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

Manufacturers 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, 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:

Inclusivity and exclusivity results showed the PathoSEEK® Total Aerobic Count method is highly specific in discriminating target organisms found in cannabis flower and infused products from non-target organisms. The SenSATIVAx® flower DNA Purification kit and PathoSEEK® Total Aerobic Count Detection Assay was validated by creating an enumeration curve against nine different bacterial species and plating on 3M™ Petrifilm™ RAC (Rapid Aerobic Count)

Plates. After creating this curve, we obtained a CRM from NSI, Quantitative APC in Hemp, and ran it using the SenSATIVAx flower DNA Purification kit and PathoSEEK Total Aerobic Count qPCR Detection Assay. After obtaining a qPCR Cq value, we converted the data to CFU/g using our conversion equation and compared it to results obtained using 3M™ Petrifilm™ RAC Plates and to the NSI value provided on the CRM Certificate of Analysis. Our result was comparable to 3M results as well as within the specifications presented by NSI.

Conclusions:

The SenSATIVAx® flower and MIP DNA Purification kits along with the PathoSEEK® Total Aerobic Count Detection Assay is a rapid, alternative method to traditional plating procedures for the detection of Total Aerobic Bacteria in cannabis flower and cannabis infused products.

Materials

Test Kit Name: PathoSEEK® Total Aerobic Bacteria Count Assay with SenSATIVAx® DNA Purification (with Optional Grim Reefer free DNA removal)

Test Kit Information

1. SenSATIVAx® Flower/Leaf DNA Purification Kit - P/N 420001
2. SenSATIVAx® MIP/Extract DNA Purification Kit - P/N 420004
3. Medicinal Genomics qPCR Master Kit v3 - P/N 420201
4. PathoSEEK® Total Aerobic Count Detection Assay - P/N 420106
5. PathoSEEK® Total Aerobic Count Assay Positive Control - P/N 420306
6. Grim Reefer Free DNA Removal Kit - P/N 420145
7. Grim Reefer Free DNA Removal Control - P/N 420144
8. Grim Reefer Free DNA Removal Assay - P/N 420143

Method Developer Validation

Wet Laboratory Methodology

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

Results

Of the 30 inclusivity strains tested, 30 were correctly detected by the PathoSEEK® 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
1	*Acinetobacter baumannii*	19606	Detected
2	*Aeromonas hydrophila*	7966	Detected
3	*Burkholderia multivorans*	17616	Detected
4	*Bacillus subtilis*	11774	Detected
5	*Citrobacter braakii*	3037	Detected
6	*Citrobacter koseri*	25408	Detected
7	*Edwardsiella tarda*	23672	Detected
8	*Enterobacter aerogenes*	13048	Detected
9	*Enterobacter cloacae*	13047	Detected
10	*Erwinia rhapontici*	29290	Detected
11	*Escherichia coli*	25922	Detected
12	*Escherichia coli O157:H7*	35150	Detected
13	*Escherichia hermannii*	700368	Detected
14	*Escherichia vulneris*	33821	Detected
15	*Hafnia alvei*	51873	Detected
16	*Klebsiella oxytoca*	51983	Detected
17	*Klebsiella pneumonia*	BAA-2146	Detected
18	*Listeria monocytogenes*	7647	Detected
19	*Morganella morganii*	25829	Detected

20	*Pantoea agglomerans*	43348	Detected
21	*Proteus mirabilis*	43071	Detected
22	*Pseudomonas aeruginosa*	15442 & 35552	Detected
23	*Pseudomonas fluorescens*	13525	Detected
24	*Pseudomonas putida*	47054	Detected
25	*Ralstonia insidiosa*	49129	Detected
26	*Rahnella species*	33991	Detected
27	*Salmonella enterica*	13311	Detected
28	*Stenotrophomonas maltophilia*	13637	Detected
29	*Staphylococcus aureus*	12600	Detected
30	*Serratia marcescens*	27137	Detected

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

#	Species	ATCC#	PathoSEEK® TAC Result
1	*Alternaria alternata*	6663	Not Detected
2	*Aspergillus alabamensis*	MYA-3633	Not Detected
3	*Aspergillus brasiliensis*	9642	Not Detected
4	*Aspergillus carbonarius*	MYA-4641	Not Detected
5	*Aspergillus caesiellus*	42693	Not Detected
6	*Aspergillus carneus*	13549	Not Detected
7	*Aspergillus clavatus*	1007	Not Detected
8	*Aspergillus deflectus*	62502	Not Detected
9	*Aspergillus flavus*	9643	Not Detected
10	*Aspergillus fumigatus*	1022	Not Detected
11	*Aspergillus japonicus*	16873	Not Detected
12	*Aspergillus nidulans*	38163	Not Detected
13	*Aspergillus niger*	13496	Not Detected
14	*Aspergillus oryzae*	42149	Not Detected
15	*Aspergillus parasiticus*	56775	Not Detected

16	*Aspergillus pseudoterreus*	10020	Not Detected
17	*Aspergillus terreus*	1012	Not Detected
18	*Aspergillus tubigensis*	1004	Not Detected
19	*Aspergillus ustus*	1041	Not Detected
20	*Aspergillus versicolor*	11730	Not Detected
21	*Candida albicans*	10231	Not Detected
22	*Cryptococcus laurentii*	18803	Not Detected
23	*Cryptococcus neoformans*	208821	Not Detected
24	*Fusarium oxysporum*	62506	Not Detected
25	*Fusarium solani*	52628	Not Detected
26	*Mucor luteus*	28932	Not Detected
27	*Penicillium chrysogenum*	18476	Not Detected
28	*Penicillium rubens*	11709	Not Detected
29	*Rhizopus stolonifera*	14037	Not Detected
30	*Talaromyces pinophilus*	11797	Not Detected

Generation of Cq to CFU Conversion Equation for flower samples

(a) The Cq to CFU/g equation was generated by running nine organisms on qPCR compared against plating on 3M Petrifilm RAC plates. qPCR was done in triplicate and plating was done in triplicate. We averaged all results before creating a scatter point graph, using the qPCR data on the x axis, and the log10 of the plating data on the y axis. We created the equation using the best fit line to these points. The resulting equation is y = -0.2383x+10.005.

(b) Use the following equation to convert Cq (X) to Log CFU (Y)

$$Y = -0.2383X + 10.005$$

(c) Perform an inverse logarithmic transformation of Y to obtain CFU/g.

(d) Multiply resulting CFU by upfront dilution factor of sample to TSB (x20).

Table 3: Cq to CFU Conversion Equations

Matrix	Microbial Test	Cq to CFU Conversion Equation
Flower	Total Aerobic Count	CFU/g = 10^((-0.2383*Cq)+10.005) Multiply resulting CFU x 20 to account for upfront dilution factor
MIP	Total Aerobic Count	IF Cq<40, Plate confirm for enumeration

Limit of Detection

The limit of detection is used to describe the smallest concentration of a species that can be reliably measured by the Medicinal Genomics (MGC) PathoSEEK® Total Aerobic Count Detection Assay. This is the point where the qPCR signal crosses the set threshold before a Cq of 40. The genomic copy number was calculated using the sample DNA concentration and the size of the genome for the species in question using the equation: number of copies = X ng*6.0221 x 10^{23} molecules/mole / (N x 650 g/mole) * 1x 10^{9} ng/g). The following data demonstrates the experiments used to calculate the limit of detection when using the PathoSEEK® v3 qPCR Master Kit and Total Aerobic Count Assay. The following organism was evaluated for LOD of the PathoSEEK® Total Aerobic Count Detection Assay in the absence of cannabis matrix, *E.coli* ATCC# 8739D-5.

Results

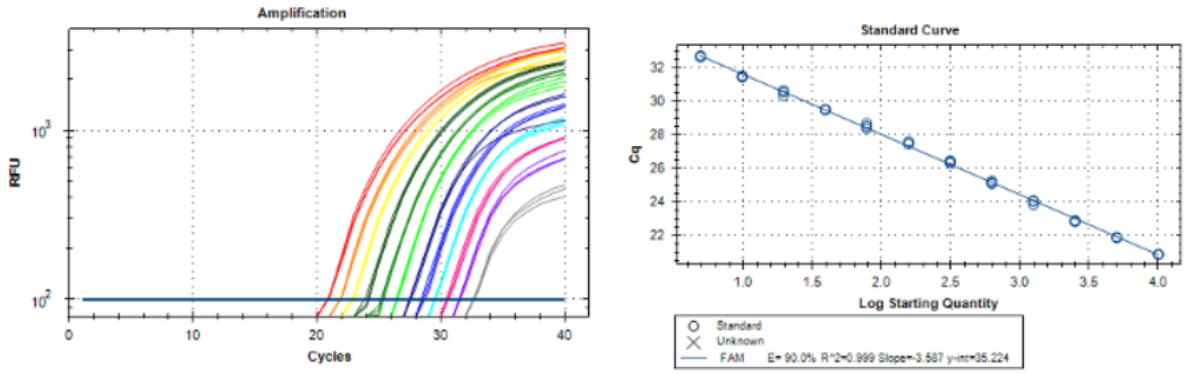
The *E. coli* organism chosen demonstrated detection down to 5 genomic copies. Table 4 summarizes this data.

Table 4: *E. coli* LOD, Total Aerobic Count Assay

Assay	DNA Copies (*E.coli*)	Cq Value	%RSD
Total Aerobic Count	10,000	20.52	0.36
Total Aerobic Count	10,000	20.38	
Total Aerobic Count	10,000	20.46	
Total Aerobic Count	5,000	21.45	0.14
Total Aerobic Count	5,000	21.39	
Total Aerobic Count	5,000	21.41	
Total Aerobic Count	2,500	22.50	0.17
Total Aerobic Count	2,500	22.45	
Total Aerobic Count	2,500	22.53	
Total Aerobic Count	1,250	23.69	0.09
Total Aerobic Count	1,250	23.67	
Total Aerobic Count	1,250	23.64	
Total Aerobic Count	625	24.73	0.18
Total Aerobic Count	625	24.80	
Total Aerobic Count	625	24.81	
Total Aerobic Count	313	26.08	0.09
Total Aerobic Count	313	26.07	
Total Aerobic Count	313	26.03	
Total Aerobic Count	156	27.22	0.16
Total Aerobic Count	156	27.14	
Total Aerobic Count	156	27.15	
Total Aerobic Count	78	28.29	0.30
Total Aerobic Count	78	28.28	

Total Aerobic Count	78	28.13	
Total Aerobic Count	39	29.43	0.07
Total Aerobic Count	39	29.41	
Total Aerobic Count	39	29.39	
Total Aerobic Count	20	30.53	0.32
Total Aerobic Count	20	30.38	
Total Aerobic Count	20	30.34	
Total Aerobic Count	10	31.42	0.35
Total Aerobic Count	10	31.51	
Total Aerobic Count	10	31.64	
Total Aerobic Count	5	32.60	0.45
Total Aerobic Count	5	32.89	
Total Aerobic Count	5	32.80	
Total Aerobic Count	NTC	ND	N/A
Total Aerobic Count	NTC	ND	
Total Aerobic Count	NTC	ND	

Figure 1: Total Aerobic Count qPCR Dilution Curves and qPCR Efficiency (E)



Action Limit Study

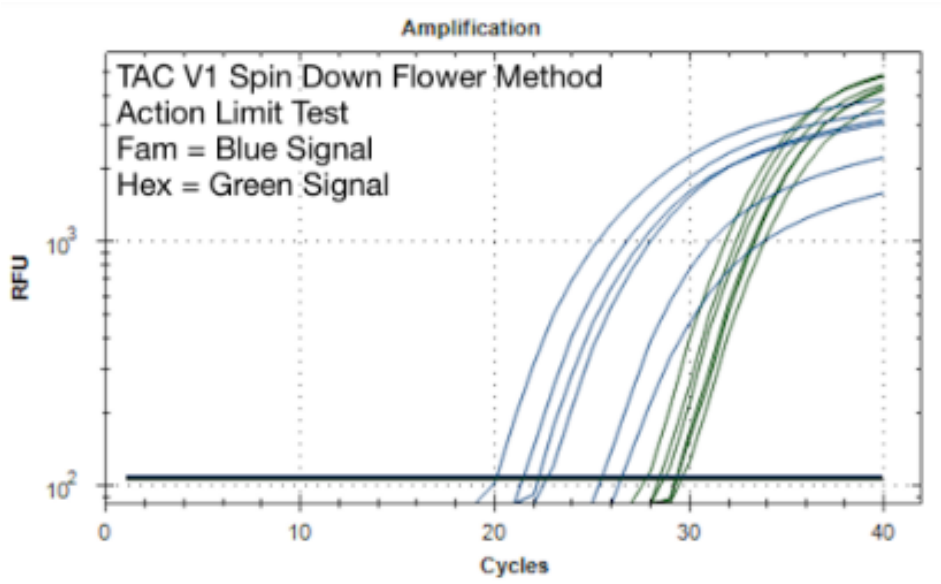
Flower Matrix

Microbiologics E-Power B. pneumonia spizizenii pellets (Catalog No. 0486E6-CRM) were resuspended and 50k, 100K and 500k CFU was spiked into 3 separate Whirl-pak bags containing 1g of cannabis flower and 19 mL of TSB. The extractions were performed in triplicate according to the Total Aerobic Count User guide and qPCR was run on each extraction.

Table 5: Flower Matrix qPCR Results

Sample	Spike Level (cfus)	TAC Cq (FAM)	Average Cq (FAM)	Cq Hex
B. Spizizenii	500,000	20.07		29.23
B. Spizizenii	500,000	21.5	20.79	29.68
B. Spizizenii	100,000	22.21		27.81
B. Spizizenii	100,000	22.81	22.51	28.76
B. Spizizenii	50,000	25.51		28.44
B. Spizizenii	50,000	26.52	26.02	29.27
Positive Control		12.22		ND
NTC		33.01		ND

Figure 2: Total Aerobic Count Detection Assay Flower Matrix Action Limit Curves



MIP Matrix

We recommend enriching non-flower (MIP) samples in TSB and processing as pass/fail as described in the updated PathoSEEK Total Aerobic Count User Guide v2.

Proficiency Testing/Certified Reference Material Results:

Flower Matrix

Material Used - NSI CRM Part Number FM-726, Quantitative APC in Hemp.

Certified Reference Material testing was performed by 2 technicians each performing four extractions from CRM vial. The identical material was plated and enumerated using 3M RAC Plates.

Table 6: NSI Quantitative APC in Hemp Results

Sample	Extraction	Avg Cq FAM (2 Analysts)	CFU/mL	CFU/g or vial (x20)	Average CFU/g or vial	3M RAC Results	NSI Acceptance Limits
APC in HEMP	1	27.97	2,186.50	43,730	35,293	49,000	32,200 - 125,000
APC in HEMP							
APC in HEMP	2	28.58	1,568.84	31,377			
APC in HEMP							
APC in HEMP	3	28.55	1,590.50	31,810			
APC in HEMP							
APC in HEMP	4	28.42	1,712.80	34,256			
APC in HEMP							