Validation of the PathoSEEK® 5-Color Aspergillus Multiplex Assays with SenSATIVAx® Extraction for the Detection of *Aspergillus*: Level 2 Method Modification

AOAC *Performance Tested Method*^{SM} 082102

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Abstract:

Background: The PathoSEEK® 5-Color Aspergillus Multiplex Assays with SenSATIVAx® Extraction Protocol is a *Performance Tested Method*^{SM} method approved for the detection of *Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus,* and *Aspergillus terreus* in dried cannabis flower [delta 9-tetrahydrocannabinol (THC) >0.3%] and THC-infused chocolate.

Objective: To extend the claim of the candidate method to cannabis concentrates, and to reduce the incubation time for dried cannabis flower to 24 h.

Methods: Dried cannabis flower and cannabis concentrates 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.

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 a wide range of cannabis matrices including dried cannabis flower (>0.3% THC), THC-infused chocolate and cannabis concentrates.

Highlights: The PathoSEEK 5-Color Aspergillus Multiplex with SenSATIVAx Extraction Protocols can simultaneously detect 4 species of *Aspergillus* after 24 h of primary enrichment.

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).

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.

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.

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), cannabis concentrate (5 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).—P/N 420148.

(b) *PathoSEEK 5-Color* Aspergillus Multiplex Assay for (CFX96).—P/N 420147.

(c) *SenSATIVAx Flower/Leaf DNA Extraction Kit*.—P/N 420001.

(d) *SenSATIVAx MIP/Extract DNA Extraction Kit*.—P/N 420004.

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

(f) *Medicinal Genomics qPCR Master Kit v3*.—P/N 420201.

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 the Tryptic Soy Broth (TSB) into 15 mL or 50 mL conical vials. For the 15 mL conical vials, ~34 vials will be needed. For 50 mL conical vials, 10 conical vials will be needed.

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 24 - 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) *Cannabis concentrates and 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 – 48 h at 37 ± 1°C. For the PTM validation, 5 g of cannabis concentrate was enriched with 12 mL TSB and 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 1.

Figure 1: 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 s then let incubate on the bench for 2 min.

(c) After 2 min incubation, spin for at least 1-3 min 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 2.

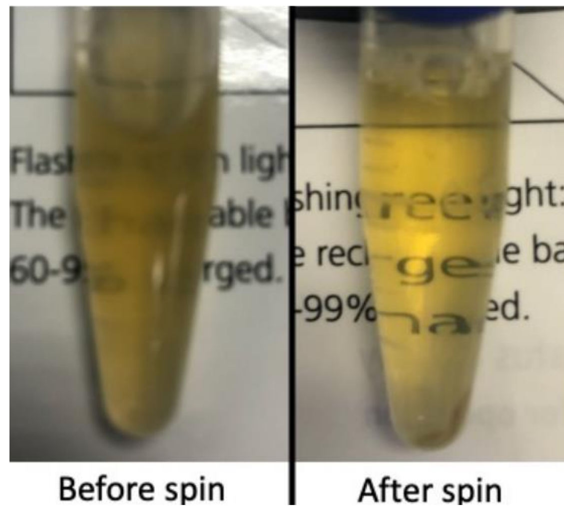

Figure 2: Example of translucent lysate after spinning.

(d) Remove the 200 µL of supernatant from the 1.5 mL 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 s.

(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 at least 5 min.

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

(i) 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. See Figure 3.

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

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

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

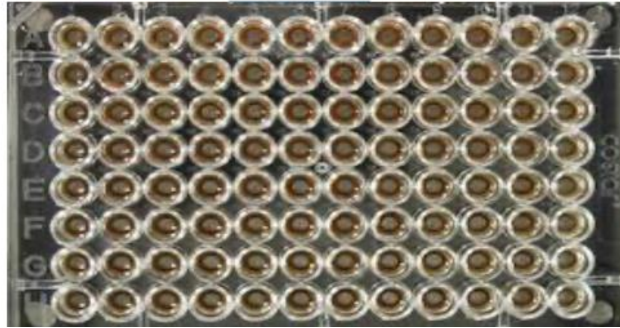

Figure 3: Extraction plate during wash step on magnetic plate.

(j) Repeat 400 µL 70% EtOH wash with the extraction plate still on the magnet plate. Wait at least 30 s 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 min.

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 4.

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

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

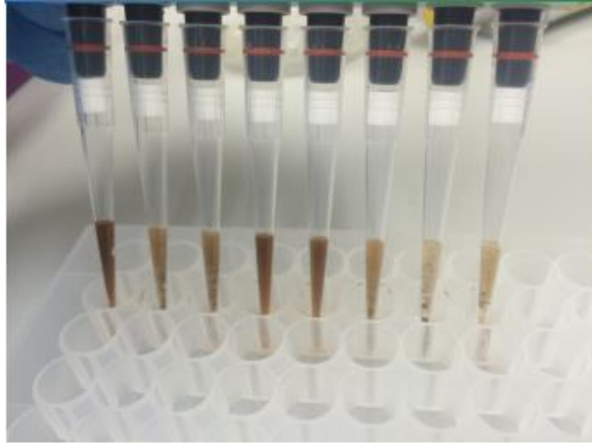

Figure 4: Multichannel pipette tips showing magnetic beads resuspended in elution buffer.

(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 Single Copy Cannabis Gene (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 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 10 µL of the SCCG internal control (1:5,000) to 1.5 mL tube and vortex to mix well.

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

(1) If no bench top centrifuge is available, centrifuge for 15 min 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 5. Example of milky sample with chloroform in it.

(h) Centrifuge for 5 min at 15,000 rcf using a bench top centrifuge.

(i) If no bench top centrifuge is available, centrifuge for 15 min 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 100 uL 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 s.

(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 at least 5 min.

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

(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% EtOH with the extraction plate still on the magnet plate.

(2) Wait at least 30 s 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 s 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 min.

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 min 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.

(s) 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 -20°C 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 1.5 mL 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 min 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: Determination of Results by Channel AriaMX

PathoSEEK™ Target	Fluor	Cq Value	Final Call	Cq Value	Final Call
Aspergillus niger	ROX	≤ 40	Positive	No Cq	Negative
Aspergillus flavus	Cy5	≤ 40	Positive	No Cq	Negative
Aspergillus fumigatus	FAM	≤ 40	Positive	No Cq	Negative
Aspergillus terreus	ATTO 425	≤ 40	Positive	No Cq	Negative
Internal Control*	HEX	≤35	*Internal control verifies the presence or absence of plant DNA		
Assay Positive Control	FAM/ROX/Cy5/ATTO 425	≤35			
Negative Control	FAM/ROX/Cy5/ATTO 425	No Cq			

Table 3: Determination of Results by Channel Bio-Rad CFX96

PathoSEEK™ Target	Fluor	Cq Value	Final Call	Cq Value	Final Call
Aspergillus niger	ROX	≤ 40	Positive	No Cq	Negative

Aspergillus flavus	Cy5	≤ 40	Positive	No Cq	Negative
Aspergillus fumigatus	FAM	≤ 40	Positive	No Cq	Negative
Aspergillus terreus	Cy5.5	≤ 40	Positive	No Cq	Negative
Internal Control*	HEX	≤35	*Internal control verifies the presence or absence of plant DNA		
Assay Positive Control	FAM/ROX/Cy5/Cy5.5	≤35			
Negative Control	FAM/ROX/Cy5/Cy5.5	No Cq			

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 modification study was conducted under the AOAC Research Institute *Performance Tested Method*℠ (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 one new matrix (cannabis concentrate) and a reduction in incubation time for dried cannabis flower (>0.3% THC). All testing was conducted by the method developer (Beverly, MA).

Matrix Studies

Methodology.— The PathoSEEK 5 - Color Aspergillus Multiplex Assays with SenSATIVAx Extraction protocol was validated for one new matrix, cannabis concentrate (5 g), and a reduced incubation time for a currently claimed matrix, dried cannabis flower (10 g, >0.3% THC). The study was conducted following guidance defined in AOAC SMPR 2019.001 and AOAC Appendix J. Each matrix was evaluated after both 24 and 48 h of enrichment by the candidate method. Regardless of the presumptive results, all matrix enrichments were culturally confirmed after 48 h enrichment.

Matrixes were 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 matrixes 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 cannabis concentrates were evaluated using a heat stressed liquid culture.

For the inoculation of the dried cannabis flower (>0.3% THC), lyophilized pellets of *Aspergillus* spp. were crushed and mixed with 10 g of finely ground matrix. This mixture was then added to a large container containing non-contaminated matrix, mixed with sterile mixing utensils and allowed to equilibrate for two weeks at room temperature (20-25°C) prior to testing.

For cannabis concentrates, the culture was grown in TSB for 24 h at 37 ± 1°C. After incubation, the solution 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/20 portions tested) and a high-level expected to yield all positive results. The inoculum was added dropwise to the concentrate 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 5 g test portions (cannabis concentrate), 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 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 matrixes 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 test portions, regardless of presumptive result, were confirmed.

Confirmation.— To confirm the absence or presence of target *Aspergillus* strains, all test portion 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 1.3 x 10^4 CFU/g, and for cannabis concentrate were 1.6 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 three matrices. Presumptive and confirmed results and statistical analyses are presented in Table 5. 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 all matrixes on either thermocycler.

Table 5: 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% Cl^g
					POD_{cp}^d	95% Cl		POD_{cc}^e	95% Cl		
Dried cannabis flower (>0.3% THC) 10 g *Aspergillus niger* ATCC 16888		NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	AriaMX – 24 h	0.58 (0.26, 1.04)	20	9	0.45	0.26, 0.66	8	0.40	0.22, 0.61	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
		NA	5	0	0.00	-0.47, 0.47	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	CFX-96 – 24 h	0.58 (0.26, 1.04)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	-0.13, 0.13
		>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
		NA	5	0	0.00	-0.47, 0.47	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	AriaMX – 48 h	0.58 (0.26, 1.04)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	-0.13, 0.13
		>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
		NA	5	0	0.00	-0.47, 0.47	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	CFX-96 – 48 h	0.58 (0.26, 1.04)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	-0.13, 0.13
		>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
Cannabis concentrate 5 g *Aspergillus flavus* ATCC MYA-4069		NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	AriaMX – 24 h	0.84 (0.44, 1.46)	20	10	0.50	0.30, 0.70	11	0.55	0.34, 0.74	-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
		NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	CFX-96 – 24 h	0.84 (0.44, 1.46)	20	10	0.50	0.30, 0.70	11	0.55	0.34, 0.74	-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
		NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	AriaMX – 48 h	0.84 (0.44, 1.46)	20	10	0.50	0.30, 0.70	11	0.55	0.34, 0.74	-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
		NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	CFX-96 – 48 h	0.84 (0.44, 1.46)	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

t 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.

1 [f]$dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
2 [g]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 cannabis concentrates. POD statistical analysis indicated no statistical difference between the candidate presumptive and confirmed results for each matrix evaluated. For dried cannabis flower, 1 false positive result was obtained at the 24 h time point on the AriaMx 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. For cannabis concentrates, 1 false negative was obtained at both 24 and 48 h for the AriaMx instrument, and at 24 h for the CFX-96 instrument.

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 cannabis concentrates (5 g) at 24 h of primary enrichment. 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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References

1. Health Canada (2011). *Pathogen Safety Data Sheets: Infectious Substances – Aspergillus spp.* https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment/aspergillus.html (Accessed March 2021)
2. Thompson, G.R., Tuscano, J.M., Dennis, M., Singapuri A., Libertini S., Gaudino R., et al. (2017). *A microbiome assessment of medical marijuana*. Clinical Microbiology and Infection. 23(4):269-70.
3. AOAC International SMPR 2019.001, Standard Method Performance Requirements for Detection of *Aspergillus* in Cannabis and Cannabis Products. http://www.eoma.aoac.org/SMPR/upload/116/SMPR%202019_001.pdf (accessed March 2021)
4. Official Methods of Analysis (2019) 21st Ed., Appendix J: AOAC INTERNATIONAL, Rockville, MD, http://www.eoma.aoac.org/app_j.pdf (Accessed March 2021)

1 5. Mycoses Study Group Education and Research Consortium (2020). Aspergillus
2 https://drfungus.org/knowledge-base/aspergillus-species (Accessed March 2021)
3 6. U. S. Food and Drug Administration *Bacteriological Analytical Manual*, Chapter 3
4 *Aerobic Plate Count* (January 2001) (Accessed March 2021)
5 https://www.fda.gov/food/laboratory-methods-food/bam-aerobic-plate-count
6 7. Least Cost Formulations, Ltd., MPN Calculator Version 1.6
7 8. Wehling, P., LaBudde, R., Brunelle, S., & Nelson, M. (2011) Probability of detection (POD) as a
8 statistical model for the validation of qualitative methods. *J. AOAC Int*., **94**, 335-347.
9 9. Payne, G.A. et. al (2006). Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. *Med.*
10 *Myco.* **44,** S9-S11.