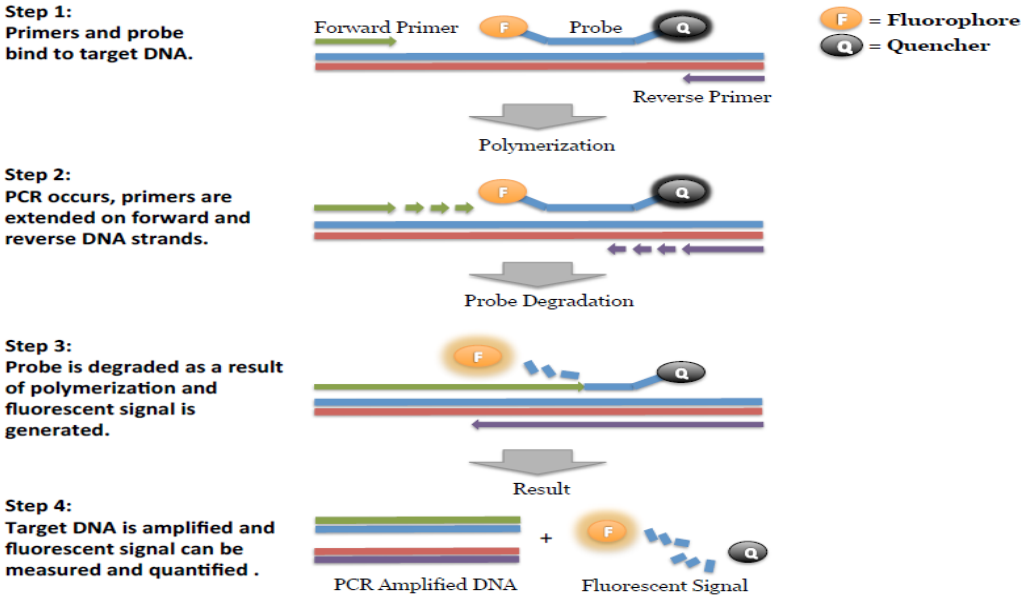

Figure 1: Overview of qPCR

Two multiplex assays are available for use, dependent on the thermocycler used for analysis. The PathoSEEK 5 – Color Aspergillus Multiplex Assay for use on the AriaMx Real-Time PCR Thermocycler (Agilent) uses the ATTO 425 Fluorophore for detection of the *Aspergillus terreus*, while the PathoSEEK 5- Color Aspergillus Multiplex Assay for use on the CFX-96 (Bio-Rad) uses the Cy5.5 Fluorophore for the detection of *Aspergillus terreus*. Fluorophores for the three additional Aspergillus species are consistent between the two thermocyclers. Both PCR assays will be validated in the study.

SenSATIVAx is a proprietary DNA isolation process that uses magnetic particles to isolate and purify both plant and microbial DNA from a raw, homogenized plant sample. The SenSATIVAx approach is designed for ease of use and minimal requirement of laboratory equipment. Large centrifuges have been replaced with lightweight minicentrifuges, magnetic particles, and magnets. The use of magnetic particles affords 8 or 96 tip automation, enabling both minimal entry costs and high throughput applications. DNA can be isolated from a single sample or a large batch in under 1 hour. Hands-on time is less than 45 minutes.

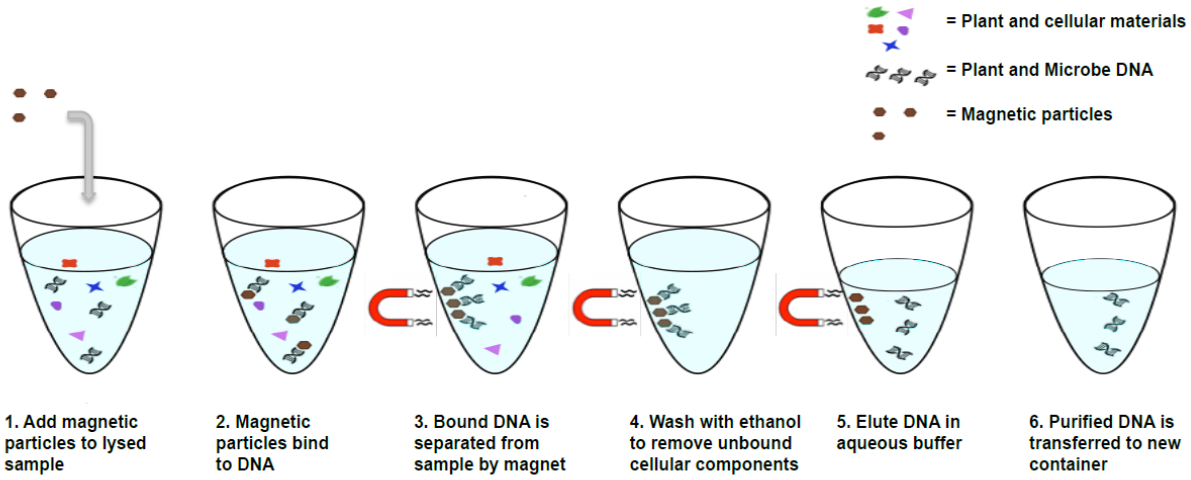

Figure 2: SenSATIVAx Extraction Protocol

General Information

Aspergillosis is a common term used to describe infections caused by over 40 different species of *Aspergillus*. *Aspergillus fumigatus* is the leading cause of cases of aspergillosis followed by *Aspergillus flavus* and *Aspergillus niger*. A fourth species, *Aspergillus terreus,* while less commonly found, has the highest mortality rate of all *Aspergillus* spp. (1). Recent research has confirmed the prevalence of *Aspergillus* in cannabis markets from several states (2). In the cannabis industry, several states require that dried cannabis flower and infused product be evaluated for the presence of these *Aspergillus* species (2).

Scope of Methods

(a) *Target organisms.*—*Aspergillus flavus, A. fumigatus, A. niger* and *A. terreus*.

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

(c) *Summary of Validated Performance Claims.*—The PathoSEEK 5-Color Aspergillus Multiplex Assays with SenSATIVAx® Extraction Protocols met the requirements of the *Standard Method Performance Requirement* (SMPR) *for Detection of Aspergillus in Cannabis and Cannabis Products* 2019.001 (3) and AOAC Appendix J: AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces (4).

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 5-Color Aspergillus Multiplex Assay with SenSATIVAx Extraction Protocol

Test Kit Components

(a) *PathoSEEK 5-Color* Aspergillus Multiplex Assay for (AriaMX)

(1) MGC P/N 420148.

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

(b) *PathoSEEK 5-Color* Aspergillus Multiplex Assay for (CFX96)

(1) MGC P/N 420147.

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

(c) *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

(d) *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

(e) *PathoSEEK Aspergillus Multiplex Positive Control*—P/N 420330 (50 reactions)

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

(f) *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) *Aspergillus* spp. includes many species, about 40 of which have been implicated in human or animal infections. Aspergillosis is a common term used to describe infections caused by different species of *Aspergillus*. Most cases of aspergillosis are caused by *A. fumigatus*, with *A. flavus* and *A. niger* being the second most common pathogenic *Aspergillus* spp. worldwide. Diseases caused by *Aspergillus* spp. include clinical allergies (allergic bronchopulmonary aspergillosis, rhinitis, Farmer's lung), superficial and local infections (cutaneous infections, otomycosis, tracheobronchitis), infections associated with damaged tissue (aspergilloma, osteomyelitis), and invasive pulmonary and extrapulmonary infections. Invasive infections due

to *Aspergillus* spp. occur mainly in immunocompromised individuals and are the most severe forms of infections caused by *Aspergillus* spp (1).

(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 pour the approximate amount of TSB into another sterile tube or container 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 48 h at 37 ± 1°C. For the PTM validation, 10 g of dried cannabis flower (>0.3% THC) was enriched with 90 mL TSB.

(i) *THC- infused chocolate, **n** grams.* — Weigh THC-infused chocolate 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 24 h at 37 ± 1°C. For the PTM validation, 25 g of THC-infused chocolate was enriched with 60 mL TSB.

(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. See flower example in Figure 3.

Figure 3: Homogenized dried cannabis flower (>0.3% THC) and TSB.

DNA Extraction – SenSATIVAx Flower/Leaf DNA Extraction

(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. See Figure 4.

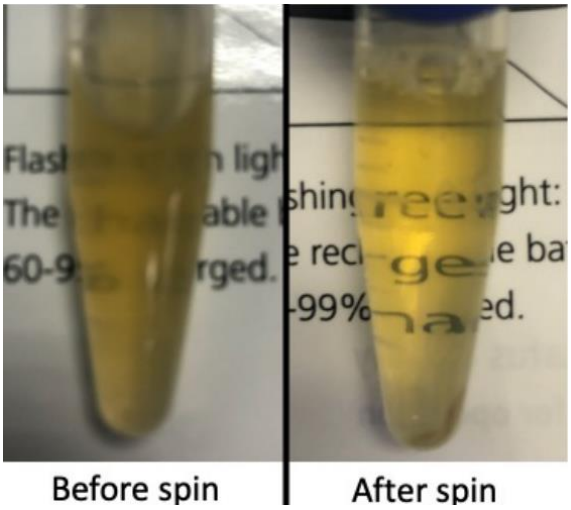

Figure 4: Example of translucent lysate after spinning.

(d) Remove the 200µL 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 200µL 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 200µL of MGC Binding Buffer to each sample, and pipette tip mix 15 times.

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

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

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

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

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

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

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

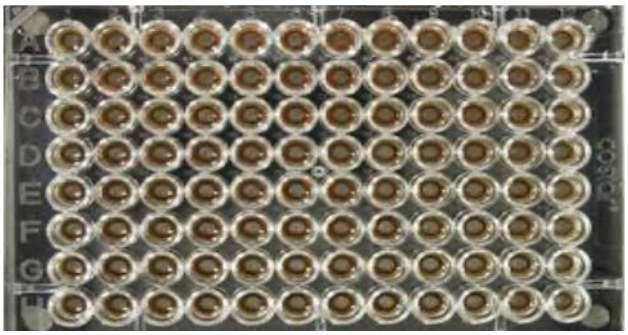

Figure 5: Extraction plate during wash step on magnetic plate.

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

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

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

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

(l) Remove the extraction plate from the magnet plate and add 25 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. See Figure 6.

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

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

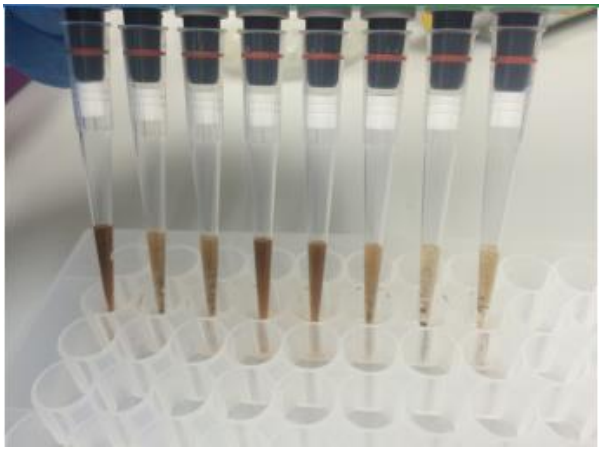

Figure 6: Multichannel pipette tips showing magnetic beads resuspended in elution buffer.

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

DNA Extraction – SenSATIVAx MIP/Extracts

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

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

the SCCG 1:50 dilution. Vortex to mix thoroughly and quick spin tube. This will result in a 1:5,000 dilution of SCCG.

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

e) Centrifuge for 10 minutes at 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 ul of the solution to a new tube. Push pipet tip through the top solid layer (if one exists), without disturbing the pellet on the bottom to aspirate the sample.

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

Note: This may require longer vortexing for some matrices

Caution: ALWAYS WEAR GLOVES WHEN HANDLING CHLOROFORM

Fig 7. Example of milky sample with chloroform in it.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

s) Remove the extraction plate from the magnet plate and add 25 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 2: 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 3: Summary of Results by Channel AriaMX

PathoSEEK™ Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g) (This may vary by state)
Aspergillus niger	≤ 40	ROX	> 40	Presence/Absence
Aspergillus flavus	≤ 40	Cy5	> 40	Presence/Absence
Aspergillus fumigatus	≤ 40	FAM	> 40	Presence/Absence
Aspergillus terreus	≤ 40	ATTO 425	> 40	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Control	≤35	FAM/ROX/Cy5/ATTO 425		

Table 4: Summary of Results by Channel Bio-Rad CFX96

PathoSEEK™ Assay	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g) (This may vary by state)
Aspergillus niger	≤ 40	ROX	> 40	Presence/Absence
Aspergillus flavus	≤ 40	Cy5	> 40	Presence/Absence
Aspergillus fumigatus	≤ 40	FAM	> 40	Presence/Absence
Aspergillus terreus	≤ 40	Cy5.5	> 40	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Control	≤35	FAM/ROX/Cy5/Cy5.5		

Confirmation of Positive Results

All positive results should be confirmed according to the following protocol. Mix enriched sample thoroughly by hand.

(a) From the primary enrichment, perform an isolation streak to a fungal specific agar (Potato Dextrose Agar [PDA] or Dichloran Rose Bengal Agar [DRBC]). Incubate at 25 ± 2°C for 5-7 days.

(b) Confirm the presence of *Aspergillus* using morphological characteristics of the colony growth and identify the typical conidial head of *Aspergillus* using a compound microscope. See Table 4 for more details

Table 4: Morphological Characteristics of *Aspergillus*

Target	Surface Colony Description (5)	Reverse Colony Description
Aspergillus flavus	Yellow-green	Goldish to red brown
Aspergillus fumigatus	Blue-green to gray	White to tan
Aspergillus niger	Black	White to yellow
Aspergillus terreus	Cinnamon to brown	White to brown

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 5-Color Aspergillus Multiplex Assays with SenSATIVAx Extraction Protocols was evaluated for two matrices (dried cannabis flower (>0.3% THC) 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 Cambium Analytica (Traverse City, MI).

Inclusivity/Exclusivity

Methodology.— For inclusivity evaluation, 50 strains of target *Aspergillus* were tested. Thirteen (13) *A. flavus*, 13 *A. fumigatus*, 11 *A. niger* and 13 *A. terreus* strains were grown for 24 hours at 37 ± 1°C in TSB. The cultures were diluted to 100 x the Limit of Detection (LOD) of the method. For the exclusivity evaluation, a total of 30 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 5 and 6. Of the 50 inclusivity strains for the *Aspergillus*, all 50 inclusivity organisms were correctly identified. For the exclusivity, 27 of the 30 exclusivity organisms were correctly excluded. *A. oryzae* (ATCC 10124) and *A. parasiticus* (ATCC 15517) tested positive for *A. flavus*, and *A. pseudoterreus* (ATCC 10020) tested positive for *A. terreus*.

Table 5. Inclusivity List: *Aspergillus* species

No.	Organism	Source[a]	Origin	Result SenSATIVax Flower AriaMx[b]	Result SenSATIVax Flower CFX-96[b]
1	*Aspergillus flavus*	ATCC 9643	Shoe sole, New Guinea	+	+
2	*Aspergillus flavus*	ATCC 26768	Dry cracower sausage, Poland	+	+
3	*Aspergillus flavus*	ATCC 16870	Butter, Japan	+	+
4	*Aspergillus flavus*	ATCC 16883	Cellophane. South Pacific	+	+
5	*Aspergillus flavus*	ATCC 11489	Not Available	+	+
6	*Aspergillus flavus*	ATCC 11497	Not Available	+	+
7	*Aspergillus flavus*	ATCC204304	Human sputum, Virginia	+	+
8	*Aspergillus flavus*	ATCC MYA-1004	Clinical Isolate, New York City, NY	+	+
9	*Aspergillus flavus*	ATCC MYA-200026	Peanut cotyledons, USA	+	+

10	*Aspergillus flavus*	ATCC 12693	Soil	+	+
11	*Aspergillus fumigatus*	ATCC 16903	Human chest cavity lining, Illinois	+	+
12	*Aspergillus fumigatus*	ATCC1022	Lung of chicken, Connecticut	+	+
13	*Aspergillus fumigatus*	ATCC 1028	Soil, Germany	+	+
14	*Aspergillus fumigatus*	ATCC 96918	Not Available	+	+
15	*Aspergillus fumigatus*	ATCC 13073	Human pulmonary lesion, Maryland	+	+
16	*Aspergillus fumigatus*	ATCC 26934	Human with Aspergillosis disease, Montana	+	+
17	*Aspergillus fumigatus*	ATCC 14110	Human sputum	+	+
18	*Aspergillus fumigatus*	ATCC 204305	Human sputum, Virginia	+	+
19	*Aspergillus fumigatus*	ATCC MYA-3626	California, USA	+	+
20	*Aspergillus fumigatus*	ATCC MYA-3627	Jacksonville, TN, USA	+	+
21	*Aspergillus niger*	ATCC 15475	Soil	+	+
22	*Aspergillus niger*	ATCC 16888	Not Available	+	+
23	*Aspergillus niger*	ATCC 9142	Not Available	+	+
24	*Aspergillus niger*	ATCC 9029	Not Available	+	+
25	*Aspergillus niger*	ATCC 10535	Painted pine board, Virginia	+	+
26	*Aspergillus niger*	ATCC 13496	Soil, Louisiana	+	+
27	*Aspergillus niger*	ATCC 1015	Not Available	+	+
28	*Aspergillus niger*	ATCC 6275	Leather	+	+
29	*Aspergillus niger*	ATCC 66295	Jerusalem artichoke tuber, Korea	+	+
30	*Aspergillus niger*	ATCC 201201	Farmland, Korea	+	+
31	*Aspergillus terreus*	ATCC 16793	Soil, Texas	+	+
32	*Aspergillus terreus*	ATCC 1012	Soil, Connecticut	+	+
33	*Aspergillus terreus*	ATCC 10690	Haversack, New Guinea	+	+
34	*Aspergillus terreus*	ATCC 16794	Not Available	+	+
35	*Aspergillus terreus*	ATCC 24839	Not Available	+	+
36	*Aspergillus terreus*	ATCC 52430	Decayed timber, India	+	+
37	*Aspergillus terreus*	ATCC 12238	Not Available	+	+
38	*Aspergillus terreus*	ATCC MYA-4897	Soil, Baghdad, Iran	+	+
39	*Aspergillus terreus*	ATCC MYA-4898	Not Available	+	+
40	*Aspergillus terreus*	ATCC 26604	Ankylosing spondylitis	+	+
41	*Aspergillus flavus*	ATCC 13697	Not Available	+	+
42	*Aspergillus flavus*	ATCC MYA-4921	Not Available	+	+
43	*Aspergillus flavus*	ATCC MYA-3631	Human biopsy, USA	+	+
44	*Aspergillus fumigatus*	ATCC MYA-4690	Not Available	+	+
45	*Aspergillus fumigatus*	ATCC MYA-4915	Human sputum, Shanghai, China	+	+
46	*Aspergillus fumigatus*	ATCC 16424	Human lung with aspergillosis, Pennsylvania	+	+
47	*Aspergillus niger*	ATCC 208815	Derived from ATCC 9029	+	+
48	*Aspergillus terreus*	ATCC 20542	Not Available	+	+
49	*Aspergillus terreus*	ATCC 20064	Not Available	+	+

50	*Aspergillus terreus*	ATCC 32587	Gamma mutant of NRRL 1960	+	+

[a]ATCC – American Type Culture Collection, Manassas, VA; [b](+) = positive, (-) = negative

Table 6. Exclusivity List

No.	Organism	Source[a]	Origin	Result SenSATIVax Flower	
				AriaMx[b]	CFX-96[b]
1	*Acinetobacter baumanii*	ATCC 19606	Urine	-	-
2	*Alternia alternata*	ATCC 6663	Not Available	-	-
3	*Aspergillus aculeatus*	ATCC 24147	Not Available	-	-
4	*Aspergillus brasiliensis*	ATCC 9642	Wireless radio equipment, New South Wales, Australia	-	-
5	*Aspergillus casiellus*	ATCC 42693	Dried chilies, New Guinea	-	-
6	*Aspergillus carbonarius*	ATCC MYA-4641	Grape berry, Brindis, Apulia, Italy	-	-
7	*Aspergillus clavatus*	ATCC 1007	Not Available	-	-
8	*Aspergillus deflectus*	ATCC 62502	Wheat, China	-	-
9	*Aspergillus fijiensis*	ATCC 20611	Not Available	-	-
10	*Aspergillus niveo-glaucus*	ATCC 10075	Not Available	-	-
11	*Aspergillus janponicus*	ATCC 16873	Soil, Panama	-	-
12	*Aspergillus nidulans*	ATCC 38163	Not Available	-	-
13	*Aspergillus oryzae*	ATCC 10124	Not Available	+[c]	+
14	*Aspergillus parasiticus* Speare	ATCC 15517	Not Available	+[c]	+
15	*Aspergillus pseudoterreus*	ATCC 10020	Not Available	+[d]	+
16	*Aspergillus tamarii*	ATCC 1005	tomato	-	-
17	*Aspergillus tubingensis*	ATCC 1004	Not Available	-	-
18	*Aspergillus tubingensis*	ATCC 10550	Not Available	-	-
19	*Aspergillus ustus*	ATCC 1041	Culture containment, USA	-	-
20	*Aspergillus versicolor*	ATCC 11730	Cellophane gas mask, India	-	-
21	*Botrytis cinerea* Persoon	ATCC 11542	Azalea flowers, Washington, D.C.	-	-
22	*Candida albicans*	ATCC 10231	Man with bronchomycosis	-	-
23	*Cryptococcus laurentii*	ATCC 18803	Palm wine, malaffou, Congo	-	-
24	*Cryptococcus neoformans*	ATCC 208821	Patient with Hodgkin's disease, New York	-	-
25	*Fusarium proliferatum*	ATCC 76097	Raw cotton, North Carolina	-	-
26	*Fusarium oxysporum*	ATCC 62506	Celery, *Apium graceolens* var. *dulce*, California, USA	-	-

27	*Fusarium solani*	ATCC 52628	Cardamom fruit pod, *Elettaria* cardamomum, Guatemala	-	-
28	*Mucor circinelloides*	ATCC 38592	N/A	-	-
29	*Mucor hiemalis*	ATCC 28935	Soil in spruce forest, Germany	-	-
30	*Penicillium chrysogenum*	ATCC 18476	Cheese?, USSR	-	-
31	*Penicillium rubens*	ATCC 11709	Selected from Wis. 48-701, after N-mustard exposure	-	-
32	*Penicillium venetum*	ATCC 16025	*Hyacinthus* sp. Bulb, England	-	-
33	*Pseudomonas aeruginosa*	ATCC 35554	Not Available	-	-
34	*Rhizopus stolonifer*	ATCC 14037	Not Available	-	-
35	*Yarrowia lipolytica*	ATCC 20390	Nonsporulating diploid	-	-

[a]ATCC – American Type Culture Collection, Manassas, VA; [b](+) = positive, (-) = negative; [c] Cross reacts with *A. flavus*; [d] Cross reacts with *A. terreus*

Matrix Studies

Methodology.— The PathoSEEK 5 - Color Aspergillus Multiplex Assays with SenSATIVAx Extraction protocol was validated for two matrices, dried cannabis flower (10 g, >0.3% THC) 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 (Cambium Analytica, Traverse City, MI). The study was conducted following guidance defined in AOAC SMPR 2019.001 and AOAC Appendix J. Dried cannabis flower (>0.3% THC) was evaluated at 48 h of enrichment and THC-infused chocolate at 24 h of enrichment by the candidate method. Regardless of the presumptive results, all matrix enrichments were culturally confirmed after 48 h enrichment.

Matrices was obtained following local state regulations and prescreened for natural contamination of *Aspergillus* with the PathoSEEK 5 - Color Aspergillus Multiplex Assay with SenSATIVAx Extraction protocols and plating onto DRBC agar. No natural contamination was found with either the rapid or culture plate method, so the matrices were artificially contaminated. Total aerobic plate count was determined following the FDA BAM Chapter 3: Aerobic Plate Count (6). Dried cannabis flower (>0.3% THC) was inoculated using a dry inoculum and THC-infused chocolate was evaluated with a heat stressed liquid culture.

For the inoculation of the dried cannabis flower (>0.3% THC), lyophilized pellets of the *A. niger* (ATCC 16888) were crushed and mixed with 10g of finely ground dried cannabis (>0.3% THC), that mixture was then added to a large container containing dried cannabis flower (>0.3% THC). The flower 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 THC-infused chocolate, *A. fumigatus* (ATCC MYA-4609) was heat stressed for 10 min at 50°C to achieve injury. To determine the level of injury, the culture was plated onto selective (DRBC) and non-selective agar (PDA) agars. 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 culture 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 inoculum were added dropwise to melted, tempered chocolate and mixed by hand. After inoculation, the chocolate was separated into test portions, allowed to harden and held for 2 weeks at 20-25°C 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 (~20 CFU/test portion). For the 10 g test portions (dried cannabis flower (>0.3% THC)), 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 (THC- infused chocolate), 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. Each test portion for MPN

determination was enriched following the alternative method protocol and confirmed following cultural procedures by plating onto DRBC. 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 (7).

PathoSEEK 5 – Color Aspergillus 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 confirmed.

Confirmation.— To confirm the absence or presence of target *Aspergillus* strains, all sample enrichments were streaked after 48 h to DRBC and PDA and incubated at 26 ± 1°C for 5-7 days. The presence of *Aspergillus* was confirmed using morphological characteristics of the colony growth on agar and identifying the typical conidial head of the specific *Aspergillus* spp. using a compound microscope by a trained and competent microscopist.

Results.— Aerobic plate count results for the dried cannabis flower (>0.3% THC) were 1.0 x 10^4 CFU/g. As per criteria outlined in Appendix J, fractional positive results were obtained at the low level of inoculation for both matrices. Method comparison results and statistical analyses are presented in Table 7. The POD comparisons (8) between the PathoSEEK 5 – Color Aspergillus Multiplex Assays with SenSATIVAx Extraction protocol presumptive and confirmed results indicated there was no significant difference at the 5% level for either matrix and on either thermocycler. POD results obtained by the independent laboratory were lower than the method developer. These results reflect variances in inoculum levels used in the study and are not a reflection of the performance of the assay.

Table 7: PathoSEEK 5 – Color Aspergillus Multiplex Assays with SenSATIVAx Extraction Presumptive vs Confirmed Results (Paired) – POD Results

Matrix and Inoculum	PCR Thermocycler	MPN_a / Test Portion	N^b	x^c	Presumptive		x	Confirmed		$dPOD_{cp}{}^f$	95% CI^g
					$POD_{cp}{}^d$	95% Cl		$POD_{cc}{}^e$	95% Cl		
Dried cannabis flower (>0.3% THC) 10g (*Aspergillus niger* ATCC 16888)	AriaMX	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.02 (0.57, 1.74)	20	11	0.55	0.34, 0.75	11	0.55	0.34, 0.75	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
	CFX-96	NA	5	0	0.00	-0.47, 0.47	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		1.02 (0.57, 1.74)	20	10	0.50	0.30, 0.70	11	0.55	0.34, 0.75	-0.05	-0.21, 0.11
		>10 (8.00, >30.0)	5	5	0.00	-0.47, 0.47	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
THC-Infused Chocolate 25g (*Aspergillus fumigatus* ATCC MYA-4609)	AriaMX	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		2.85 (1.64, 4.96)	20	14	0.70	0.48, 0.86	12	0.60	0.39, 0.78	0.10	-0.08, 0.28
		>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
	CFX-96	NA	5	0	0.00	-0.47, 0.47	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		2.85 (1.64, 4.96)	20	13	0.65	0.43, 0.82	12	0.60	0.39, 0.78	0.05	-0.11, 0.21
		>10 (8.00, >30.0)	5	5	0.00	-0.47, 0.47	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Dried cannabis flower (>0.3% THC) 10g	AriaMX	NA	5	0	0.00	-0.47, 0.47	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.48 (0.24,0.80)	20	6	0.30	0.14, 0.52	5	0.25	0.11, 0.47	0.05	-0.16, 0.26

(*Aspergillus niger* ATCC 16888*)* - Independent Laboratory		>10 (8.00, >30.0)	5	5	1.00	-0.47, 0.47	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
	CFX-96	NA	5	0	0.00	-0.47, 0.47	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.48 (0.24,0.80)	20	6	0.30	0.14, 0.52	5	0.25	0.11, 0.47	0.05	-0.16, 0.26
		>10 (8.00, >30.0)	5	5	1.00	-0.47, 0.47	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.

Robustness Study

Methodology.—All the assays were carried out by the method developer. Parameters varied for the SenSATIVAx Plant/Leaf DNA Extraction protocol followed by PathoSEEK analysis included: lysis buffer volume (40 µL, 60 µL), MGC Binding Buffer volume (180 µL, 220 µL) and master mix: probe volume (3.80:0.8 µL, 3.70:1.2 µL), along with one assay of with the nominal values (50 µL, 200 µL, 2.75/1 µL).

Parameters varied for the SenSATIVAx MIP/Extract DNA Extraction protocol followed by PathoSEEK analysis included: Chloroform volume (280 µL, 320 µL), MGC Binding Buffer volume (180 µL, 220 µL) and master mix: probe volume (3.80:0.8 µL, 3.70:1.2 µL), along with one assay of with the nominal values (300 µL, 200 µL, 2.75/1 µL).

Each parameter was evaluated in dried cannabis flower (>0.3% THC) using both thermocyclers by analyzing the candidate method with ten replicates of a target strain (*A. niger* ATCC 16888) diluted to achieve fractional positive results and ten replicates of non-target organism (*Penicillium chrysogenum* ATCC 18476). POD values and confidence intervals were calculated, 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 the SenSATIVAx Plant/Leaf DNA Extraction, the results for the target organism evaluation combinations 7 and 8 produced statistically significant results with both thermocyclers and combination 4 produced a statistically significant result with the CFX-96 thermocycler. Each of these parameters incorporates a high volume of MGC binding buffer, which may be the cause of the statistical difference.

For the SenSATIVAx MIP/Extract DNA Extraction, the results for the target organism evaluation combinations 5, 7 and 8 produced statistically significant results with the AriaMx thermocycler and combinations 4, 6 and 8 produced a statistically significant result with the CFX-96 thermocycler. A majority of these parameters incorporates a high volume of MGC binding buffer, which may be the cause of the statistical difference. Based on this data, ensuring that correct volumes of MGC binding buffer are transferred during the PCR process is viewed as a critical step.

For the non-target organism, there were 0 presumptive positives out of 10 replicates for test combinations 1-8. For the nominal combination 9, there were 0 presumptive positives out of 10 replicates. No confirmations were performed with target or non-target organism. POD analysis was performed with 95% confidence interval and no statistically significant difference was observed (Tables 8-11).

Table 8: Robustness study of the SenSATIVAx Flower/Leaf DNA Extraction & PathoSEEK – 5 Color Aspergillus AriaMx, POD comparison

	Parameters													
Parameter test combination[a]	Lysis Buffer (µL)	MGC Binding Buffer (µL)	Master Mix/Probe 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 cannabis flower (>0.3% THC) inoculated with *A. niger* ATCC 16888														
1	40 µL	180 µL	3.80:0.8 µL	10	6	0.60	0.31, 0.83	9	10	8	0.80	0.49, 0.94	-0.20	-0.52, 0.19
2	40 µL	180 µL	3.70:1.2 µL	10	7	0.70	0.40, 0.89	9	10	8	0.80	0.49, 0.94	-0.10	-0.44, 0.26
3	40 µL	220 µL	3.80:0.8 µL	10	5	0.50	0.24, 0.76	9	10	8	0.80	0.49, 0.94	-0.30	-0.60, 0.11
4	40 µL	220 µL	3.70:1.2 µL	10	5	0.50	0.24, 0.76	9	10	8	0.80	0.49, 0.94	-0.30	-0.60, 0.11
5	60 µL	180 µL	3.80:0.8 µL	10	7	0.70	0.40, 0.89	9	10	8	0.80	0.49, 0.94	-0.10	-0.44, 0.26
6	60 µL	180 µL	3.70:1.2 µL	10	4	0.40	0.17, 0.69	9	10	8	0.80	0.49, 0.94	-0.40	-0.67, 0.02
7	60 µL	220 µL	3.80:0.8 µL	10	2	0.20	0.06, 0.51	9	10	8	0.80	0.49, 0.94	-0.60	-0.80, -0.16
8	60 µL	220 µL	3.70:1.2 µL	10	3	0.30	0.11, 0.60	9	10	8	0.80	0.49, 0.94	-0.50	-0.74, -0.07
9	50 µL	200 µL	3.75:1 µL	10	8	0.80	0.49, 0.94	9	10	8	0.80	0.49, 0.94	0.00	-0.34, 0.34
Non-target analyte: dried cannabis flower (>0.3% THC) inoculated with *Penicillium chrysogenum* ATCC #18476														
1	40 µL	180 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
2	40 µL	180 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
3	40 µL	220 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
4	40 µL	220 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
5	60 µL	180 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
6	60 µL	180 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
7	60 µL	220 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
8	60 µL	220 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
9	50 µL	200 µL	3.75:1 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 9: Robustness study of the SenSATIVAx Flower/Leaf DNA Extraction & PathoSEEK – 5 Color Aspergillus CFX-96, POD comparison

	Parameters													
Parameter test combination[a]	Lysis Buffer (µL)	MGC Binding Buffer (µL)	Master Mix/Probe 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 cannabis flower (>0.3% THC) inoculated with *A. niger* ATCC 16888														
1	40 µL	180 µL	3.80:0.8 µL	10	5	0.50	0.24, 0.76	9	10	6	0.60	0.31, 0.83	-0.10	-0.45, 0.29
2	40 µL	180 µL	3.70:1.2 µL	10	5	0.50	0.24, 0.76	9	10	6	0.60	0.31, 0.83	-0.10	-0.45, 0.29
3	40 µL	220 µL	3.80:0.8 µL	10	4	0.40	0.17, 0.69	9	10	6	0.60	0.31, 0.83	-0.20	-0.53, 0.21
4	40 µL	220 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	6	0.60	0.31, 0.83	-0.60	-0.83, -0.20
5	60 µL	180 µL	3.80:0.8 µL	10	9	0.90	0.60, 1.00	9	10	6	0.60	0.31, 0.83	0.30	-0.09, 0.60
6	60 µL	180 µL	3.70:1.2 µL	10	4	0.40	0.17, 0.69	9	10	6	0.60	0.31, 0.83	-0.20	-0.53, 0.21
7	60 µL	220 µL	3.80:0.8 µL	10	1	0.10	0.00, 0.40	9	10	6	0.60	0.31, 0.83	-0.50	-0.75, -0.09
8	60 µL	220 µL	3.70:1.2 µL	10	1	0.10	0.00, 0.40	9	10	6	0.60	0.31, 0.83	-0.50	-0.75, -0.09
9	50 µL	200 µL	3.75:1 µL	10	6	0.60	0.31, 0.83	9	10	6	0.60	0.31, 0.83	0.00	-0.37, 0.37
Non-target analyte: dried cannabis flower (>0.3% THC) inoculated with *Penicillium chrysogenum* ATCC #18476														
1	40 µL	180 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
2	40 µL	180 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
3	40 µL	220 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
4	40 µL	220 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
5	60 µL	180 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
6	60 µL	180 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
7	60 µL	220 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
8	60 µL	220 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
9	50 µL	200 µL	3.75:1 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 SenSATIVax MIP/Extract DNA Extraction & PathoSEEK – 5 Color Aspergillus AriaMx, POD comparison

	Parameters													
Parameter test combination[a]	Lysis Buffer (µL)	MGC Binding Buffer (µL)	Master Mix/Probe 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 cannabis flower (>0.3% THC) inoculated with *A. niger* ATCC 16888														
1	40 µL	180 µL	3.80:0.8 µL	10	10	1.00	0.72, 1.00	9	10	7	0.70	0.40, 0.89	0.30	-0.04, 0.60
2	40 µL	180 µL	3.70:1.2 µL	10	9	0.90	0.57,1.00	9	10	7	0.70	0.40, 0.89	0.20	-0.16, 0.52
3	40 µL	220 µL	3.80:0.8 µL	10	9	0.90	0.57,1.00	9	10	7	0.70	0.40, 0.89	0.20	-0.16, 0.52
4	40 µL	220 µL	3.70:1.2 µL	10	6	0.60	0.31, 0.83	9	10	7	0.70	0.40, 0.89	-0.10	-0.45, 0.28
5	60 µL	180 µL	3.80:0.8 µL	10	2	0.20	0.06, 0.51	9	10	7	0.70	0.40, 0.89	-0.50	-0.74, -0.07
6	60 µL	180 µL	3.70:1.2 µL	10	4	0.40	0.17, 0.69	9	10	7	0.70	0.40, 0.89	-0.30	-0.60, 0.12
7	60 µL	220 µL	3.80:0.8 µL	10	1	0.10	0.00, 0.40	9	10	7	0.70	0.40, 0.89	-0.60	-0.82, -0.17
8	60 µL	220 µL	3.70:1.2 µL	10	1	0.10	0.00, 0.40	9	10	7	0.70	0.40, 0.89	-0.60	-0.82, -0.17
9	50 µL	200 µL	3.75:1 µL	10	7	0.70	0.40, 0.89	9	10	7	0.70	0.40, 0.89	0.00	-0.36, 0.36
Non-target analyte: dried cannabis flower (>0.3% THC) inoculated with *Penicillium chrysogenum* ATCC #18476														
1	40 µL	180 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
2	40 µL	180 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
3	40 µL	220 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
4	40 µL	220 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
5	60 µL	180 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
6	60 µL	180 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
7	60 µL	220 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
8	60 µL	220 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
9	50 µL	200 µL	3.75:1 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 11: Robustness study of the SenSATIVax MIP/Extract DNA Extraction & PathoSEEK – 5 Color Aspergillus CFX-96, POD comparison

	Parameters													
Parameter test combination[a]	Lysis Buffer (µL)	MGC Binding Buffer (µL)	Master Mix/Probe 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 cannabis flower (>0.3% THC) inoculated with *A. niger* ATCC 16888														
1	40 µL	180 µL	3.80:0.8 µL	10	10	1.00	0.72, 1.00	9	10	8	0.80	0.49, 0.94	0.20	-0.11, 0.51
2	40 µL	180 µL	3.70:1.2 µL	10	9	0.90	0.57,1.00	9	10	8	0.80	0.49, 0.94	0.10	-0.24, 0.43
3	40 µL	220 µL	3.80:0.8 µL	10	7	0.70	0.40, 0.89	9	10	8	0.80	0.49, 0.94	-0.10	-0.44, 0.26
4	40 µL	220 µL	3.70:1.2 µL	10	3	0.30	0.11, 0.60	9	10	8	0.80	0.49, 0.94	-0.50	-0.74, -0.07
5	60 µL	180 µL	3.80:0.8 µL	10	9	0.90	0.57,1.00	9	10	8	0.80	0.49, 0.94	0.10	-0.24, 0.43
6	60 µL	180 µL	3.70:1.2 µL	10	2	0.20	0.06, 0.51	9	10	8	0.80	0.49, 0.94	-0.60	-0.80, -0.16
7	60 µL	220 µL	3.80:0.8 µL	10	8	0.80	0.49, 0.94	9	10	8	0.80	0.49, 0.94	0.00	-0.34, 0.34
8	60 µL	220 µL	3.70:1.2 µL	10	1	0.10	0.00, 0.40	9	10	8	0.80	0.49, 0.94	-0.70	-0.88, -0.27
9	50 µL	200 µL	3.75:1 µL	10	8	0.80	0.49, 0.94	9	10	8	0.80	0.49, 0.94	0.00	-0.34, 0.34
Non-target analyte: dried cannabis flower (>0.3% THC) inoculated with *Penicillium chrysogenum* ATCC #18476														
1	40 µL	180 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
2	40 µL	180 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
3	40 µL	220 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
4	40 µL	220 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
5	60 µL	180 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
6	60 µL	180 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
7	60 µL	220 µL	3.80:0.8 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
8	60 µL	220 µL	3.70:1.2 µL	10	0	0.00	0.00, 0.28	9	10	0	10	0.00, 0.28	0.00	-0.28, 0.28
9	50 µL	200 µL	3.75:1 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 5 – Color Aspergillus Multiplex Assays and three lots of SenSATIVAx Extraction kits were evaluated for lot-to-lot consistency. One target strain, *A. flavus* ATCC 9643, and 1 non-target strain, *P. chrysogenum* ATCC 18476, 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 of diluted *A. flavus* strain and 10 replicates of undiluted *P. chrysogenum* 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 test portions, there were 0 presumptive positives out of 10 replicates. For the evaluation, there were no observed discrepant results observed for all lots evaluated (Tables 12 and 13). For the low inoculation level, results obtained by POD analysis demonstrated that there were no statistically significant differences between the lots evaluated.

Table 12: Product consistency (lot-to-lot) of the PathoSEEK 5 – Color Aspergillus Multiplex Assays with SenSATIVAx Flower/Leaf DNA Extraction, Paired Lot POD comparison

Extraction Lot	Thermocycler / PCR Lot	N[a]	x[b]	POD [c]	95% CI	Extraction/ Thermocycler Lots	N	x	POD	95% CI	dPOD[d]	95% CI[e]
Target analyte: *A. flavus* ATCC 9643												
A 17719100	AriaMx (C)	10	4	0.40	0.17, 0.69	B/A	10	4	0.40	0.17, 0.69	0.00	-0.37, 0.37
	CFX-96 (A)	10	5	0.50	0.24, 0.77	B/B	10	6	0.60	0.31, 0.83	-0.10	-0.45, 0.29
B A0102180	AriaMx (A)	10	4	0.40	0.17, 0.69	C/B	10	5	0.50	0.24, 0.77	-0.10	-0.45, 0.29
	CFX-96 (B)	10	6	0.60	0.31, 0.83	C/C	10	5	0.50	0.24, 0.77	0.10	-0.29, 0.45
C 18367700	AriaMx (B)	10	5	0.50	0.24, 0.77	A/C	10	4	0.40	0.17, 0.69	0.10	-0.29, 0.45
	CFX-96 (C)	10	5	0.50	0.24, 0.77	A/A	10	5	0.50	0.24, 0.77	0.00	-0.37, 0.37
Non-target analyte: *Penicillium chrysogenum* ATCC #18476												
A 17719100	AriaMx (C)	10	0	0.00	0.00, 0.28	B/A	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
	CFX-96 (A)	10	0	0.00	0.00, 0.28	B/B	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
B A0102180	AriaMx (A)	10	0	0.00	0.00, 0.28	C/B	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
	CFX-96 (B)	10	0	0.00	0.00, 0.28	C/C	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
C 18367700	AriaMx (B)	10	0	0.00	0.00, 0.28	A/C	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
	CFX-96 (C)	10	0	0.00	0.00, 0.28	A/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.

Table 13: Product consistency (lot-to-lot) and Stability of the PathoSEEK 5 – Color Aspergillus Multiplex Assays with SenSATIVAx MIP/Extracts DNA Extraction, Paired Lot POD comparison

Extraction Lot	Thermocycler / PCR Lot	N[a]	x[b]	POD [c]	95% CI	Extraction/ Thermocycler Lots	N	x	POD	95% CI	dPOD[d]	95% CI[e]
Target analyte: *A. flavus* ATCC 9643												
A 17719100	AriaMx (C)	10	5	0.50	0.24, 0.77	B/A	10	6	0.60	0.31, 0.83	-0.10	-0.45, 0.29
	CFX-96 (A)	10	7	0.70	0.40, 0.89	B/B	10	6	0.60	0.31, 0.83	0.10	-0.29, 0.45
B A0102180	AriaMx (A)	10	6	0.60	0.31, 0.83	C/B	10	5	0.50	0.24, 0.77	0.10	-0.29, 0.45
	CFX-96 (B)	10	5	0.50	0.24, 0.77	C/C	10	5	0.50	0.24, 0.77	0.00	-0.37, 0.37
C 18367700	AriaMx (B)	10	5	0.50	0.24, 0.77	A/C	10	4	0.40	0.17, 0.69	0.10	-0.29, 0.45
	CFX-96 (C)	10	5	0.50	0.24, 0.77	A/A	10	5	0.50	0.24, 0.77	0.00	-0.37, 0.37
Non-target analyte: *Penicillium chrysogenum* ATCC #18476												
A 17719100	AriaMx (C)	10	0	0.00	0.00, 0.28	B/A	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
	CFX-96 (A)	10	0	0.00	0.00, 0.28	B/B	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
B A0102180	AriaMx (A)	10	0	0.00	0.00, 0.28	C/B	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
	CFX-96 (B)	10	0	0.00	0.00, 0.28	C/C	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
C 18367700	AriaMx (B)	10	0	0.00	0.00, 0.28	A/C	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
	CFX-96 (C)	10	0	0.00	0.00, 0.28	A/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.

Stability Study

Methodology.— One lot of PathoSEEK 5 – Color Aspergillus Multiplex Assay (AriaMx and CFX-96) and one lot of SenSATIVAx Flower/Leaf DNA Extraction Kit and SenSATIVAx MIP/Extract DNA Extraction were evaluated for stability. One target strain, *A. flavus* ATCC 9643, and 1 non-target strain, *P. chrysogenum* ATCC 18476, 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/DNA Extraction assay was tested with 10 replicates each of diluted *A. flavus* strain and 10 replicates of undiluted *P. chrysogenum* strain in a randomized blind coded fashion. POD values and confidence intervals were calculated, and data analyzed for potential variable detection in during stability

Results.—For the 10 non-target test portions, there were 0 presumptive positives out of 10 replicates. For the evaluation, there were no observed discrepant results observed for all lots evaluated (Tables 14 and 15). For the low inoculation level, results obtained by POD analysis demonstrated that there were no statistically significant differences between the age of the assays evaluated. Note* Stability testing is ongoing and additional data will be provided upon method renewal.

Table 14: Real-Time Stability of the SenSATIVax Protocol Flower & PathoSEEK 5 – Color Aspergillus Assays, Paired Lot POD comparison

Testing Month	Thermocycler	N[a]	x[b]	POD[c]	95% CI[d]
Target analyte: *A. flavus* ATCC #9643					
0	AriaMx	10	6	0.60	0.31, 0.83
	CFX-96	10	5	0.50	0.24, 0.77
3	AriaMx	10	6	0.60	0.31, 0.83
	CFX-96	10	6	0.60	0.31, 0.83
6	AriaMx	10			
	CFX-96	10			
9	AriaMx	10			
	CFX-96	10			
12	AriaMx	10			
	CFX-96	10			
Non-target analyte: *Penicillium chrysogenum* ATCC #18476					
0	AriaMx	10	0	0.00	0.00, 0.28
	CFX-96	10	0	0.00	0.00, 0.28
3	AriaMx	10	0	0.00	0.00, 0.28
	CFX-96	10	0	0.00	0.00, 0.28
6	AriaMx	10			
	CFX-96	10			
9	AriaMx	10			
	CFX-96	10			
12	AriaMx	10			
	CFX-96	10			

[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]95% CI = the confidence interval
[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.

Table 15: Real-Time Stability of the SenSATIVax Protocol MIP/Extract & PathoSEEK 5 – Color Aspergillus Assays, Paired Lot POD comparison

Testing Month	Thermocycler	N[a]	x[b]	POD[c]	95% CI[d]
Target analyte: *A. flavus* ATCC #9643					
0	AriaMx	10	6	0.60	0.31, 0.83
	CFX-96	10	6	0.60	0.31, 0.83
3	AriaMx	10	6	0.60	0.31, 0.83
	CFX-96	10	7	0.70	0.40, 0.89
6	AriaMx	10			
	CFX-96	10			
9	AriaMx	10			
	CFX-96	10			
12	AriaMx	10			
	CFX-96	10			
Non-target analyte: *Penicillium chrysogenum* ATCC #18476					
0	AriaMx	10	0	0.00	0.00, 0.28
	CFX-96	10	0	0.00	0.00, 0.28
3	AriaMx	10	0	0.00	0.00, 0.28
	CFX-96	10	0	0.00	0.00, 0.28
6	AriaMx	10			
	CFX-96	10			
9	AriaMx	10			
	CFX-96	10			
12	AriaMx	10			
	CFX-96	10			

[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]95% CI = the confidence interval

[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.

Instrument Variation Study

Methodology.—- This study was carried out with pure cultures and examined results between three AriaMx and CFX-96 instruments manufactured according to specifications. One target strain, *A. flavus* ATCC 9643, and 1 non-target strain, *P. chrysogenum* ATCC 18476, were incubated in TSB for 24 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 instrument was evaluated with 10 replicates each of diluted *A. flavus* strain and 10 replicates of undiluted *P. chrysogenum* strain in a randomized blind coded fashion. POD values and confidence intervals were calculated, and data analyzed for potential variable detection.

Results.—Results were decoded, and the POD and confidence intervals were calculated for results. The POD analysis indicated that there was no significant difference, with 95% confidence, between instruments. A summary of the POD analysis is presented in Tables 16 and 17.

Table 16: Instrument Variation of PathoSEEK 5 – Color Aspergillus Method, Paired Lot POD comparison

Instrument	Lot age	N[a]	x[b]	POD[c]	95% CI	Instrument	N	x	POD	95% CI	dPOD[d]	95% CI[e]
Target analyte: *Aspergillus flavus* ATCC 9463												
AriaMx – 1		10	8	0.80	0.49, 0.94	2	10	7	0.70	0.40, 0.89	0.10	-0.26, 0.44
AriaMx – 2		10	7	0.70	0.40, 0.89	3	10	7	0.70	0.40, 0.89	0.00	-0.36, 0.36
AriaMx – 3		10	7	0.70	0.40, 0.89	1	10	8	0.80	0.49, 0.94	-0.10	-0.44, 0.26
Non-target analyte: *Penicillium chrysogenum* ATCC #18476												
AriaMx – 1		10	0	0.00	0.00, 0.28	2	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
AriaMx – 2		10	0	0.00	0.00, 0.28	3	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
AriaMx – 3		10	0	0.00	0.00, 0.28	1	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.

Table 17: Instrument Variation of PathoSEEK 5 – Color Aspergillus Method, Paired Lot POD comparison

Instrument	Lot age	N[a]	x[b]	POD[c]	95% CI	Instrument	N	x	POD	95% CI	dPOD[d]	95% CI[e]
Target analyte: *Aspergillus flavus* ATCC 9463												
CFX-96 – 1		10	8	0.80	0.49, 0.94	2	10	7	0.70	0.40, 0.89	0.10	-0.26, 0.44
CFX-96 – 2		10	7	0.70	0.40, 0.89	3	10	7	0.70	0.40, 0.89	0.00	-0.36, 0.36
CFX-96 – 3		10	7	0.70	0.40, 0.89	1	10	8	0.80	0.49, 0.94	-0.10	-0.44, 0.26
Non-target analyte: *Penicillium chrysogenum* ATCC #18476												
CFX-96 – 1		10	0	0.00	0.00, 0.28	2	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
CFX-96 – 2		10	0	0.00	0.00, 0.28	3	10	0	0.00	0.00, 0.28	0.00	-0.28, 0.28
CFX-96 – 3		10	0	0.00	0.00, 0.28	1	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.

Discussion

The PathoSEEK 5-Color Aspergillus Multiplex Assays with SenSATIVAx Extraction Protocol successfully detected *Aspergillus* species from dried cannabis flower (>0.3% THC) and THC-infused chocolate. POD statistical analysis indicated no difference between the candidate presumptive and confirmed results for the matrix evaluated. For THC-infused chocolate, 2 false positive results were obtained using the AriaMx instrument and 1 false positive result obtained using the CFX-96 instrument. Due to the 2 week equilibration time, the target organisms may have become nonviable, but the presence of the organisms DNA detected by the candidate method resulting in the false positive results. This is a limitation of spiking studies and may not be observed in real-world testing. In the independent laboratory study, 2 false positive results and 1 false negative result were observed. A low level of inoculum (0.48 CFU/test portion) was obtained in the independent laboratory study and is not indicative of contamination levels observed in real-world settings. This low level may have led to the discrepancies observed in the study.

In the inclusivity and exclusivity evaluations, all inclusivity organisms were correctly identified. Three exclusivity organisms (*A. oryzae* ATCC 10124, *A. parasiticus* ATCC 15517 and *A. pseudoterreus* ATCC 10020) were detected by the candidate method; however, these strains are recognized as being close neighbors to the target strains (9). For the lot-to-lot consistency and stability study, results indicated no statistical significant differences observed between lots and the results of shelf life data obtained to this point indicate the claimed shelf life is appropriate. Using POD analysis, the robustness study indicated the volume of the MGC Binding Buffer is critical to the performance of the assay and laboratories performing testing should be made aware.

Conclusion

The data from these studies, within their statistical uncertainty, support the product claims of the PathoSEEK 5-Color Aspergillus Multiplex Assays with SenSATIVAx Extraction Protocol for dried cannabis flower (10 g, >0.3% THC) 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 and the confirmed results. The PathoSEEK 5-Color Aspergillus Multiplex Assays with SenSATIVAx Extraction Protocols is a rapid and accurate procedure allowing for the detection of the four target *Aspergillus* species within a couple of hours post enrichment.

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