PathoSEEK® Total Aerobic Bacteria Count Assay v2 with SenSATIVAx® DNA Purification Protocol for Detection in Cannabis Flower and MIP Matrices

Method Developer Validation

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Abstract

Background:

Total Aerobic Bacteria encompasses many species. Bacteria can cause deterioration and decomposition of cannabis, and certain species of aerobic bacteria, such as Shiga Toxin producing *E. coli,* can cause infections in humans. Current regulations allow cannabis flower and cannabis products to contain a limit of total aerobic bacteria. The PathoSEEK® Total Aerobic Bacteria Count, or Total Aerobic Count (TAC), Assay with SenSATIVAx® DNA Purification Protocol is designed to detect all aerobic bacteria in a single qPCR (Quantitative Polymerase Chain Reaction) in cannabis flower, hemp flower, cannabis concentrates, infused edibles, and infused non-edibles.

Objective:

To evaluate the PathoSEEK® Total Aerobic Count Detection Assay v2, using the SenSATIVAx® Flower and MIP DNA purification protocols for the enumeration of total aerobic bacteria in cannabis flower (delta 9-tetrahydrocannabinol >0.3%; 1g), and for presence/absence detection in marijuana-infused products (MIP).

Results:

The inclusivity and exclusivity results demonstrated the high specificity of the PathoSEEK® Total Aerobic Count method in differentiating target organisms, prevalent in cannabis flower and infused products, from non-target organisms. The SenSATIVAx® flower DNA Purification kit and PathoSEEK® Total Aerobic Count Detection Assay v2 was validated by constructing an enumeration curve and conversion equation using eleven distinct bacterial species and plating on 3M™ Petrifilm™ RAC (Rapid Aerobic Count) Plates. Subsequent to the generation of this curve, a Certified Reference Materials (CRMs) from NSI, Quantitative APC in Hemp and EB in Hemp, were procured and analyzed utilizing the SenSATIVAx flower DNA Purification kit and PathoSEEK Total Aerobic Count qPCR Detection Assay v2. Resulting Cq values were converted to CFU/g utilizing the conversion equation and were compared to results obtained via Neogen Petrifilm™ RAC Petrifilms and to the NSI value provided on the CRM Certificate of Analysis. Results generated by qPCR were comparable to the RAC Petrifilm results and aligned with the specifications presented by NSI.

Materials

SenSATIVAx® Flower & Leaf DNA Purification Kit Components - **P/N 420001**

Component Name	Qty Provided	Storage Conditions
MGC Cell Lysis Buffer	1 Bottle (12 mL)	RT (20–28°C)
MGC Binding Buffer	1 Bottle (48 mL)	Refrigerate (2-8 ºC)
MGC Elution Buffer	1 Bottle (12 mL)	RT (20–28°C)

SenSATIVAx® Infused Product DNA Purification Kit Components - **P/N 420004**

Component Name	Qty Provided	Storage Conditions
SenSATIVAx® Solution A	1 Bottle (350 mL)	RT (20–28°C)
SenSATIVAx® Solution B	1 Bottle (25 mL)	RT (20–28°C)
MGC Binding Buffer	1 Bottle (48 mL)	Refrigerate (2-8 ºC)
MGC Elution Buffer	1 Bottle (12 mL)	RT (20–28°C)

PathoSEEK® Internal Control - **P/N 420337**

Component Name	Qty Provided	Storage Conditions
Internal Control	1 Tube (50 µL)	-15 to -20 ºC

PathoSEEK® Total Aerobic Bacteria Count (TAC) Detection Assay v3 Kit - **P/N 420541**

Component Name	Qty Provided	Storage Conditions
PathoSEEK® Total Aerobic Count Detection Assay v2	1 Tube (200 µL)	-15 to -20 ºC
qPCR Master Mix Kit v3 - Reaction Buffer	1 Tube (160 µL)	-15 to -20 ºC
qPCR Master Mix Kit v3 - Nuclease Free Water	2 Tubes (1 mL)	-15 to -20 ºC
qPCR Master Mix Kit v3 - Master Mix v3	1 Tube (750 µL)	-15 to -20 ºC

Optional: Grim Reef Free DNA Removal Kit - **P/N 420145**

Component Name	Qty Provided	Storage Conditions
GR Enzyme	1 Bottle (2.5 mL)	-15 to -20 ºC
GR Buffer	1 Bottle (12.5 mL)	-15 to -20 ºC

Note: Actual fill volumes include overage

Inclusivity and Exclusivity

Methodology

For the inclusivity evaluation, 30 bacterial strains were assessed. Target strains were either cultured in Tryptic Soy Broth for 24 hours at 37°C, followed by DNA extraction, or purified DNA from ATCC was utilized. For exclusivity, 30 organisms were evaluated. Target strains were either cultured under optimal growth conditions for the organism, followed by DNA extraction, or purified DNA from ATCC was utilized. Inclusivity and exclusivity cultures were randomized, blind-coded, and analyzed using the PathoSEEK® Total Aerobic Count method.

Results

Of the 30 inclusivity strains tested, 30 were correctly detected by the method. Of the 30 exclusivity strains tested, all 30 were correctly excluded. Tables 1 and 2 present a summary of the results.

Table 1: Inclusivity Results, PathoSEEK® Total Aerobic Count Assay

Species	ATCC#	PathoSEEK® TAC Result
Acinetobacter baumannii	19606	Detected
Aeromonas hydrophila	7966	Detected
Burkholderia multivorans	17616	Detected
Bacillus subtilis	11774	Detected
Citrobacter braakii	3037	Detected
Citrobacter koseri	25408	Detected
Edwardsiella tarda	23672	Detected

Enterobacter aerogenes	13048	Detected
Enterobacter cloacae	13047	Detected
Erwinia rhapontici	29290	Detected
Escherichia coli	25922	Detected
Escherichia coli O157:H7	35150	Detected
Escherichia hermannii	700368	Detected
Escherichia vulneris	33821	Detected
Hafnia alvei	51873	Detected
Klebsiella oxytoca	51983	Detected
Klebsiella pneumonia	BAA-2146	Detected
Listeria monocytogenes	7647	Detected
Morganella morganii	25829	Detected
Pantoea agglomerans	43348	Detected
Proteus mirabilis	43071	Detected
Pseudomonas aeruginosa	15442 & 35552	Detected
Pseudomonas fluorescens	13525	Detected
Pseudomonas putida	47054	Detected
Ralstonia insidiosa	49129	Detected
Rahnella species	33991	Detected
Salmonella enterica	13311	Detected
Stenotrophomonas maltophilia	13637	Detected
Staphylococcus aureus	12600	Detected
Serratia marcescens	27137	Detected

Table 2: Exclusivity Results, PathoSEEK® Total Aerobic Count Assay

Species	ATCC#	PathoSEEK® TAC Result
Alternaria alternata	6663	Not Detected
Aspergillus alabamensis	MYA-3633	Not Detected
Aspergillus brasiliensis	9642	Not Detected
Aspergillus carbonarius	MYA-4641	Not Detected
Aspergillus caesiellus	42693	Not Detected
Aspergillus carneus	13549	Not Detected
Aspergillus clavatus	1007	Not Detected
Aspergillus deflectus	62502	Not Detected
Aspergillus flavus	9643	Not Detected
Aspergillus fumigatus	1022	Not Detected
Aspergillus japonicus	16873	Not Detected
Aspergillus nidulans	38163	Not Detected

Aspergillus niger	13496	Not Detected
Aspergillus oryzae	42149	Not Detected
Aspergillus parasiticus	56775	Not Detected
Aspergillus pseudoterreus	10020	Not Detected
Aspergillus terreus	1012	Not Detected
Aspergillus tubigensis	1004	Not Detected
Aspergillus ustus	1041	Not Detected
Aspergillus versicolor	11730	Not Detected
Candida albicans	10231	Not Detected
Cryptococcus laurentii	18803	Not Detected
Cryptococcus neoformans	208821	Not Detected
Fusarium oxysporum	62506	Not Detected
Fusarium solani	52628	Not Detected
Mucor luteus	28932	Not Detected
Penicillium chrysogenum	18476	Not Detected
Penicillium rubens	11709	Not Detected
Rhizopus stolonifera	14037	Not Detected
Talaromyces pinophilus	11797	Not Detected

Cq to CFU Equation for Flower Samples

1. The equation utilized for quantifying colony-forming units per gram (CFU/g) was established through quantitative polymerase chain reaction (qPCR) analysis of eleven bacterial organisms, followed by their subsequent cultivation on Petrifilm Coliform Count (CC) plates. Both qPCR and plating protocols were executed in triplicate across a series of six serial dilutions. Mean values were computed and depicted on a scatter graph, with qPCR cycle quantification (Cq) data presented on the x-axis and the base-10 logarithm of plating data on the y-axis. A linear regression analysis was performed to determine the best-fit line, resulting in the equation: **y = -0.2383x + 10.005,** where y is the log10 CFU/g and x is the Cq value.
2. Utilize the following linear equation to convert Cq (x) values to Log CFU (y): y = -0.2383x + 10.005.
3. Perform an inverse logarithmic transformation of Y to obtain CFU/g.
4. Multiply the derived CFU/g value by the sample's upfront dilution factor (x 20) in TSB to determine the final CFU/g.

Empirical validation has confirmed that this derived equation produces comparable results across the

Agilent AriaMX, BioRad CFX96, and BioMolecular Systems MIC quantitative PCR instruments.

Proficiency Testing / Certified Reference Materials

Methodology - Flower Matrix

Two CRMs from NSI, APC in Hemp (catalog # FM-725) and EB in Hemp (catalog # FM-730), were analyzed following the PathoSEEK Total Aerobic Count User Guide v3. qPCR was performed in triplicate on an Agilent Aria, Bio-Rad CFX96, and BioMolecular Systems MIC. Cq values were converted into CFU/g values and compared with the expected range of the CRMs provided by NSI.

Table 3: CRM Results Flower, PathoSEEK® Total Aerobic Count Assay

Content	Instrument	Average Cq Fam	CFU
APC in Hemp	Agilent Aria	27.92	45,029
APC in Hemp	Biorad CFx96	28.62	30,565
APC in Hemp	BMS MIC	28.19	38,730
EB in Hemp	Agilent Aria	27.68	51,273
EB in Hemp	Biorad CFx96	28.24	37,764
EB in Hemp	BMS MIC	27.88	45,826

Methodology, Non–Flower Matrices

To ascertain our ability to detect Total Aerobic Bacteria in MIPs, we acquired a S. arizonae EZ Accushot from Microbiologics (Ref # 0901A). Following the provided instructions, we inoculated 1g of Oil with 100 cfu of S. arizonae, 2.4 mL TSB and enriched the sample at 37°C for 16 hours. Post enrichment, three samples were processed and analyzed on all three qPCR instruments to determine if this concentration of Total Aerobic Bacteria could be detected.

Results

Each instrument detected the presence of Total Aerobic Bacterial DNA.

Table 4: Detection of 100 cfu in Oil, PathoSEEK® Total Aerobic Count Assay

Content	Instrument	Detected
S. arizonae in Oil	Agilent Aria	Yes
S. arizonae in Oil	Biorad CFx96	Yes
S. arizonae in Oil	BMS MIC	Yes

Methodology, Non-Flower Matrices Continued

To further test the assays ability to detect aerobic bacteria in non flower matrices, two CRMs from NSI, S. aureus in Hemp Oil (catalog # FM-832) and STEC in Edible (catalog # CMQC-029), were analyzed following the PathoSEEK Total Aerobic Count User Guide v3. Samples were enriched in TSB for 16 hours at 37°C followed by DNA purification and qPCR. qPCR was performed in triplicate on an Agilent Aria, Bio-Rad CFX96, and BioMolecular Systems MIC. A Cq value indicates a positive result and the sample material should be plated for enumeration.

Results

Each instrument called within the expected range of each CRM.

Table 5: CRM Results MIP, PathoSEEK® Total Aerobic Count Assay

Content	Instrument	Detected
S. aureus in Hemp oil	Agilent Aria	Yes
S. aureus in Hemp oil	Biorad CFx96	Yes
S. aureus in Hemp oil	BMS MIC	Yes
STEC in Edible	Agilent Aria	Yes
STEC in Edible	Biorad CFx96	Yes
STEC in Edible	BMS MIC	Yes

Results

Each instrument detected the presence of Total Aerobic Bacterial DNA.

Conclusions

The PathoSEEK® Total Aerobic Count qPCR Assay v2 with SenSATIVAx® DNA Purification is a rapid, alternative method to traditional plating procedures for the detection of aerobic bacteria in cannabis flower and cannabis infused products. The method produced comparable results to 3M Petrifilm EB and CC plates for the enumeration of aerobic bacteria in cannabis flower.

REVISION HISTORY

Version	Date	Description
v1	September, 2021	1. Update to User Guide Format 2. Updated sample to media ratio used for homogenization of flower 3. Update to qPCR Master Mix v3 4. Update to conversion equations
v2	November 2022	1. Updated sample to media ratio used for homogenization of flower 2. Update to conversion equation for flower matrix 3. Update MIP detection to a presence absence, removal of conversion equation for MIP matrices.
v3	August 2025	1. New kitted packaging format 2. New assay version (v2) to include use with BMS Mic and Myra 3. BMS Mic and Myra data analysis section 4. Update to Internal Control from SCCG to IC

DISCLAIMER

This test was developed, and its performance characteristics determined by Medicinal Genomics Company, for laboratory use. Any deviations from this protocol are not supported by MGC.

This test has not been validated on remediated (irradiated, ozone treated, acid treated, hydrogen peroxide treated, etc.) samples. Samples that have undergone remediation may cause discordant results between plating methods and PathoSEEK® methods. When remediated samples produce a result above the action limit on qPCR, we recommend confirming viability with an approved plating method.

Results may vary based on laboratory conditions. Altitude and humidity are factors known to affect the growth of bacterial and fungal species.

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