Validation of the PathoSEEK® Total Yeast and Mold Count Assay with SenSATIVAx® TLP DNA Purification and Grim Reefer® Free DNA Removal for Detection and Enumeration of Yeast and Mold in Dried Cannabis Flower AOAC *Performance Tested Method*℠ 062401

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Abstract

Background: PathoSEEK® Total Yeast and Mold Detection Assay combined with use of SenSATIVAx® Thaumatin-Like Protein (TLP) Extraction Enzyme purification protocol and use of Grim Reefer® Free DNA Removal (PathoSEEK TYM method) is a real-time quantitative PCR (qPCR) method to detect and enumerate yeast and mold in cannabis matrixes. SenSATIVAx is a proprietary DNA isolation process that purifies both plant and microbial DNA from dried cannabis flower, delta 9-tetrahydrocannabinol (THC)

>0.3%.

Objective: The objective is to validate PathoSEEK TYM as both a qualitative threshold method and an enumeration method for yeast and mold in dried cannabis flower (THC, >0.3%) for AOAC *Performance Tested Methods*^{SM} (PTM) certification.

Methods: Inclusivity/exclusivity testing and matrix studies for dried cannabis flower were conducted according to the AOAC *Standard Method Performance Requirements* 2021.009 for Viable Yeast and Mold Count Enumeration in Cannabis and Cannabis Products. Robustness, product consistency and stability testing were performed to satisfy PTM requirements.

Results: In the inclusivity testing, 51 target strains were correctly detected, while four strains were not, *Arthrinium arundinis, Phytophthora infestans, Botrytis cinerea,* and *Scopulariopsis acremonium*. All 31 exclusivity strains were not detected. In the matrix study, no significant differences were detected by probably of detection analysis between presumptive and confirmed results for two Real-time qPCR systems and two confirmatory agars. In the quantitative analysis, the PathoSEEK TYM assay gave equivalent results to the confirmatory agars. No significant differences were seen in the product consistency testing, and a 12-month shelf life was validated in the stability study.

Conclusions: This study demonstrates that the PathoSEEK TYM Assay is a reliable method for total yeast and mold detection and enumeration in dried cannabis flower at contamination levels >10^3 cfu/g.

Highlights: The data were reviewed by the AOAC PTM Program and approval was granted for certification of the PathoSEEK TYM method as PTM 062401.

Introduction

Yeasts and molds are known to cause deterioration and decomposition of cannabis. Certain species of yeast and mold, such as *Aspergillus fumigatus* can produce toxins and infect immunocompromised patients with fatal Aspergillosis. The Medicinal Genomics PathoSEEK® Total Yeast and Mold Count (TYM)

Detection Assay combined with use of SenSATIVAx® Thaumatin-Like Protein (TLP) Extraction Enzyme purification protocol is a DNA Purification and qPCR method for the rapid detection and enumeration of yeasts and molds in cannabis flower. This method provides results within hours of sample acquisition, compared to plating methods where results are obtained in 5–7 days. The TLP is a beta-glucanase that digests the glucan cell wall of some yeasts that are difficult to lyse. In 2020, *Candida albicans* became a commonly utilized TYM-cannabis reference standard for cannabis testing. *C. albicans* is an excellent example of a yeast with a thick glucan cell wall that is difficult to lyse. While *C. albicans* has not been found on cannabis, it is reasonable to assume other yeast and molds may similarly produce thick glucan cell walls and robust lysis methods will be required for concordance with colony forming unit (CFU) based regulations.

Most states target 10,000 cfu/g of total yeast and mold in cannabis flower as their action limit. A few states utilized 1000 cfu/g yeast and mold as their action limit. As a result, both action limit levels will be targets in this study.

The PathoSEEK process includes real-time qPCR assays using a multiplex system of primers to detect yeast and mold within the dried cannabis flower. Two real-time qPCR systems are available for use with this assay: the PathoSEEK TYM Assay may be used on the AriaMx G8830A Real-Time PCR system (Agilent, Santa Clara, CA) and on the CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad, Hercules, CA).

Principle of the Method

PathoSEEK TYM Detection Assay combined with use of the Grim Reefer® Free DNA Removal process and the SenSATIVAx TLP Extraction Enzyme purification protocol is a novel, real-time qPCR method that uses a multiplex system of primers to enumerate yeast and molds in cannabis matrixes.

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 use of magnetic particles affords eight tip 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 h. Hands-on time is less than 45 min.

The TYM Count Detection Assay and all components required to perform the method are intended for use by trained personnel familiar with laboratory techniques associated with pathogenic organism detection.

Scope of Study

(a) *Analyte.*—Total yeast and mold.

(b) *Matrix.*—Dried cannabis flower, delta 9-tetrahydrocannabinol (THC) >0.3%.

(a) *Summary of validated performance claims.*—The study data were unable to find a statistically detectable difference from zero between the PathoSEEK Total Yeast and Mold Assay combined with SenSATIVAx TLP purification protocol and Grim Reefer Free DNA Removal method for qualitative presumptive and confirmed results, based on the AOAC *Standard Method Performance Requirements* (SMPR) 2021.009 for Viable Yeast and Mold Count Enumeration in Cannabis and Cannabis Products (1) and AOAC Appendix J: AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces (2). In addition, the study data indicate with 95% confidence that the results of the PathoSEEK Total Yeast and Mold Assay combined with SenSATIVAx TLP purification protocol and Grim Reefer Free DNA Removal are equivalent to results on dichloran rose bengal chloramphenicol (DRBC) agar and acidified potato dextrose (PDA) agar spread plates for yeast and mold contamination levels >10^3 cfu/g.

Method

Test Kit Information

(a) *Kit Name*.—PathoSEEK Total Yeast and Mold Count Assay.

(1) SenSATIVAx Flower/Leaf DNA Purification Kit.—Part No. 420001.

(2) SenSATIVAx TLP Purification Enzyme.—2 mL, Part No. 420206.

(3) PathoSEEK qPCR Master Kit v3.—Part No. 420201.

(4) PathoSEEK Total Yeast and Mold Count Detection Assay.—Part No. 420103.

(5) PathoSEEK Total Yeast and Mold Count Assay Positive Control.—Part No. 420303.

(6) Grim Reefer Free DNA Removal Kit.—Part No. 420145.

(7) Grim Reefer Free DNA Removal Control.—Part No. 420144.

(8) Grim Reefer Free DNA Removal Assay.—Part No. 420143.

(b) *Ordering information*.—https://store.medicinalgenomics.com/search.

Test Kit Components

(a) *SenSATIVAx Flower /Leaf DNA Purification Kit*.—Part No. 420001 (200 tests).

(1) MGC Lysis Buffer.—One bottle.

(2) MGC Binding Buffer.—One bottle.

(3) MGC Elution Buffer.—One bottle.

(b) *SenSATIVAx TLP Purification Enzyme*.—One vial (2 mL), Part No. 420206 (50 purifications).

(c) *PathoSEEK qPCR Master Kit v3*.—Part No. 420201 (200 tests).

(1) Reaction Buffer (10x).—One tube.

(2) Nuclease Free Water.—Two tubes.

(3) qPCR Master Mix (5x).—One tube.

(d) *PathoSEEK Total Yeast and Mold Count Detection Assay.*—One tube, Part No. 420103 (200 tests).

(e) *PathoSEEK Total Yeast and Mold Count Assay Positive Control*.—One tube, Part No. 420303.

(f) *Grim Reefer Free DNA Removal Kit*.—Part No. 420145 (125 – 250 tests).

(1) MGC Grim Reefer Buffer.

(2) MGC Grim Reefer Enzyme.

(g) *Grim Reefer Free DNA Removal Control*.—Part No. 420144 (50 tests).

(h) *Grim Reefer Free DNA Removal Assay*.—Part No. 420143 (200 tests).

Additional Supplies and Reagents

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

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

(c) *Crushed ice.*

(d) *96 well PCR Cryogenic rack.*—VWR No. 89004-570.

(e) *1.5 µL Tube Benchtop Cryogenic rack.*—VWR No. 89004-558 or equivalent.

(f) *Stomacher-type Filter bags.*—Whirl-Pak No. B01385WA or Medicinal Genomics Part No. 10008.

(g) *Ziplock storage bag.*—One gallon.

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

(i) *Tryptic Soy Broth.*—MGC Part No. 420205. Store media at 2–8°C.

(j) *1.5 mL sterile Eppendorf tubes.*

(k) *15 mL or 50 mL sterile polypropylene conical tubes.*

(l) *96 Well Plate Magnet.*—Medicinal Genomics Part No. 420202.

(m) SenSATIVAx® 96 *Well Extraction Plate.*—Medicinal Genomics Part No. 100298.

(n) *Eppendorf Tube rack.*

(o) *25 mL Serological pipette*—VWR No. 89130-890 or No. 89130-900 or equivalent.

(p) *10% bleach*.

(q) *70% Ethanol*.—Medicinal Genomics Part No. 420030.

Apparatus

(a) *Real-Time PCR System.*—Agilent AriaMx G8830A thermocycler, containing the following Optical Channels: FAM, HEX, and Cy5 (if using optional Grim Reefer Free DNA Removal Control, hereafter called GR Positive Control).

(1) AriaMx Version 2.1 software.

(2) PC.—With Microsoft Windows 10, able to run Agilent software, Agilent HP Notebook PC or equivalent.

(3) 96 Well Optical qPCR plate.—Medicinal Genomics Part No. 100164.

(4) Adhesive optical seal for qPCR plates.—Medicinal Genomics Part No. 100177.

(5) Optical Strip Caps (optional).—Agilent Part No. 401425.

***Note**: If using adhesive seals instead of strip caps, use Applied Biosystems MicroAmp Optical Film Compression Pad, Fisher Scientific, No. 43-126-39 to prevent evaporation and cross contamination between wells. Medicinal Genomics will provide pad at no charge if AriaMx is purchased through Medicinal Genomics.*

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

(1) CFX Manager Version 3.1 software or CFX Maestro Version 2.2 software.

(2) PC.—With Microsoft Windows 10, able to run Bio-Rad software, Bio-Rad Personal PC or equivalent .

(3) 96 Well Optical qPCR plate.— Bio-Rad No. HSP-96601 or Medicinal Genomics Part No. 100164.

(4) Adhesive optical seal for qPCR plates.—Bio-Rad No. MSB-1001, or Medicinal Genomics Part No. 100177.

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

(d) *Table Top Mini Plate Centrifuge.*—Fisher Scientific No. 14-100-143 or equivalent.

(e) *Table Top Mini Centrifuge.*—VWR No. 10067-588, No. 2631-0006 or equivalent.

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

(g) *High Speed centrifuge.*—To accommodate 1.5 mL tubes such as Eppendorf model 5414R or similar, with ability to spin up to speeds of 14,000–19,300 RCF.

(h) *Incubator.*—Capable of maintaining 37 ± 2°C and 25 ± 1°C, VWR No. 97025-630 or equivalent.

(i) *Scientific balance.*—Capable of measuring to milligrams.

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

Safety Precautions

The PathoSEEK Total Yeast and Mold Count Assay is a qPCR detection assay for the rapid detection and enumeration of yeast and mold in cannabis flower .

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

(b) It is the responsibility of each laboratory to handle waste and effluents processed according to their nature and degree of hazardousness. Waste and effluents processed must be treated and disposed of in accordance with all applicable local, state, and federal regulations.

(c) 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.

(d) Environment. The quality of results depends on the strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR.

(1) Never circulate lab equipment from one workstation to another.

(2) Always use a positive and negative control for each series of amplification reactions.

(3) Periodically verify the accuracy and precision of pipettes, as well as correct functioning of the instruments.

(4) Change gloves often, especially if you suspect contamination.

(5) Clean workspaces periodically with 10% bleach and other decontaminating agents.

(6) Use powder-free gloves.

(7) If using qPCR reaction strip tubes instead of plates, avoid fingerprints and writing on caps because both can interfere with data acquisition.

Sample Preparation (Cannabis Flower)

(a) Aliquot sterile Tryptic Soy Broth (TSB) ahead into 15 mL or 50 mL conical vials, depending on matrix type.

***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 source bottle. Inspect stock of TSB for flocculants or signs of growth prior to aliquoting. Return TSB to the 2–8°C refrigerator immediately after use.*

(b) Wipe down the workspace with a 10% bleach solution, including the benchtop 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 1.5 mL centrifuge tubes needed “[sample name]”.

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

(g) Remove the GR positive control from the -20°C freezer and allow to thaw at room temperature. O nce thawed keep on ice before using. Dilute GR Positive control (GRC) to 1:10,000.

(1) Label a new 1. 5 mL Eppendorf tube (GRC 1:100), add 1 µL of GR Positive Control into 99 µL of sterile distilled water (dH_2O). Vortex to mix thoroughly and quickly spin tube. Label another 1.5 mL Eppendorf tube (GRC 1:10,000), add 99 µL of dH_2O, then add 1 µL of the GRC 1:100 dilution. Vortex to mix thoroughly and quickly spin the tube. This will result in a 1:10,000 dilution of the GR Positive Control.

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

(2) Place GRC on ice until use.

(h) Weigh cannabis flower test portions ahead into Whirl-pak bag, 1-gallon zip lock storage bag, or conical tubes:

*(1) Cannabis flower**, 10** grams* —Weigh 10 g flower test portion material into one side of the mesh liner inside the Whirl-Pak bag. Add 190 mL of TSB to each test portion. This is a 1:20 initial dilution of the sample. Close the Filter bag by folding the top over three times. Homogenize for 1 min by hand.

(i) If processing multiple samples, be sure to change gloves between each test portion to ensure that there is no cross contamination between test portions during the weighing process.

(j) Allow a 1.5 mL tube rack to come to temperature in a 37 ± 2°C incubator.

SenSATIVAx for Flower/Leaf DNA Purification

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

(b) Spin tubes at 14,000–19,300 RCF for 5 min.

(c) Use a pipette to remove and discard 950 µL of the supernatant without disturbing the pellet.

(d) Resuspend the pellet by adding 200 µL of Nuclease Free Water to each tube.

(e) Pipette mix and then vortex tubes well to resuspend the pellet.

(f) Add 28 µL of MGC Grim Reefer Buffer and 5 µL of MGC Grim Reefer Enzyme to the resuspended pellet in tubes. The buffer and enzyme are found in the Grim Reefer Free DNA Removal Kit.

(g) Vortex tubes for 10 s. Incubate tubes in tube rack in a 37°C ± 2°C incubator for 10 min.

(h) Remove tubes from the incubator and add 12.5 µL of MGC Lysis Buffer into tubes. Vortex tubes for 30 s.

(i) Allow tubes to incubate for 5 min at room temperature.

***Note**: The addition of MGC Lysis Buffer deactivates the Grim Reefer Enzyme and should be done as quickly as possible.*

(j) Add 2.5 µL of prepared 1:10,000 dilution of GR Positive Control into tubes.

(k) Add 12 µL of SenSATIVAx TLP Purification Enzyme into tubes. Vortex tubes for 30 s.

(l) Incubate tubes in 37°C ± 2°C incubator for 30 min.

(m) Remove tubes from the incubator and vortex tubes for 30 s.

(n) Let tubes incubate on the bench for 2–5 min.

(1) After incubation, spin tubes for a minimum of 1–3 min in a bench top mini centrifuge or high-speed centrifuge to pellet cellular debris.

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

(o) Remove 200 µL of lysate from the tubes containing the centrifuged sample, being careful not to disturb the pellet at the bottom of the tube. Dispense supernatant into the desired well of the previously labeled 96 well extraction plate.

Note: Pellet size will vary depending on trichome density.

(p) Vortex MGC Binding Buffer thoroughly before use for a minimum of 30 s. Be sure that the magnetic particles are completely re-suspended in the buffer .

(q) Add 200 µL MGC Binding Buffer to each supernatant, and gently pipette mix 15 times.

Note*: Be careful to avoid adding too many bubbles by pipetting up and down gently when tip mixing to avoid contamination of other wells within the extraction plate.*

(r) Incubate extraction plate on the bench top for at least 5 min.

(s) Place the extraction plate atop the 96 Well Plate Magnet for at least 5 min.

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

(u) Add 400 µL of 70% ethanol (EtOH) into each well with the extraction plate still on the Plate Magnet.

(v) Wait at least 30 s and then remove all the EtOH from the wells.

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

(w) Repeat addition of 400 µL of 70% ethanol wash with the extraction plate still on the Plate Magnet . Wait at least 30 s and then remove all EtOH.

(x) After all the EtOH has been removed, let the beads dry at room temperature on the Plate Magnet plate for up to 15 min. Required drying time will vary based on complete removal of the second ethanol wash, as well as the laboratory environment. Visually inspect beads for residual ethanol before the elution step.

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

***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.*

(y) Remove the extraction plate from the Plate Magnet. Add 50 µL of MGC Elution Buffer to each well.

(1) Mix beads in buffer with tips, 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. It may be helpful to allow the elution buffer to soak the beads prior to tip mixing.*

(2) Incubate the extraction plate for at least 1 min on the bench, then return the extraction plate to the Plate Magnet .

(3) Let the extraction plate sit on the magnet for at least 1 min. Use a pipette to transfer the eluant (50 µL) to a new extraction plate labeled with "Final Extract [date]".

(4) Seal the extraction plate containing eluants 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 the plate at -20°C until ready to perform qPCR protocol.

Real-Time Quantitative PCR (qPCR) Setup Protocol

(a) Remove qPCR reagents including qPCR Master Mix, Nuclease Free Water, Reaction Buffer, PathoSEEK Total Yeast and Mold Count Detection Assay, PathoSEEK Total Yeast and Mold (TYM) Count Positive Control, and Grim Reefer Free DNA Removal Assay from the -20°C freezer. Place qPCR Master Mix tube 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 master mix, mix reagent tubes.

(1) Total Yeast and Mold Count Detection Assay, Reaction Buffer, Total Yeast and Mold Count Positive Control, Grim Reefer Free DNA Removal Assay and Nuclease Free Water – Vortex tubes

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 sufficient for all test reactions being run. The Total Yeast and Mold Count Detection Assay contains the internal plant control (ICC), probe mix, and the probes for all microbial targets. Grim Reefer Free DNA Removal Assay contains the Grim Reefer Control detection probe mix. Label new master mix tube with TYM MM (Master Mix). Always prepare enough master mix for 1 or 2 additional reactions over the total number of tests to account for pipetting and dead volumes. An example of the TYM Master Mixes can be found in Tables 1 and 2.

***Note**: It is best to add the largest volume reagent first, in this case water. Add qPCR master mix last*. *It is also important to avoid small volume errors as those errors could lead to errors in the results.*

Table 1. TYM Master Mix Reagent Volumes: Include Grim Reefer Free DNA Removal Assay

Reagents	1 Reaction	24 Reactions (Plus 1 excess rxn)	48 Reactions (Plus 2 excess rxns)
qPCR Master Mix	3.75 µL	93.75 µL	187.5 µL
Total Yeast and Mold Count Detection Assay	1 µL	25 µL	50 µL
Grim Reefer Free DNA Removal Assay	0.5 µL	12.5 µL	25 µL
Reaction Buffer	0.8 µL	20 µL	40 µL
Nuclease Free Water	7.7 µL	192.5 µL	385 µL
TYM Master Mix	13.75 µL	343.75 µL	687.5 µL

***Note**: The Grim Reefer Assay is detected in the Cy5 Channel of the qPCR instrument. Be sure to select*

the Cy5 channel when setting up the detection plate.

Table 2. TYM Master Mix Reagent Volumes: No Grim Reefer Free DNA Removal Assay

Reagents	1 Reaction	24 Reactions (Plus 1 excess rxn)	48 Reactions (Plus 2 excess rxns)
qPCR Master Mix	3.75 µL	93.75 µL	187.5 µL
Total Yeast and Mold Count Detection Assay	1 µL	25 µL	50 µL
Reaction Buffer	0.8 µL	20 µL	40 µL
Nuclease Free Water	8.2 µL	205 µL	410 µL
TYM Master Mix	13.75 µL	343.75 µL	687.5µL

(d) Once combined, gently tip mix or invert the tube 5 times to combine the master mix together. Pulse spin-down tube in microcentrifuge.

(e) Place TYM Master Mix on ice until used.

(f) For the positive control, make a 1:10 dilution of TYM Count Positive Control.

(1) Vortex the TYM Positive Control tube and pulse spin-down. Add 1 µL of TYM Positive Control dilute with 9 µL of Nuclease Free Water (found in the kit) into a 1.5 mL tube and vortex to mix.

***Note**: It is best to add the largest volume reagent first, in this case the 9 µL water, then the 1 µL of TYM Positive Control. Pipette mix well to ensure control DNA is in solution.*

(g) For the negative control, use Nuclease Free Water only.

(h) Place the extraction plate on the Plate Magnet. This is to ensure no magnetic beads are transferred into the qPCR reactions if there are some left over from the purification elution process.

(i) Use a new 96 Well Optical qPCR plate and label the plate "qPCR Plate_[date]".

(j) If frozen, let the DNA thaw completely. Spin the extraction plate in mini plate centrifuge before removing the seal, to avoid cross-contamination between the samples. Carefully remove the seal from the extraction plate containing DNA samples.

(k) Pipette mix the DNA in wells and place the extraction plate onto the Plate Magnet for 1 min.

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

(l) Transfer 5 µL of each DNA sample into the corresponding well on the Optical qPCR plate, keeping the extraction plate on the Plate Magnet when aspirating the liquid .

(m) Add 5 µL of the diluted TYM Positive Control into the corresponding positive control well.

(n) Add 5 µL of Nuclease Free Water to the corresponding negative control well.

(o) Add 13.75 µL of the TYM Master Mix to each corresponding sample wells, positive control well, and negative control well in the Optical qPCR plate. Gently tip mix the contents a few times after each addition of successive TYM master mix to plate wells. Be careful not to introduce bubbles during this mixing step.

(p) Seal the Optical qPCR plate with strip caps or adhesive seal.

(q) Spin down Optical qPCR plate for at least 1 min in mini plate centrifuge to bring well contents to the bottom of wells and help 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.*

(r) For the Agilent AriaMx: If using adhesive seal on Optical qPCR plate, place the reusable MicroAmp Optical Film Compression Pad (gray side down) on the plate directly lining up the holes in the pad with the wells in the plate.

(s) Place the sealed Optical qPCR plate onto the PCR instrument, positioning the A1 well in the top left corner of the system.

(t) Follow the software-specific instructions to initiate the run.

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

Interpretation

<u>Agilent AriaMx</u>

(a) The following species will be detected on the following Fluorophores channels.

(1) Total yeast and mold: FAM.

(2) Internal Plant Control: HEX (Internal control detects presence of cannabis plant DNA).

(3) Grim Reefer Control: Cy5.

Total Yeast and Mold Control Wells (POS and NEG):

(b) Assay positive control (well) – **Passing** - On the FAM Fluorophore, has a Quantification Cycle (Cq) value ≤35.

(1) Visually confirm result with the curve on the graph. If FAM for positive well has a Cq value > 35 or a negative Cq result, then the qPCR run sample results are inconclusive and the qPCR must be re-run from the already extracted DNA.

(c) Assay negative control (well) – **Passing** - On the FAM Fluorophore, has no Cq value.

(1) Visually confirm result with the curve on the graph. If FAM signal for the negative well is observed, then this indicates that the results are inconclusive, and the qPCR must be re-run from the already extracted DNA. Be sure to use all new qPCR reagents as a positive result for a negative well indicates a contaminant.

Internal Control:

(d) **Passing:** Internal Control, on the HEX Fluorophore, has a Cq value ≤ 35 for flower samples and <40 for all other matrices.

(1) Visually confirm result with the curve on the graph.

(2) If no HEX signal or HEX signal has a Cq value >35 for flower sample, the DNA purification for sample should be repeated from beginning. This result is an indication that purification process was not successful, or that the sample contains very little cannabis DNA.

Grim Reefer FREE DNA:

(e) **Passing:** GR Positive Control, on the Cy5 Fluorophore, has a Cq value between 22 and 30.

(1) If the Cq value is between 22–30 for Cy5, then the Grim Reefer Enzyme was properly deactivated.

(2) If the Cq value is less than 22:

a. This may indicate a dilution error of the spiked GR Positive Control, or it may indicate that your flower sample is contaminated with russet mites.

b. If the Cq is less than 22, repeat the flower extraction from beginning, but now when repeat PCR analysis do not spike in the GR Positive Control.

c. Once the samples have been re-extracted, analyze the newly extracted samples with the TYM assay including the Grim Reefer Assay probe. After repeat analysis, if there is still a Cy5 signal without the spiked GR Positive Control, this indicates the sample has russet mites.

d. If the sample does have russet mites, the Grim Reefer treatment can be performed, but the Grim Reefer Positive Control should not be spiked into the sample during the extraction, and no Grim Reefer components should be included in the qPCR setup (see Table 2, qPCR Reagent Volumes – No Grim Reefer Assay Probe Mix Included).

e. When not using the GR Positive Control, incorporate a TSB blank to ensure that the Grim Reefer Enzyme was properly deactivated by the addition of MGC Lysis Buffer. A TSB blank sample should be taken through the extraction process. The TSB blank should be spiked with the Grim Reefer Positive Control after the addition of lysis buffer. Your samples should NOT be spiked with the GR Positive Control. This sample should also be run with the addition of the Grim Reefer Free DNA Removal Assay to the qPCR master mix. If the Cy5 signal is between the Cq range of 22–30, the Grim Reefer Enzyme was properly deactivated.

(3) If the Cq value is greater than 30: this may indicate a dilution error of the spiked GR Positive Control, or it may indicate that the Grim Reefer Enzyme was not properly deactivated with the MGC Lysis Buffer. The entire DNA purification from beginning should be repeated.

Unknown Total Yeast and Mold targets:

(f) Unknown Total Yeast and Mold targets.

(1) A low CFU count or **passing** result (under threshold cfu for usage by vendor) for the unknown Total Yeast and Mold targets.

a. Passing Sample Estimated CFU: Check Cq Value on the FAM Fluorophore. Use Cq to cfu conversion equation to determine approximate cfu/g. The equation employs an experimentally generated best fit line to correlate Cq to estimated cfu/g. See Table 3.

Table 3. Cq to CFU Conversion Equation for Flower

Matrix	Microbial Test	Cq to CFU Conversion Equation
Flower	Total Yeast and Mold	CFU/g = 10^[(-0.1267*Cq) + 6.6781] Multiply resulting CFU x 20 to account for upfront dilution factor

b. Visually confirm with the curve on the graph.

(2) A high CFU count or **failing** result (over threshold cfu for disallowance by vendor) for the unknown Total Yeast and Mold targets.

a. Failing Sample Estimated CFU: Check Cq value on the FAM Fluorophore. Use Cq to cfu conversion equation {10^[(-0.1267*Cq)+6.6781]}*20 to determine approximate cfu/g.

b. Visually confirm with the curve on the graph. It is very important to confirm with the amplification curve when a high CFU count occurs. Sometimes the background amplification will give a potential false-positive curve, especially when Cq is <15. A potential false-positive curve will appear to lack an exponential growth phase. Linear data view does not display a sigmoidal shaped curve. Raw data view shows a baseline that drifts upward throughout the run. These factors indicate that the results are inconclusive, and the qPCR must be re-run from the already extracted DNA.

BioRad CFX96

(a) The following species will be detected on the following Fluorophores.

(1) Total yeast and mold: FAM.

(2) Internal Plant Control: HEX.

(3) Grim Reefer Control: Cy5.

Total Yeast and Mold Control Wells (POS and NEG):

(b) Assay positive control (well) – **Passing** - on the FAM Fluorophore, has a Cq value ≤35.

(1) Visually confirm result with the curve on the graph. If FAM for positive well has a Cq value >35 or negative Cq result, then the qPCR run sample results are inconclusive and the qPCR must be re-run from the already extracted DNA.

(c) Assay negative control (well) – **Passing** - on the FAM Fluorophore, has no Cq value.

(1) Visually confirm result with the curve on the graph. If FAM signal for negative well is observed, then this indicates that the results are inconclusive, and the qPCR must be re-run from the already extracted DNA. Be sure to use all new qPCR reagents as a positive result for a negative well indicates a contaminant

(d) **Passing**: Internal Control, on the HEX Fluorophore, has a Cq value ≤35 for flower samples and <40 for all other matrices.

(1) Visually confirm result with the curve on the graph.

(2) If no HEX signal or HEX signal has a Cq value >35 for flower sample, the DNA purification for sample should be repeated. This result is an indication that the purification process was not successful or that the sample contains very little cannabis DNA.

Grim Reefer FREE DNA:

(e) Passing: GR Control, on the Cy5 Fluorophore, has a Cq value between 22 and 30.

(1) If the Cq value is between 22–30, then the Grim Reefer Enzyme was properly deactivated.

(2) If the Cq value is less than 22:

a. This may indicate a dilution error of the spiked GR Positive Control, or it may indicate that your flower sample is contaminated with russet mites.

b. If the Cq value is less than 22, repeat the flower extraction, but now for repeat PCR analysis do not spike in the GR Positive Control.

c. Once the samples have been re-extracted run the newly extracted samples with the TYM assay including the Grim Reefer Assay probe. After repeat analysis, if there is still a Cy5 signal without the spiked GR Positive Control, this indicates the sample has russet mites.

d. If the sample does have russet mites, the Grim Reefer treatment can be performed, but the Grim Reefer Positive Control should not be spiked into the sample during the extraction, and no Grim Reefer components should be included in the qPCR setup (see Table 2, qPCR Reagent Volumes – No Grim Reefer Assay Probe Mix Included).

e. When not using the GR Positive Control incorporate a TSB blank to ensure that the Grim Reefer Enzyme was properly deactivated by the addition of MGC Lysis Buffer. A TSB blank sample should be taken through the extraction process. The TSB blank should be spiked with the GR Positive Control after the addition of lysis buffer. Your samples should NOT be spiked with the GR Positive Control. This sample should also be run with the addition of the Grim Reefer Free DNA Removal Assay to the qPCR master mix. If the Cy5 signal is between the Cq range of 22–30 the Grim Reefer Enzyme was properly deactivated.

(3) If the Cq value is greater than 30, this may indicate a dilution error in the spiked GR Positive Control, or it may indicate that the Grim Reefer Enzyme was not properly deactivated with the MGC Lysis Buffer. The entire DNA purification from beginning should be repeated.

Unknown Total Yeast and Mold targets:

(f) Unknown Total Yeast and Mold targets.

(1) A low CFU count or **passing** result (under threshold for cfu for usage by vendor) for the unknown Total Yeast and Mold targets.

a. Passing Sample Estimated CFU: Check Cq Value on the FAM Fluorophore. Use Cq to CFU conversion equation to determine approximate cfu/g. The equation employs an experimentally generated best fit line to correlate Cq to estimated cfu/g. See Table 3.

b. Visually confirm with the curve on the graph.

(2) A high CFU count or **failing** result (over threshold cfu for disallowance by vendor) for the unknown Total Yeast and Mold targets.

a. Failing Sample Estimated CFU: Check Cq value on the FAM Fluorophore. Use Cq to cfu conversion equation {10^[(-0.1267*Cq)+6.6781]}*20 to determine approximate cfu/g.

b. Visually confirm with the curve on the graph. It is very important to confirm with the amplification curve when a high CFU count occurs. Sometimes the background amplification will give a potential false-positive curve, especially when Cq is <15. A potential false-positive curve will appear to lack an exponential growth phase. Linear data view does not display a sigmoidal shaped curve. Raw data view shows a baseline that drifts upward throughout the run. These factors indicate that the results are inconclusive, and the qPCR must be re-run from the already extracted DNA.

Confirmation

Test portions can be confirmed using dichloran rose bengal chloramphenicol (DRBC) agar or acidified potato dextrose agar (PDA) spread plates. Dilute portions as necessary to obtain 10–150 colonies/plate. Incubate plates at 25 ± 1°C. Do not stack plates higher than 3 and keep plates one or two inches apart.

Do not invert plates. Enumerate colonies after 5 days. If no growth is present on the plate, return to incubator for 2 additional days and enumerate (7 total days). Record results from plates with 10–150 colonies, multiply by the dilution factor, and report results in cfu/g.

Experimental

This validation study was conducted under the AOAC Research Institute *Performance Tested Method*[SM] program, *Standard Method Performance Requirements* (SMPR) 2021.009 for Viable Yeast and Mold Count Enumeration in Cannabis and Cannabis Products, and Appendix J: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*. Method developer studies were conducted in the laboratories of Medicinal Genomics (Beverly, MA) and included the inclusivity/exclusivity, matrix, robustness, and product consistency and stability studies. The independent laboratory study was conducted by Cambium Analytica (Traverse City, MI) and included a matrix study for cannabis flower (THC > 0.3%) matrix.

Method Developer Studies

Inclusivity/Exclusivity Study

Methodology.—For the inclusivity evaluation, 55 strains of yeast and mold, as identified by the AOAC SMPR 2021.009, were evaluated. Target strains were cultured in TSB for 48 h at room temperature (20–28°C). After incubation, inclusivity cultures were diluted, if necessary, in TSB to levels of 100–1,000 cfu/mL. After making 1:10, 1:100, and 1:1,000 dilutions, 100 µL of each dilution was plated onto PDA and incubated at 25 ± 1°C for up to 5 days to determine the plate count. Thirty-one exclusivity organisms were cultured in non-selective broth under optimal conditions for growth, depending on the strain. All exclusivity strains, except three, grew to turbid state in non-selective broth at 36 ± 2°C. *Pantoa agglomerans* was regrown at a temperature of 26 ± 2°C, *Aeromonas hydrophila* and *Rahnella*

aquatics were both regrown at a temperature of 30 ± 2°C. These organisms were run with the Medicinal Genomics Total Aerobic Count qPCR Assay to confirm that they did in fact grow. Exclusivity cultures were analyzed undiluted.

Inclusivity and exclusivity cultures were randomized, blind coded, and analyzed by the PathoSEEK TYM Method. All isolates were tested on both Real-time PCR systems, AriaMx and CFX-96, and also tested with or without the Grim Reefer components.

Results.—Of the 55 inclusivity strains tested, 51 were correctly detected by the PathoSEEK TYM Method. Two inclusivity isolates, *Arthrinium arundinis* and *Phytophthora infestans* were not detected by either the PathoSEEK TYM Method or PDA agar. The other two isolates not detected by the PathoSEEK TYM Method were *Botrytis cinerea* and *Scopulariopsis acremonium,* but these isolates were able to be recovered on PDA agar. Of the 31 exclusivity strains tested, all 31 were correctly excluded. Tables 4 and 5 present a summary of the results.

Table 4. Inclusivity Results: PathoSEEK Total Yeast and Mold Count Assay

No.	Species	ATCC[a] Strain	Origin	PathoSEEK TYM Result[b]
1	*Alternaria alternata*	6663	No origin listed[c]	Detected
2	*Arthrinium arundinis*	96021	Bing cherry fruit	Not Detected (No growth on PDA)
3	*Aspergillus aculeatus*	24147	Fragaria sp., Brazil	Detected
4	*Aspergillus brasiliensis*	9642	Wireless radio equipment	Detected
5	*Aspergillus brasiliensis*	16404	Blueberry	Detected
6	*Aspergillus carbonarius*	MYA-4 641	Grape berry, Brindis, Apulia, Italy	Detected
7	*Aspergillus caesiellus*	42693	Dried chilies, New Guinea	Detected
8	*Aspergillus carneus*	13549	France	Detected
9	*Aspergillus clavatus*	1007	No origin listed	Detected
10	*Aspergillus deflectus*	62502	Wheat, China	Detected
11	*Aspergillus flavus*	9643	Shoe sole, New Guinea	Detected
12	*Aspergillus fijiensis Varga et al*	20611	No origin listed	Detected
13	*Aspergillus fumigatus*	204305	Human sputum, Virginia	Detected

No.	Species	ATCC[a] Strain	Origin	PathoSEEK TYM Result[b]
14	*Aspergillus japonicus*	16873	Soil, Panama	Detected
15	*Aspergillus nidulans*	38163	No origin listed	Detected
16	*Aspergillus niger*	16888	No origin listed	Detected
17	*Aspergillus niveus glaucus*	10075	No origin listed	Detected
18	*Aspergillus ochraceus*	18500	Rubber sheet	Detected
19	*Aspergillus oryzae*	42149	Cereal	Detected
20	*Aspergillus parasiticus*	56775	No origin listed	Detected
21	*Aspergillus tamarii*	1005	Tomato	Detected
22	*Aspergillus terreus*	1012	Soil, Connecticut	Detected
23	*Aspergillus tubingensis*	1004	No origin listed	Detected
24	*Aspergillus ustus*	1041	Culture Contaminant, USA	Detected
25	*Aspergillus versicolor*	11730	Cellophane gas mask, India	Detected
26	*Aureobasidium species*	62921	No origin listed	Detected
27	*Beauveria bassiana*	44860	Soil, Georgia	Detected
28	*Botrytis cinerea*	11542	Azalea flowers, Washington, DC	Not Detected
29	*Candida albicans*	10231	Man with bronchomycosis	Detected
30	*Candida tropicalis*	13803	No origin listed	Detected
31	*Cladosporium sphaerospermum*	11288	Human nails	Detected
32	*Cryptococcus laurentii*	18803	Palm wine, Malaffou, Congo	Detected
33	*Cryptococcus neoformans*	208821	Patient with Hodgkin's Disease, New York	Detected
34	*Fusarium proliferatum*	76097	Raw cotton, North Carolina	Detected
35	*Fusarium oxysporum*	62506	Celery, California	Detected
36	*Fusarium solani*	52628	Cardamom fruit pod, Guatemala	Detected
37	*Fusarium sporotrichioides*	24631	Corn, USA	Detected
38	*Fusarium verticillioides*	MYA4922	Maize, Visalia, CA, USA	Detected
39	*Mucor circinelloides*	38592	No origin listed	Detected
40	*Mucor hiemalis*	28935	Soil in Spruce Forest, Germany	Detected
41	*Paecilomyces species*	13435	Soil, Japan	Detected
42	*Penicillium brevicompactum*	9056	No origin listed	Detected
43	*Penicillium citrinum*	10105	Egypt	Detected

No.	Species	ATCC[a] Strain	Origin	PathoSEEK TYM Result[b]
44	*Penicillium chrysogenum*	18476	Cheese, USSR	Detected
45	*Penicillium expansum*	28885	Grape berry, California	Detected
46	*Penicillium rubens*	11709	Selected from Wis. 48-701, ATCC 11707, after N-mustard exposure	Detected
47	*Penicillium simplicissimum*	48706	No origin listed	Detected
48	*Penicillium venetum*	16025	Acidic soil, England	Detected
49	*Phytophthora infestans*	MYA1113	Hyacinthus sp. bulb, England	Not Detected (No growth on PDA)
50	*Purpureocillium lilacinum*	10114	Potato tuber, Glasston, MN	Detected
51	*Rhizopus oryzae*	52748	Soil, Ithaca, NY	Detected
52	*Rhizopus stolonifer*	14037	No origin listed	Detected
53	*Scopulariopsis acremonium*	58636	No origin listed	Not Detected
54	*Yarrowia lipolytica*	20390	Chicken house soil, Alberta, Canada	Detected
55	*Talaromyces pinophilus*	11797	Non-sporulating diploid	Detected

[a] ATCC = American Type Culture Collection, Manassas, VA.

[b] The PathoSEEK TYM results were identical between the AriaMx and CFX96 Real-time PCR systems, analysis with or without the Grim Reefer components.

[c] No origin listed on the ATCC website.

Table 5. Exclusivity Results: PathoSEEK Total Yeast and Mold Count Assay

No.	Species	ATCC[a] Strain	Origin	PathoSEEK TYM Result[b]
1	*Acinetobacter baumannii*	19606	Urine	Not Detected
2	*Aeromonas hydrophila*	7966	From a tin of milk with fishy odor	Not Detected
3	*Burkholderia multivorans*	17616	Soil enriched with anthranilate at 41°C, Berkeley, CA	Not Detected
4	*Bacillus subtilis*	11774	No origin listed[c]	Not Detected
5	*Citrobacter braakii*	3037	No origin listed	Not Detected
6	*Citrobacter koseri*	25408	Throat	Not Detected
7	*Edwardsiella tarda*	23672	No origin listed	Not Detected
8	*Enterobacter aerogenes*	13048	Sputum, South Carolina Dept. of Health, and Environmental Control	Not Detected
9	*Enterobacter cloacae*	13047	Spinal fluid	Not Detected
10	*Erwinia rhapontici*	29290	English pink wheat grains, England	Not Detected

No.	Species	ATCC[a] Strain	Origin	PathoSEEK TYM Result[b]
11	*Escherichia coli*	25922	Clinical isolate	Not Detected
12	*Escherichia coli* O157:H7	35150	Human feces	Not Detected
13	*Escherichia hermannii*	700368	No origin listed	Not Detected
14	*Escherichia vulneris*	33821	Human wound, Bethesda MD	Not Detected
15	*Hafnia alvei*	51873	Human feces, Netherlands	Not Detected
16	*Klebsiella oxytoca*	51983	Human blood, Albany NY, USA	Not Detected
17	*Klebsiella pneumonia*	BAA-2146	Human urine	Not Detected
18	*Listeria monocytogenes*	7647	Bovine	Not Detected
19	*Morganella morganii*	25829	Stool of infant, Providence City	Not Detected
20	*Pantoea agglomerans*	43348	Gypsophila paniculata galls, California	Not Detected
21	*Proteus mirabilis*	43071	Rectum, Georgia	Not Detected
22	*Pseudomonas aeruginosa*	15442	Animal room water bottle	Not Detected
23	*Pseudomonas aeruginosa*	35554	No origin listed	Not Detected
24	*Pseudomonas fluorescens*	13525	Pre-filter tanks, England	Not Detected
25	*Pseudomonas putida*	47054	No origin listed	Not Detected
26	*Ralstonia insidiosa*	49129	Clinical isolate	Not Detected
27	*Rahnella aquatilis*	33991	Soil	Not Detected
28	*Salmonella enterica*	13311	Feces, human, 1911	Not Detected
29	*Stenotrophomonas maltophilia*	13637	Oropharyngeal region of patient with mouth cancer	Not Detected
30	*Staphylococcus aureus*	12600	Pleural fluid	Not Detected
31	*Serratia marcescens*	27137	Human isolate	Not Detected

[a] ATCC = American Type Culture Collection, Manassas, VA.

[b] The PathoSEEK TYM results were identical between the AriaMx and CFX96 Real-time PCR systems, analysis with or without the Grim Reefer components.

[c] No origin listed on the ATCC website.

Matrix Study

Methodology.—In the matrix study, naturally dried cannabis flower (>0.3% THC) was evaluated. The study design followed SMPR 2021.009, Table 6 for qualitative threshold detection. Four sets of contamination levels were prepared to evaluate action limits of $<10^3$ cfu/g, $\geq 10^3$ cfu/g, $\geq 10^4$ cfu/g, and

$>10^4$ cfu/g. The contamination levels were targeted at $<10^3$ cfu/g, approximately 10^3 cfu/g), approximately 10^4 cfu/g, and $>10^4$ cfu/g. The target contamination levels were designed to produce all negative threshold results (5 test portions) at the lowest level, fractional positive threshold results (5–15 positive results/20 portions tested) at the low and high action limits, and all positive results (5 test portions) above the highest threshold. There were two threshold limits for this study. The threshold limits for this study were 1000 and 10,000 cfu, used for the approximately 10^3 cfu/g and approximately 10^4 cfu/g contamination levels, respectively.

Dried cannabis flower was obtained from a local grower. The cannabis materials were screened for total yeast and mold counts using the PathoSEEK TYM method and DRBC and PDA plating. The dried cannabis flower materials were found to be naturally contaminated with yeast and mold at various levels, from <500 cfu/g to >50,000 cfu/g. The contaminated flowers were combined as needed to create the target contamination levels. The flower was crushed by gloved hands, and then aliquoted into 1-gallon zip lock type bags. Two Real-time qPCR systems were evaluated in the study, AriaMx and CFX96.

On the day of testing, test portions were randomized and blind-coded for the study. Each test portion was prepared as described in the Sample Preparation section of this report. To each 10 g test portion, 190 mL of TSB was added, and then the bag was homogenized for 1 min by hand. From each homogenate, an aliquot was removed and processed according to the SenSATIVAx for Flower/Leaf DNA Purification method. Realtime PCR analyses on each test portion was performed using each system (AriaMx and CFX96) as described in the method section. To confirm, each homogenate was also spread-plated onto DRBC and PDA agar plates. Dilutions were made to each homogenate as needed to obtain spread counts between 10–150 colonies/plate. Plates were incubated at 25 ± 1°C for a total of 7 days, counted and cfu calculated.

For each test portion, the PathoSEEK TYM method result (Cq) was converted to cfu/g according to the equation provided in the instructions for use (IFU):

Total yeast and mold cfu/g = {10^[(-0.1267*Cq) + 6.6781]}*20

For the qualitative threshold analysis, test portions producing cfu results above the threshold limits were classified as positive, and test portions producing cfu results below the threshold limits were classified as negative. The corresponding DRBC and PDA agar plates for each test portion were counted, and then the counts were multiplied by the appropriate dilution factor to determine the final count in cfu/g for each agar plate result. The PCR results were considered the presumptive results, and the agar plate results were considered the confirmed results.

For the quantitative analysis, the PCR cfu/g calculated result for each test portion was compared to the corresponding cfu/g result for each agar plate, irrespective qualitative threshold results.

Results.—For the qualitative threshold evaluation, the Least Cost Formulations (LCF) AOAC Binary Workbook v5-2 (Virginia Beach, VA) was used to calculate the POD results using paired analysis. For the quantitative evaluation, the LCF Quantitative Analysis for Micro Methods v1.2 (Virginia Beach, VA) was used for paired analysis. Results are presented in Table 5 for the qualitative threshold evaluation and in Table 6 for the quantitative evaluation.

In the qualitative study, test portions that contained less than lowest threshold, 1000 cfu/g (either by Cq conversion or agar plate counts), were considered negative for purpose of statistical calculations; these were classified for PCR result as a “non-detect response”. In the qualitative threshold study, all test portions (five) at the $<10^3$ target (lowest) contamination level were negative by PCR (AriaMx and CFX96) and $<10^3$ cfu/g for each agar plate type (DRBC and PDA). All test portions (five) at the $>10^4$ target (highest) contamination level were qualitative threshold (calculated $>10^4$ cfu/g) positive by PCR (AriaMx and CFX96) and $>10^4$ cfu/g for each agar plate type (DRBC and PDA). For the low action limit (approximately 10^3 cfu/g), there were 15 qualitative threshold positive test portions and 5 qualitative threshold negative test portions by both PCR systems, which matched the DRBC and PDA count plate threshold results. For the high action limit (approximately 10^4 cfu/g), there were 13 qualitative threshold

positive test portions and 7 qualitative threshold negative test portions by both PCR systems, which also matched the DRBC and PDA count plate threshold results. Therefore, no statistical differences were detected in the qualitative threshold evaluations between PCR systems (AriaMx and CFX96) and agar count plates (DRBC and PDA). See Table 6.

In the quantitative study, there were test portions that PCR result was a "non-detect response". For this case, an AOAC Statistical Advisor was consulted for an approach to evaluate the statistical comparison. The recommendation was to base the LOQ on a Cq value of 40 cycles. Using the conversion equation, 850 was calculated and then divided by the square root of 2. The resulting number, 601 cfu, was then used to substitute for any negative (non-detect response) PCR value. The cfu/g results were $\log_{10}$ transformed using the equation in the LCF Quantitative Analysis for Micro Methods v1.2 workbook, $\log_{10}$[cfu/g + (0.1)f], and then analyzed using the paired comparison.

All five quantitative test portions at the target $<10^3$ (lowest) contamination level were threshold negative by PCR (i.e., gave a non-detect response by both AriaMx and CFX96), and thus a cfu/g could not be determined. The plate count results for both agar plates also were $<10^3$ cfu/g for each portion. At this level, quantitative analysis is not applicable. For the low action limit ($\geq 10^3$ cfu/g) level, there were five test portions that were threshold negative (i.e., gave a non-detect response by both AriaMx and CFX96). For the high action limit ($\geq 10^4$ cfu/g) level, there were seven test portions that were below the 10,000 cfu/g action limit (i.e., yet still gave a target detection response by both AriaMx and CFX96).

In this evaluation, the PCR results (AriaMx and CFX96) gave equivalent results to the DRBC and PDA plate counts at the approximately 10^3 cfu/g, approximately 10^4 cfu/g, and $>10^4$ cfu/g) contamination levels based on the acceptance criterion that the 90% confidence interval (CI) on the difference of means (DOM) is within -0.5 and 0.5. In all comparisons, 90% CIs were well within this limit. All DOMs were below 0.120 $\log_{10}$. See Table 7.

1 Table 6. Matrix study: PathoSEEK Total Yeast and Mold Count Assay qualitative threshold evaluation - presumptive vs confirmed results

Matrix	PCR System/ confirmation agar	Material[a]	Test Threshold (cfu/g)[b]	N[c]	PathoSEEK TYM presumptive results			Agar confirmed results				
					x[d]	POD_{CP}[e]	95% CI	x	POD_{CC}[f]	95% CI	$dPOD_{CP}$[g]	95% CI[h]
Naturally contaminated cannabis flower (THC >0.3%)	AriaMx/DRBC[i]	1	$<10^3$	5	0	0.000	0.000, 0.434	0	0.000	0.000, 0.434	0.000	-0.469, 0.469
		2	$\geq 10^3$	20	15	0.750	0.531, 0.888	15	0.750	0.531, 0.888	0.000	-0.132, 0.132
		3	$\geq 10^4$	20	13	0.650	0.433, 0.819	13	0.650	0.433, 0.819	0.000	-0.132, 0.132
		4	$>10^4$	5	5	1.000	0.566, 1.000	5	1.000	0.566, 1.000	0.000	-0.469, 0.469
	AriaMx/PDA[j]	1	$<10^3$	5	0	0.000	0.000, 0.434	0	0.000	0 .000, 0.434	0.000	-0.469, 0.469
		2	$\geq 10^3$	20	15	0.750	0.531, 0.888	15	0.750	0.531, 0.888	0.000	-0.132, 0.132
		3	$\geq 10^4$	20	13	0.650	0.433, 0.819	13	0.650	0.433, 0.819	0.000	-0.132, 0.132
		4	$>10^4$	5	5	1.000	0.566, 1.000	5	1.000	0.566, 1.000	0.000	-0.469, 0.469
	AriaMx/DRBC[k]	A	$<10^3$	5	1	0.200	0.000, 0.624	0	0.000	0.000, 0.434	0.200	-0.360, 0.760
		B	$\geq 10^3$	20	17	0.850	0.640, 0.948	15	0.750	0.531, 0.888	0.100	-0.083, 0.283
		C	$\geq 10^4$	20	6	0.300	0.145, 0.519	4	0.200	0.081, 0.416	0.100	-0.083, 0.283
		D	$>10^4$	5	5	1.000	0.566, 1.000	5	1.000	0.566, 1.000	0.000	-0.469, 0.469
	CFX96/DRBC	1	$<10^3$	5	0	0.000	0.000, 0.434	0	0.000	0.000, 0.434	0.000	-0.469, 0.469
		2	$\geq 10^3$	20	15	0.750	0.531, 0.888	15	0.750	0.531, 0.888	0.000	-0.132, 0.132
		3	$\geq 10^4$	20	13	0.650	0.433, 0.819	13	0.650	0.433, 0.819	0.000	-0.132, 0.132
		4	$>10^4$	5	5	1.000	0.566, 1.000	5	1.000	0.566, 1.000	0.000	-0.469, 0.469
	CFX96/PDA	1	$<10^3$	5	0	0.000	0.000, 0.434	0	0.000	0.000, 0.434	0.000	-0.469, 0.469
		2	$\geq 10^3$	20	15	0.750	0.531, 0.888	15	0.750	0.531, 0.888	0.000	-0.132, 0.132
		3	$\geq 10^4$	20	13	0.650	0.433, 0.819	13	0.650	0.433, 0.819	0.000	-0.132, 0.132

	4	$>10^4$	5	5	1.000	0.566, 1.000	5	1.000	0.566, 1.000	0.000	-0.469, 0.469

[a] There were four naturally contaminated cannabis flower materials evaluated for each PCR system/confirmation agar per laboratory, one for each threshold. In the method developer laboratory, materials are labeled 1, 2, 3, 4, and in the independent laboratory, materials are labeled A, B, C, D.

[b] Based on dilution and volume of sample tested. A positive result indicates contamination above the test threshold level.

[c] N = Number of test portions.

[d] x = Number of positive test portions.

[e] POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.

[f] POD_{CC} = Candidate method presumptive positive outcomes confirmed positive divided by the total number of trials.

[g] $dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

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

[i] DRBC = Dichloran rose bengal chloramphenicol agar.

[j] PDA = Acidified potato dextrose agar.

[k] Matrix tested in the independent laboratory.

Table 7. Matrix study: PathoSEEK Total Yeast and Mold Count Assay quantitative evaluation

Matrix	PCR System/ Confirmatory agar	Material[a]	n	PathoSEEK TYM result		Confirmatory agar[d]		DOM[e]	SE[f]	90% CI[g]	95% CI
				Mean[b]	s_r[c]	Mean	s_r				
Naturally contaminated cannabis flower (THC >0.3%)	AriaMx/DRBC[h]	1	5	<3.000	NA	<3.000	NA	NA	NA	NA	NA
		2	20	3.093	0.215	3.188	0.200	-0.095	0.027	-0.141, -0.048	-0.151, -0.039
		3	20	4.050	0.250	4.098	0.186	-0.048	0.035	-0.109, 0.013	-0.122, 0.026
		4	5	4.598	0.056	4.617	0.031	-0.019	0.034	-0.092, 0.054	-0.114, 0.076
	AriaMx/PDA[i]	1	5	<3.000	NA	<3.000	NA	NA	NA	NA	NA
		2	20	3.093	0.215	3.090	0.311	0.003	0.041	-0.068, 0.074	-0.083, 0.089
		3	20	4.050	0.250	4.076	0.241	-0.026	0.022	-0.064, 0.012	-0.072, 0.020
		4	5	4.598	0.056	4.717	0.094	-0.119	0.050	-0.226, -0.012	-0.259, 0.021
	AriaMx/DRBC[j]	A	5	<3.000	NA	<3.000	NA	NA	NA	NA	NA
		B	20	3.450	0.386	3.052	0.175	0.397	0.070	0.275, 0.519	0.250, 0.545
		C	20	3.972	0.219	3.889	0.101	0.083	0.040	0.014, 0.151	0.000, 0.166
		D	5	4.492	0.066	4.581	0.121	-0.089	0.062	-0.220, 0.043	-0.260, 0.083
	CFX96/DRBC	1	5	<3.000	NA	<3.000	NA	NA	NA	NA	NA
		2	20	3.126	0.233	3.188	0.200	-0.062	0.031	-0.116, -0.008	-0.127, 0.003
		3	20	4.082	0.198	4.098	0.186	-0.016	0.026	-0.061, 0.029	-0.071, 0.038
		4	5	4.640	0.055	4.617	0.031	0.023	0.033	-0.047, 0.093	-0.069, 0.115
	CFX96/PDA	1	5	<3.000	NA	<3.000	NA	NA	NA	NA	NA
		2	20	3.126	0.233	3.090	0.311	0.036	0.039	-0.032, 0.104	-0.046, 0.118
		3	20	4.082	0.198	4.076	0.241	0.006	0.026	-0.040, 0.051	-0.049, 0.061
		4	5	4.640	0.055	4.717	0.094	-0.077	0.054	-0.193, 0.039	-0.228, 0.074

[a] There were four naturally contaminated cannabis flower materials evaluated for each PCR system/confirmation agar per laboratory, one for each threshold. In the method developer laboratory, materials are labeled 1, 2, 3, 4, and in the independent laboratory, materials are labeled A, B, C, D.

[b] Mean of n test portions, after logarithmic transformation: $Log_{10}[CFU/g + (0.1)f]$.

[c] s_r = Standard deviation of repeatability.

[d] Confirmatory agar result = For the quantitative analysis, the PCR estimated cfu/g result for each test portion was compared to the corresponding cfu/g result for each agar plate.

[d] DOM = Difference of means; $mean_{cand}$ - $mean_{ref}$

[e] SE = Standard Error of DOM.

[f] CI = Confidence interval for DOM.

[I] DRBC = Dichloran rose bengal chloramphenicol agar.

[J] PDA = Acidified potato dextrose agar.

[K] Matrix tested in the independent laboratory.

Robustness Study

Methodology.—Robustness testing (qualitative) was conducted to evaluate the ability of the PathoSEEK TYM method to remain unaffected by small variations in method parameters that might occur when the method is performed by an end user. Three of the most critical parameters were tested, two of which impact the extraction kit, and one which impacts the PCR assay. The parameters were TLP enzyme volume, MGC Lysis Buffer volume, and Master Mix:Probe volume ratio. Each parameter was varied by approximately 10% above and below the nominal test condition. The varied test parameters were mixed into eight combinations using a factorial design. The test combinations can be found in Tables 8 and 9. Cannabis flower (THC >0.3%) was inoculated with *A. niger* (ATCC 16888) at a low action limit level ($\geq 10^3$ cfu/g, 1000 cfu/g) to produce fractional threshold positive results (2–8 positive results) from 10 replicate test portions tested. Ten inoculated replicate 10 g test portions were tested for each test combination. In addition, ten replicate 10 g test portions were tested using the nominal method parameters. Cannabis flower (THC >0.3%) with a yeast and mold concentration at $<10^3$ cfu/g was used as the negative control. Ten replicate 10 g test portions of the negative control were tested for each test combination and the nominal method parameters. Per the PathoSEEK TYM method protocol, 190 mL of TSB Broth was added to each test portion and then homogenized. Each homogenate was then processed according to the test combinations and nominal method parameters (including Grim Reefer components). Lysates were then tested on both the AriaMx and CFX96 Real-time PCR systems.

Results.—The results of the robustness testing are shown in Tables 8 and 9. The LCF AOAC Binary Workbook v5-2 was used to calculate the POD results using paired analysis. For the CFX96, there were no significant differences detected between any of the test combinations and the nominal conditions, for either the inoculated cannabis flower or the non-inoculated cannabis flower. For the AriaMx, significant differences were detected for at least two test combinations; Combination 2, 10 µL TLP enzyme, 10 µL MGC Lysis Buffer, 3.70:1.2 µL Master Mix/Probe volume, and Combination 5, 14 µL TLP

enzyme, 10 µL MGC Lysis Buffer, 3.70:1.2 µL Master Mix/Probe volume. In both cases, the MGC Lysis Buffer volume and Master Mix/Probe volume were the same and differed from the nominal condition, 12 µL MGC Lysis Buffer, 3.75:1 µL Master Mix/Probe volume respectively. Changing both conditions at the same time had an effect on the AriaMx system, however there were no other significant differences detected otherwise.

Table 8: Robustness study: PathoSEEK Total Yeast and Mold Count Assay for AriaMx results

Parameter test combination[a]	Parameters: TLP Enzyme	Parameters: MGC Lysis Buffer	Parameters: Master Mix:Probe volume	N[b]	x[c]	POD_E[d]	95% CI[e]	Nominal condition[f] result	POD_N[g]	95% CI	$dPOD_{EN}$[h]	95% CI[i]
Cannabis flower inoculated with *A. niger* ATCC[j] 16888												
1	10 µL	10 µL	3.80:0.8 µL	10	8	0.800	0.409, 0.943	8	0.800	0.490, 0.943	0.000	-0.253, 0.253
2	10 µL	10 µL	3.70:1.2 µL	10	4	0.400	0.168, 0.687	8	0.800	0.490, 0.943	-0.400	-0.787, -0.013
3	10 µL	15 µL	3.80:0.8 µL	10	8	0.800	0.409, 0.943	8	0.800	0.490, 0.943	0.000	-0.253, 0.253
4	10 µL	15 µL	3.70:1.2 µL	10	6	0.600	0.313, 0.832	8	0.800	0.490, 0.943	-0.200	-0.543, 0.143
5	14 µL	10 µL	3.80:0.8 µL	10	9	0.900	0.596, 1.000	8	0.800	0.490, 0.943	0.100	-0.206, 0.406
6	14 µL	10 µL	3.70:1.2 µL	10	2	0.200	0.057, 0.510	8	0.800	0.490, 0.943	-0.600	-1.000, -0.200
7	14 µL	15 µL	3.80:0.8 µL	10	7	0.700	0.397, 0.892	8	0.800	0.490, 0.943	-0.100	-0.406, 0.206
8	14 µL	15 µL	3.70:1.2 µL	10	6	0.600	0.313, 0.832	8	0.800	0.490, 0.943	-0.200	-0.543, 0.143
Cannabis flower with $<10^3$ CFU/g of TYM												
1	10 µL	10 µL	3.80:0.8 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
2	10 µL	10 µL	3.70:1.2 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
3	10 µL	15 µL	3.80:0.8 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
4	10 µL	15 µL	3.70:1.2 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
5	14 µL	10 µL	3.80:0.8 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
6	14 µL	10 µL	3.70:1.2 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
7	14 µL	15 µL	3.80:0.8 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
8	14 µL	15 µL	3.70:1.2 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253

[a] Each parameter test combination is being compared to the nominal test condition.
[b] N = Number of test portions per test combination.
[c] x = Number of positive test portions per combination.
[d] POD_E = Positive outcomes divided by the total number of trials per experimental combination.
[e] 95% CI = Confidence interval on the POD.
[f] Nominal condition = TLP enzyme 12 µL, MGC lysis buffer 12.5 µL, and Master mix/Probe volume 3.75:1 µL.
[g] POD_N = Positive outcomes divided by the total number of trials per nominal test condition.
[h] $dPOD_{EN}$ = Difference in POD between the nominal condition and experimental combinations.
[i] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[j] ATCC = American Type Culture Collection.

Table 9: Robustness study: PathoSEEK Total Yeast and Mold Count Assay for CFX96 results

Parameter test combination[a]	Parameters: TLP Enzyme	Parameters: MGC Lysis buffer	Parameters: Master Mix:Probe volume	N[b]	x[c]	POD_E[d]	95% CI[e]	Nominal condition[f] result	POD_N[g]	95% CI	$dPOD_{EN}$[h]	95% CI[i]
Cannabis flower inoculated with *A. niger* ATCC[j] 16888												
1	10 µL	10 µL	3.80:0.8 µL	10	9	0.900	0.596, 1.000	8	0.800	0.490, 0.943	0.100	-0.206, 0.406
2	10 µL	10 µL	3.70:1.2 µL	10	10	1.000	0.722, 1.000	8	0.800	0.490, 0.943	0.200	-0.143, 0.543
3	10 µL	15 µL	3.80:0.8 µL	10	8	0.800	0.409, 0.943	8	0.800	0.490, 0.943	0.000	-0.253, 0.253
4	10 µL	15 µL	3.70:1.2 µL	10	9	0.900	0.596, 1.000	8	0.800	0.490, 0.943	0.100	-0.206, 0.406
5	14 µL	10 µL	3.80:0.8 µL	10	8	0.800	0.409, 0.943	8	0.800	0.490, 0.943	0.000	-0.253, 0.253
6	14 µL	10 µL	3.70:1.2 µL	10	9	0.900	0.596, 1.000	8	0.800	0.490, 0.943	0.100	-0.206, 0.406
7	14 µL	15 µL	3.80:0.8 µL	10	8	0.800	0.409, 0.943	8	0.800	0.490, 0.943	0.000	-0.253, 0.253
8	14 µL	15 µL	3.70:1.2 µL	10	8	0.800	0.409, 0.943	8	0.800	0.490, 0.943	0.000	-0.253, 0.253
Cannabis flower with $<10^3$ CFU/g of TYM												
1	10 µL	10 µL	3.80:0.8 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
2	10 µL	10 µL	3.70:1.2 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
3	10 µL	15 µL	3.80:0.8 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
4	10 µL	15 µL	3.70:1.2 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
5	14 µL	10 µL	3.80:0.8 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
6	14 µL	10 µL	3.70:1.2 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
7	14 µL	15 µL	3.80:0.8 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
8	14 µL	15 µL	3.70:1.2 µL	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253

[a] Each parameter test combination is being compared to the nominal test condition.
[b] N = Number of test portions per test combination.
[c] x = Number of positive test portions per combination.
[d] POD_E = Positive outcomes divided by the total number of trials per experimental combination.
[e] 95% CI = Confidence interval on the POD.
[f] Nominal condition = TLP enzyme 12 µL, MGC lysis buffer 12.5 µL, and Master mix/Probe volume 3.75:1 µL.
[g] POD_N = Positive outcomes divided by the total number of trials per nominal test condition.
[h] $dPOD_{EN}$ = Difference in POD between the nominal condition and experimental combinations.
[i] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[j] ATCC = American Type Culture Collection.

Product Consistency and Stability Studies

Methodology.—The product consistency and the product stability were evaluated in separate studies. For the product consistency, three unique production lots of Extraction Components (SenSATIVAx Flower/Leaf DNA Purification Kit and SenSATIVAx TLP Purification Enzyme) and three unique production lots of Primer/Probe (PathoSEEK TYM PCR Mold Count Detection Assay) were tested for lot-to-lot consistency. Lots for each extraction kit and each PCR kit were mixed to create new lots A, B, and C as outlined in Table 9. The study was carried out with pure culture. *Aspergills flavus* (target strain, ATCC 16883) and *Pseudomonas aeruginosa* (non-target strain, ATCC 15442), which were cultured in TSB for 24 h at 37°C. The *A. flavus* was diluted in sterile TSB to create a low action limit level ($\geq 10^3$ cfu/g, 1000 cfu/g) yielding fractional threshold positive results (2–8 positive results/10 replicate portions tested). The *P. aeruginosa* strain was used without dilution. Ten replicates of each strain were randomized and blind-coded, and then tested as described in Table 10.

For the stability study, one production lot each of Extraction Components (SenSATIVAx Flower/Leaf DNA Purification Kit and SenSATIVAx TLP Purification Enzyme) and Primer/Probe (PathoSEEK TYM PCR Mold Count Detection Assay) were tested at 5 time points: 0, 3, 6, 9, and 12 months. The method components were kept at their recommended storage conditions for the 12-month duration. *Penicillium chrysogenum* (target strain, ATCC 18476) and *Escherichia coli* (non-target strain, ATCC 25922) were grown in TSB for 24 h at 37°C. The *P. chrysogenum* was diluted in sterile TSB to create a concentration that would yield a fractional threshold response (2–8 positive results/10 replicate portions tested). The *E. coli* strain was used without dilution. Ten replicates of each strain were randomized and blind-coded, and then tested as described in Table 11. The lysates generated for the 0-month time point were stored frozen and used for the testing at 3, 6, 9 and 12-month timepoints. Results from each storage timepoint was compared to the original 0 timepoint results.

Results.—The data for both product consistency and stability studies were examined using the LCF AOAC

Binary Workbook v5-2 paired POD statistical analysis. There were no statistical differences detected between production lots or storage time points. The shelf-life was proven to be at least 12 months.

Table 10. Product consistency study: PathoSEEK Total Yeast and Mold Count Assay results

Extraction Lot	Thermocycler/ PCR Lot	N[a]	x[b]	POD[c]	95% CI	Extraction/ PCR Lots	x	POD	95% CI	dPOD[d]	95% CI[e]
Target strain: *Penicillium chrysogenum* ATCC[f] 18476											
A	AriaMx (B)	10	7	0.700	0.397, 0.892	B/C	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
	CFX-96 (C)	10	6	0.600	0.313, 0.832	B/A	7	0.700	0.397, 0.892	-0.100	-0.406, 0.206
B	AriaMx (C)	10	7	0.700	0.397, 0.892	C/A	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
	CFX-96 (A)	10	7	0.700	0.397, 0.892	C/B	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
C	AriaMx (A)	10	7	0.700	0.397, 0.892	A/B	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
	CFX-96 (B)	10	7	0.700	0.397, 0.892	A/C	6	0.600	0.313, 0.832	0.100	-0.206, 0.406
Non-target strain: *Escherichia coli* ATCC 25922											
A	AriaMx (B)	10	0	0.000	0.000, 0.278	B/C	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
	CFX-96 (C)	10	0	0.000	0.000, 0.278	B/A	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
B	AriaMx (C)	10	0	0.000	0.000, 0.278	C/A	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
	CFX-96 (A)	10	0	0.000	0.000, 0.278	C/B	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
C	AriaMx (A)	10	0	0.000	0.000, 0.278	A/B	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
	CFX-96 (B)	10	0	0.000	0.000, 0.278	A/C	0	0.000	0.000, 0.278	0.000	-0.253, 0.253

[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 = Difference in POD between the lots.
[e] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[f] ATCC = American Type Culture Collection, Manassas, VA.

Table 11. Stability study: PathoSEEK Total Yeast and Mold Count Assay results

Age (Months)	PCR Instrument	N[a]	x[b]	POD[c]	95% CI[d]	x[e] (0)	POD[f]	95% CI	dPOD[g]	95% CI[h]
Target analyte: *Penicillium chrysogenum* ATCC[i] 18476										
3	AriaMx	10	7	0.700	0.397, 0.892	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
	CFX-96	10	7	0.700	0.397, 0.892	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
6	AriaMx	10	7	0.700	0.397, 0.892	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
	CFX-96	10	7	0.700	0.397, 0.892	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
9	AriaMx	10	7	0.700	0.397, 0.892	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
	CFX-96	10	7	0.700	0.397, 0.892	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
12	AriaMx	10	7	0.700	0.397, 0.892	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
	CFX-96	10	7	0.700	0.397, 0.892	7	0.700	0.397, 0.892	0.000	-0.253, 0.253
Non-target analyte: *Escherichia coli* ATCC 25922										

3	AriaMx	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
	CFX-96	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
6	AriaMx	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
	CFX-96	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
9	AriaMx	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
	CFX-96	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
12	AriaMx	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253
	CFX-96	10	0	0.000	0.000, 0.278	0	0.000	0.000, 0.278	0.000	-0.253, 0.253

1 [a] N = Number of test portions.
2 [b] x = Number of positive test portions at 3, 6, 9, and 10 months.
3 [c] POD = Positive outcomes divided by the total number of trials for the test timepoints.
4 [d] 95% CI = Confidence interval on the POD.
5 [e] x(0) = Number of positive test portions at 0 months.
6 [f] POD = Positive outcomes divided by the total number of trials for the 0 timepoint.
7 [d] POD = Difference between the test timepoint and the 0 timepoint POD values.
8 [h] 95% CI = Confidence interval on the dPOD.
9 [I] ATCC = American Type Culture Collection, Manassas, VA.
10
11

12 **Independent Laboratory Study**

13 *Matrix Study*

14 *Methodology*.—The Independent Laboratory study was conducted by Cambium Analytica (Traverse City,
15 MI). The purpose of the study was to demonstrate transferability of the PathoSEEK TYM method to a
16 new end user. For this study, a matrix study was performed on dried cannabis flower (THC>0.3%). The
17 study design followed that of the method developer's matrix study (see above). Four materials were
18 prepared targeting contamination levels below the lowest threshold (<10^3 cfu/g), the low and high
19 action limits (approximately 10^3 and 10^4 cfu/g), and above the highest threshold (>10^4 cfu/g). The
20 laboratory had naturally contaminated dried cannabis flower in-house that they were able to use for the
21 study. The dried cannabis flower materials were screened for total yeast and mold counts using DRBC,
22 found to be naturally contaminated, and then product was mixed with other low-level contaminated
23 (<10^3 cfu/g) dried cannabis flower as needed to create the target contamination levels. All negative
24 results were expected at the lowest level, fractional positive results (5–15 positive results/20 portions
25 tested) were expected at the low and high action threshold limits, and all positive results were expected

above the highest threshold. One Real-time PCR system was evaluated in the study, AriaMx, and one agar plate, DRBC, for confirmation and agar counts.

The test portions were randomized and blind-coded before the start of testing. Each 10 g test portion was weighed into a Whirl-Pak bag with a mesh liner, and then 190 mL of TSB was added. The cannabis with broth was mixed by hand for 1 min. An aliquot was removed from each test homogenate and processed according to the PathoSEEK TYM IFU. Each homogenate was also spread-plated onto DRBC agar plates for counts. Dilutions were made to each homogenate as needed to obtain counts between 10–150 colonies/plate. Plates were incubated at 25 ± 1°C for a total of 7 days.

For each test portion, the PathoSEEK TYM method result was converted to cfu/g according the equation provided in the IFU, cfu/g = {10^[(-0.1267*Cq)+6.6781]}*20. For the qualitative threshold analysis, test portions producing results above the threshold limits were classified as positive, and test portions producing results below the threshold limits were classified as negative. The corresponding DRBC agar plates for each test portion were counted, and then the counts were multiplied by the appropriate dilution factor to determine the final count in cfu/g. The PCR results were considered the presumptive results, and the agar plate results were considered the confirmed results.

For the quantitative analysis, the PCR cfu/g result for each test portion was compared to the corresponding cfu/g result for the DRBC agar plate.

Results.—The results for the independent laboratory matrix study are included in Tables 6 and 7. The LCF AOAC Binary Workbook v5-2 and the LCF Quantitative Analysis for Micro Methods v1.2 were used for the paired statistical analyses for the qualitative and quantitative evaluations, respectively.

In the qualitative threshold study, one test portion at the $<10^3$ target contamination level gave a positive PCR result (AriaMx), but a negative ($<10^3$ cfu/g) DRBC result negative at the $<10^3$ cfu/g level. The four other test portions at that level were threshold negative ($<10^3$ cfu/g) for both PCR and DRBC. All test portions (five) at the highest threshold ($>10^4$) target contamination level were qualitatively

threshold (calculated >10^4 cfu/g) positive by PathoSEEK TYM PCR (AriaMx) and >10^4 cfu/g for on DRBC. For the low action limit (approximately 10^3 cfu/g), there were 17 positive test portions and 3 negative test portions (i.e., gave a non-detect response) by PCR. There were 15 test portions that plate counts were >10^3 cfu/g on DRBC.

For the high action limit (≥10^4 cfu/g) level, there were 6 positive test portions and 14 test portions that were threshold negative. There were 4 test portions that were threshold positive (≥10^4 cfu/g) on DRBC plates. Based on the POD analysis, no significant statistical differences were detected. See Table 6.

In the quantitative study, one of the five test portions at the target <10^3 contamination level was positive by PCR (AriaMx), but the results for the DRBC plates were <10^3 cfu/g for each portion. Applying the same recommendation from the AOAC statistical advisor to substitute the value 601 in the quantitative spreadsheet for values that are below the detection limit. The results in cfu/g were $\log_{10}$ transformed as described in the method developer study for the analysis. The mean transformed result for the 5 replicate test portions at that level was <3.000 $\log_{10}$. At this level, quantitative analysis is not applicable.

For the low action limit (≥10^3 cfu/g), there were three test portions that were negative. Same as previously utilized for the in-house studies, the 601 cfu value was used to substitute for negative threshold test portions in the analysis. The 90% CI for this level was (0.275, 0.519), which was just outside the acceptance criterion for equivalence (-0.5, 0.5). The DOM was 0.397 for this level. For the ≥10^4 cfu/g and >10^4 cfu/g contamination levels, the PCR results (AriaMx) and the DRBC plate results were determined to be equivalent based on the acceptance criterion, and the DOMs at these levels were below 0.100 $\log_{10}$. See Table 7.

Discussion

In the inclusivity study, four strains out of the 55 yeasts and mold tested were not detected by the

PathoSEEK TYM method: *Arthrinium arundinis, Phytophthora infestans, Botrytis cinerea, and Scopulariopsis acremonium* (Table 4). Both *A. arundinis* and *P. infestans* were also not detected by PDA or DRBC, and these strains did not grow in the PathoSEEK TYM medium used for the inclusivity study (TSB). *B. cinerea* and *S. acremonium* were recovered on PDA, but the *B. cinera* grew slowly on the DRBC. The ATCC website recommends using potato dextrose broth for *S. acremonium*, and this strain may not have grown well in TSB. All 31 exclusivity strains tested were correctly excluded (Table 5).

In the matrix studies, the PathoSEEK TYM method was evaluated as both a qualitative threshold method and as a quantitative method. Using the conversion equation provided in the IFU, cfu/g = {10^[(-0.1267*Cq)+6.6781]}*20, the Cq values were converted to estimated cfu/g. For the qualitative threshold study in the method developer's laboratory, no statistical differences were detected by POD analysis (95% CI on the dPOD contains 0) between the PathoSEEK TYM results using either Real-time qPCR system (AriaMx and CFX96) compared to both plating media (DRBC and PDA) at any of the test levels: lowest threshold ($<10^3$ cfu/g), the low and high action limits (approximately 10^3 and 10^4 cfu/g), and above the highest threshold ($>10^4$ cfu/g). In the independent laboratory, no statistical differences were detected between the PathoSEEK TYM results using the AriaMx system and DRBC agar plate. There were two results at the low action limit ($\geq 10^3$ cfu/g) that were positive by the PathoSEEK TYM and negative by DRBC, and two results at the high action limit ($\geq 10^4$ cfu/g) that were positive by the PathoSEEK TYM and negative by DRBC. For the two test portions at the low action limit that were positive by PCR but negative by plating, the results for both were just over the action limit, 1,211 and 1,060 cfu/g for PCR, while the plating result was just under at 760 and 720 cfu/g. For the two test portions at the high action limit, that were threshold positive by PCR, with 14,809 and 20,000 cfu/g for PCR, but threshold negative by plating, with 5,720 and 7,900 cfu/g for plating. In both cases, the positive responses would err on the side of caution (the cannabis flower being tested would be considered over the action limit and thus fail to be released). There were no results that were negative by PCR and positive by plating (Table 6).

For the quantitative analysis in the method developer's laboratory, equivalent results were demonstrated (90% CI on the DOM within -0.5, 05) between the PathoSEEK TYM method and the agar plating methods for both PCR systems (AriaMx and CFX96) and both agars (DRBC and PDA) at all applicable levels. In the independent laboratory, the 90% CI on the DOM for the low action limit ($\geq 10^3$ cfu/g) was (0.275, 0.519), which is just barely above the acceptance criterion, and the DOM was 0.397. This DOM was higher than the rest, as all others in both laboratories were approximately 0.1 or lower (Table 7). Considering the difference in technologies (PCR estimation vs plating), the DOMs and 90% CIs are well under the acceptance criterion expectations.

In the robustness testing, TLP enzyme, MGC Lysis Buffer, and Master Mix/Probe volume were varied using a factorial design and then compared to the nominal test condition to evaluate any effects on the PathoSEEK TYM method. When using the AriaMx system, there were statistical differences detected in two test combinations using the paired POD analysis, where the test combinations had fewer positive results than the nominal condition. In both cases, the MGC Lysis Buffer volume and Master Mix/Probe volume were the same in the test combinations and differed from the nominal condition. There was less lysis buffer and more probe volume in the test combinations (Table 8). It is possible that this proportion is not optimal for the AriaMx system performance, however using the CFX96 system, the results were not affected. For the CFX96 system, there were no statistical differences detected in any of the test combinations compared to the nominal test condition (Table 9).

In the product consistency and stability testing, there were no statistical differences detected between production lots or storage time points based on the paired POD analysis (Tables 10 and 11). The shelf-life was proven to be 12 months (Table 11).

Conclusion

The data from these studies, within their statistical uncertainty, support the performance claims of the of the PathoSEEK TYM method for qualitative threshold detection and enumeration of total yeast and molds in dried cannabis flower (THC >0.3%).

The study data have been evaluated in the AOAC Research Institute *Performance Tested Methods*^{SM} Program and support certification of the PathoSEEK TYM method (AOAC PTM 062401) for total yeast and mold detection and enumeration in dried cannabis flower (>0.3% THC) at contamination levels >10^3 cfu/g within the scope indicated in Tables 12–14.

Table 12. PathoSEEK TYM Method: Method Performance Claims – Qualitative

Matrix	Test Portion	Diluent	SMPR	Claim
Dried cannabis flower (>0.3% THC)	10 g	TSB[b]	2021.009[c]	NSDD[d]
Dried cannabis flower (>0.3% THC)[d]	10 g	TSB	2021.009	NSDD

[a] Matrix tested by the method developer and the independent laboratory.

[b] TSB = Tryptic soy broth.

[c] *Standard Method Performance Requirements* (SMPRs) for Viable Yeast and Mold Count Enumeration in Cannabis and Cannabis Products (AOAC SMPR 2021.009).

[d] NSDD = No statistical difference detected using SLV study design from OMA Appendix J (2012) is not intended to demonstrate statistical equivalence. Expert opinion is that the method is appropriate for its intended use. For cannabis matrixes, comparison is only between presumptive and confirmed candidate method results. Confirmation by plating onto dichloran rose bengal chloramphenicol and acidified potato dextrose agar spread plates for 5–7 days as 25°C.

Table 13. PathoSEEK TYM Method: Method Performance Claims – Quantitative

Matrix	Test Portion	Diluent	SMPR	Claim
Dried cannabis flower (>0.3% THC)[a]	10 g	TSB[b]	2021.009[c]	Eq[d]
Dried cannabis flower (>0.3% THC)	10 g	TSB	2021.009	Eq

[a] Matrix tested by the method developer and the independent laboratory.

[b] TSB = Tryptic soy broth.

[c] *Standard Method Performance Requirements* (SMPRs) for Viable Yeast and Mold Count Enumeration in Cannabis and Cannabis Products (AOAC SMPR 2021.009).

[d] Eq = Equivalence of candidate method vs. plate counts (dichloran rose bengal chloramphenicol and acidified potato dextrose agar spread plates for 5–7 days as 25°C) demonstrated by 90% confidence interval on the difference of means contained entirely within -0.5 to 0.5 $\log_{10}$, for contamination levels >10^3 cfu/g.

Table 10. PathoSEEK TYM Method: Method Selectivity

Enrichment	Inclusivity Strains	Exclusivity Species

Broth	Temp., °C	No. Tested	No. Positive	No. Tested	No. Positive
TSB[a]	20–28°C	55[b]	51[c]	31[d]	0

[a] TSB = tryptic soy broth.

[b] Comprising 55 unique yeast and mold species.

[c] *Arthrinium arundinis* and *Phytophthora infestans* were not detected by either the PathoSEEK TYM Method or PDA agar. The other two isolates not detected by the PathoSEEK TYM Method were *Botrytis cinerea* and *Scopulariopsis acremonium,* but these isolates were able to be recovered on PDA agar.

[d] Comprising 31 non-target species. Exclusivity organisms were cultured under optimal conditions for growth.

Acknowledgments

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Conflict of Interest

The authors declare that they work for Medicinal Genomics and receive a salary for this work. Cambium Analytica received payment from Medicinal Genomics to perform the independent laboratory work.

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