PathoSEEK® Total Coliform and Enterobacteriaceae Detection Assay with SenSATIVAx® Extraction Protocol for Detection in Cannabis Flower and MIP Matrices

Manufacturers Validation Document

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Abstract

Background:

Coliforms and Enterobacteriaceae can cause deterioration and decomposition of cannabis, and certain species of bacteria, such as *Shiga Toxin producing E. coli,* can cause infections in humans. Coliforms and Enterobacteriaceae are good indicator organisms for the assessment of overall quality of a finished product. The PathoSEEK® Total Coliform and Enterobacteriaceae Detection assay is a qPCR detection assay for the rapid detection and/or enumeration of these bacteria in cannabis matrices.

Objective:

To evaluate the PathoSEEK® Total Coliform and Enterobacteriaceae Detection Assay, using the SenSATIVAx® flower and MIP extraction protocols for the enumeration of total Coliform and Enterobacteriaceae 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® Coliform and Enterobacteriaceae method is highly specific in discriminating target organisms found in cannabis flower and infused products from non-target organisms. The SenSATIVAx® flower extraction kit and PathoSEEK® Total Coliform and Enterobacteriaceae Detection Assay was validated by creating an enumeration curve against ten different bacterial species and plating on 3M™ Petrifilm™ Enterobacteriaceae Count Plates. After creating this curve, we obtained a CRM from NSI, Quantitative EB in Hemp, and ran it using the SenSATIVAx flower extraction kit and PathoSEEK Coliform and Enterobacteriaceae 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™ Coliform Count 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 extraction kits along with the PathoSEEK® Total Coliform and Enterobacteriaceae Detection Assay is a rapid, alternative method to traditional plating procedures for the detection of Coliform and Enterobacteriaceae in cannabis flower and cannabis infused products.

Materials

Test Kit Name: PathoSEEK® Total Coliform and Enterobacteriaceae Detection Assay with SenSATIVAx® Extraction (with Optional Grim Reefer free DNA removal)

Test Kit Information

1. SenSATIVAx® Flower/Leaf DNA Extraction Kit - P/N 420001
2. SenSATIVAx® MIP/Extract DNA Extraction Kit - P/N 420004
3. Medicinal Genomics qPCR Master Kit v3 - P/N 420201
4. PathoSEEK Total Coliform/Entero Assay - P/N 420153
5. PathoSEEK BTGN/Coliform/Entero Positive Control - P/N 420353
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, 34 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 the exclusivity, 14 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 Coliform and Enterobacteriaceae method.

Results

Of the 34 inclusivity strains tested, 34 were correctly detected by the PathoSEEK® Method. Of the 14 exclusivity strains tested, all 14 were correctly excluded. Tables 1 and 2 present a summary of the results.

Table 1: Inclusivity Results, PathoSEEK® Total Coliform and Enterobacteriaceae Assay

#	**Species**	**ATCC#**	Pathoseek Coliform/Entero Result
1	*Aeromonas hydrophila*	7965 DNA	Detected
2	*Aeromonas hydrophila*	7966	Detected
3	*Aeromonas hydrophila*	7966 DNA	Detected
4	*Citrobacter braakii*	3037	Detected
5	*Citrobacter freundii*	8090	Detected
6	*Citrobacter koseri*	25408	Detected
7	*Cronobacter sakazakii*	BAA-894	Detected
8	*Enterobacter aerogenes*	15038 DNA	Detected
9	*Escherichia hermannii*	700368	Detected
10	*Escherichia coli Strain 2005-3287 O145*	BAA-2223	Detected
11	*Escherichia coli Strain 2000-3039 O45:H2*	BAA-2193 DNA	Detected
12	*Escherichia coli Strain 2002-3211 O121-H19*	BAA-2219 DNA	Detected
13	*Escherichia coli Strain 2003-3014 O26:H11*	BAA 2196 DNA	Detected
14	*Escherichia coli Strain 2006-3008 O103:H11*	BAA 2215 DNA	Detected
15	*Escherichia coli Strain 99-3311 O145*	BAA 2192 DNA	Detected
16	*Escherichia coli Strain O111*	BAA 2440 DNA	Detected
17	*Hafnia alvei*	51873	Detected
18	*Klebsiella pneumonia*	200721 DNA	Detected
19	*Klebsiella oxytoca*	51983	Detected

20	*Morganella morganii*	25829	Detected
21	*Pantoea agglomerans*	43348	Detected
22	*Proteus mirabilis*	43071	Detected
23	*Proteus vulgaris*	8427	Detected
24	*Rahnella aquatilis*	33991	Detected
25	*Salmonella bongori*	43975D-5	Detected
26	*Escherichia hermannii*	6962	Detected
27	*Salmonella enterica subsp. arizonae*	BAA-731D-5	Detected
28	*Salmonella enterica subsp. diarizonae*	BAA-1579D-5	Detected
29	*Salmonella enterica subsp. houtene*	BAA-1580D-5	Detected
30	*Salmonella enterica subsp. indica*	BAA-15780D-5	Detected
31	*Salmonella enterica subsp. Salamae*	BAA-1582D-5	Detected
32	*Shigella flexneri*	29903D-5	Detected
33	*Vibrio cholerae*	39315D-5	Detected
34	*Yersinia enterocolitica*	9610	Detected

Table 2: Exclusivity Results, PathoSEEK® Total Coliform and Enterobacteriaceae Assay

#	**Species**	**ATCC#**	Pathoseek Coliform/Entero Result
1	*Bacillus subtilis*	11774	Not Detected
2	*Clostridium sporogenes*	11437	Not Detected
3	*Lactobacillus acidophilus*	4357	Not Detected
4	*Listeria monocytogenes*	19115D-5	Not Detected
5	*Listeria seeligeri*	35967D-5	Not Detected
6	*Listeria wilshire*	35897D-5	Not Detected
7	*Staphylococcus aureus*	6538	Not Detected
8	*Aspergillus niger*	1015	Not Detected
9	*Aspergillus flavus*	9643	Not Detected
10	*Aspergillus terreus*	20542	Not Detected

11	*Candida albicans*	10231	Not Detected
12	*Penicillium chrysogenum*	10160 DNA	Not Detected
13	*Penicillium rubens*	11709	Not Detected
14	*Pseudomonas aeruginosa*	9027	Not Detected

Generation of Cq to CFU Conversion Equation for flower samples

(a) The Cq to CFU/g equation was generated by running ten organisms on qPCR compared against plating on Petrifilm EB 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 = -0.2847x+11.672.

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

$$Y = -0.2847X + 11.672$$

(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 5: Cq to CFU Conversion Equations

Matrix	Microbial Test	Cq to CFU Conversion Equation
Flower	Total Coliform and Enterobacteriaceae	CFU/g = 10^((-0.2847*Cq)+11.672) Multiply resulting CFU x 20 to account for upfront dilution factor
MIP	Total Coliform and Enterobacteriaceae	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 Coliform and Enterobacteriaceae 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 Coliform and Enterobacteriaceae Assay. The following organism was evaluated for LOD of the PathoSEEK® Total Coliform and Enterobacteriaceae Detection Assay in the absence of cannabis matrix, *E.coli* ATCC# BAA-23326D-5.

Results

The *E. coli* organism chosen demonstrated detection down to 5 genomic copies. Table 3 summarizes this data.

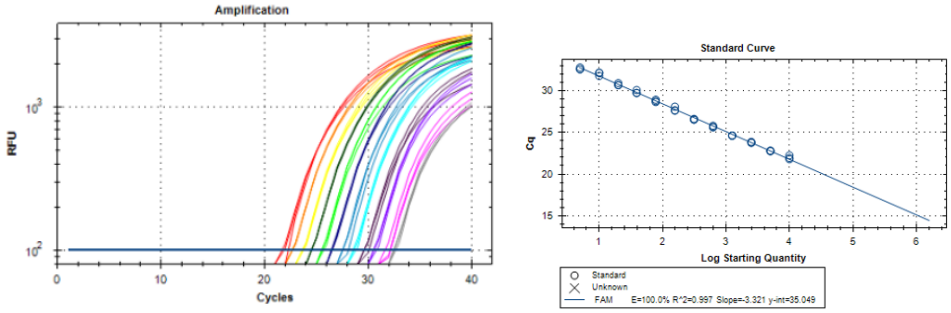
Table 3: *E. coli* LOD, Coliform and Enterobacteriaceae Assay

Assay	DNA Copies (*E.coli)*	Cq Value	%RSD
Coliform_Entero	10,000	21.79	1.05
Coliform_Entero	10,000	21.93	
Coliform_Entero	10,000	22.24	
Coliform_Entero	5,000	22.74	0.14
Coliform_Entero	5,000	22.77	
Coliform_Entero	5,000	22.80	
Coliform_Entero	2500	23.76	0.24

Coliform_Entero	2500	23.86	
Coliform_Entero	2500	23.76	
Coliform_Entero	1250	24.60	0.03
Coliform_Entero	1250	24.61	
Coliform_Entero	1250	24.61	
Coliform_Entero	625	25.79	0.39
Coliform_Entero	625	25.59	
Coliform_Entero	625	25.65	
Coliform_Entero	313	26.52	0.19
Coliform_Entero	313	26.54	
Coliform_Entero	313	26.61	
Coliform_Entero	156	28.07	0.94
Coliform_Entero	156	27.60	
Coliform_Entero	156	27.64	
Coliform_Entero	78	28.90	0.48
Coliform_Entero	78	28.62	
Coliform_Entero	78	28.74	
Coliform_Entero	39	29.71	0.67
Coliform_Entero	39	29.71	
Coliform_Entero	39	30.05	
Coliform_Entero	20	30.88	0.44
Coliform_Entero	20	30.65	
Coliform_Entero	20	30.64	
Coliform_Entero	10	32.13	0.67
Coliform_Entero	10	32.10	
Coliform_Entero	10	31.75	
Coliform_Entero	5	32.55	0.37
Coliform_Entero	5	32.55	
Coliform_Entero	5	32.76	

Positive Control	Positive Control	14.45	
NTC	NTC	ND	

Figure 1: Total Coliform and Enterobacteriaceae qPCR Dilution Curves and qPCR Efficiency (E)



Action Limit Study

Flower Matrix

Microbiologics E-Power K. pneumonia pellets (Catalog No. 0684E7-CRM) were resuspended and 250, 500, 1000, 2500 and 5,000 cfu was spiked into 5 separate Whirl-pak bags containing 1g of cannabis flower and 19 mLs TSB. The extractions were performed in triplicate according to the Coliform and Enterobacteriaceae User guide and qPCR was run on each extraction.

Table 4: Flower Matrix qPCR Results

Sample	Spike level (cfu)	Total Coliform - Entero Cq Fam	Avergae cq Fam)	Cq Hex
Klebsiella pneumoniae	5K	27.34		32.56
Klebsiella pneumoniae	5K	28.20		33.72
Klebsiella pneumoniae	5K	26.84	27.46	33.63
Klebsiella pneumoniae	2.5K	28.64		32.77
Klebsiella pneumoniae	2.5K	28.18		32.37
Klebsiella pneumoniae	2.5K	27.98	28.27	33.11
Klebsiella pneumoniae	1K	30.11		32.72
Klebsiella pneumoniae	1K	30.32		33.03
Klebsiella pneumoniae	1K	29.14	29.86	32.57
Klebsiella pneumoniae	500	31.38		32.61
Klebsiella pneumoniae	500	31.25		33.19
Klebsiella pneumoniae	500	30.75	31.13	32.47
Klebsiella pneumoniae	250	31.66		32.98
Klebsiella pneumoniae	250	32.17		32.71
Klebsiella pneumoniae	250	32.38	32.07	32.35
Positive Control		13.65		ND
NTC		ND		ND

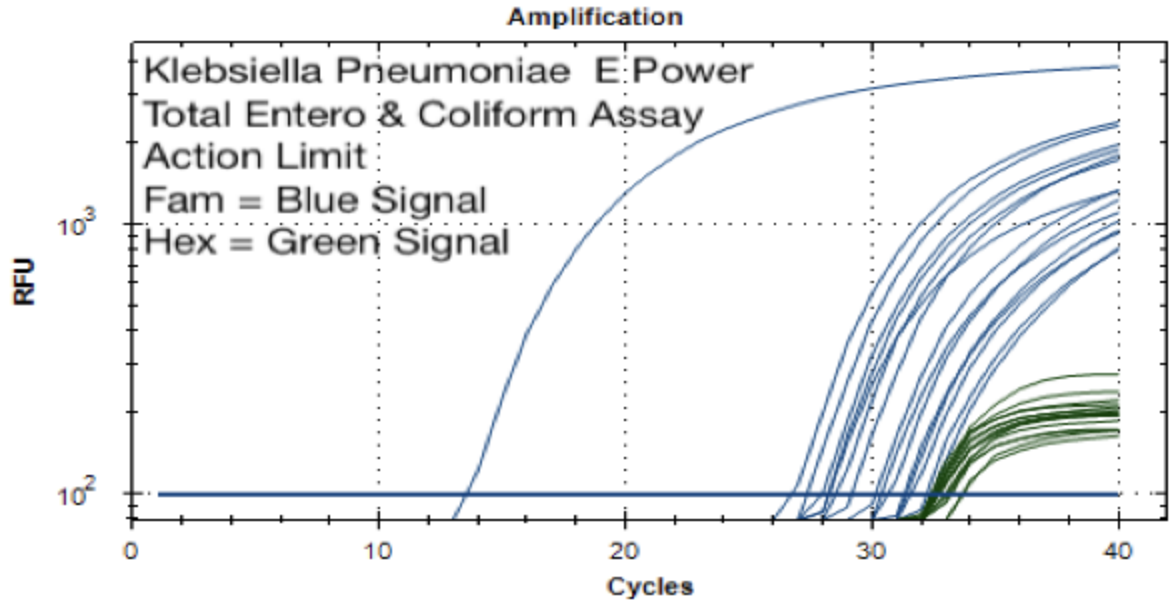


Figure 2: Total Coliform and Enterobacteriaceae Detection Assay Flower Matrix Action Limit Curves

MIP Matrix

Due to the low action limit for MIP samples of 100 cfu/g in most jurisdictions, we recommend enriching non flower (MIP) samples in TSB and processing as pass/fail as described in the updated PathoSEEK Total Coliform and Enterobacteriaceae User Guide.

Proficiency Testing/Certified Reference Material Results:

Flower Matrix

Material Used - NSI CRM Part Number FM-730, Quantitative EB in Hemp (lot number 210928). Certified Reference Material testing was performed by two technicians each performing three extractions from CRM vial. The identical material was plated and enumerated using 3M CC Plates.

Table 7: NSI Quantitative EB in Hemp Results

Sample	Extraction	Cq FAM	CFU/g or vial (qPCR)	NSI CRM FM-730 lot 210928 CoA reported Acceptable Range CFU/g
EB in HEMP	Extraction 1	26.41	241,699	235,000 - 939,000
EB in HEMP	Extraction 2	26.30	259,663	
EB in HEMP	Extraction 3	26.30	260,093	
		AVG CFU/g qPCR	**253,818**	
		AVG CFU/g 3M CC	**428,750**	
		NSI EB CRM COA CFU/g	**587,000**	

MIP Matrix

Matrix Used - NSI CRM Salmonella in Hemp Oil. Part Number FM-611 (Lot numer 210921) Certified Reference Material testing was performed by one technician. Three extractions were performed from CRM vial after enrichment for 16 hours at 37 ℃. Each extraction was run in duplicate with PathoSEEK® Coliform and Enterobacteriaceae Detection Assay.

Table 8: NSI Salmonella in Oil CRM results

Sample	Spike Level (CFU)	Col/Entero Cq Fam	Average Cq Fam	Cannabis Cq Hex
Salmonella in Hemp Oil CRM Extraction 1	Enriched	18.06	17.99	26.31
Salmonella in Hemp Oil CRM Extraction 1	Enriched	18.18		26.13
Salmonella in Hemp Oil CRM Extraction 2	Enriched	17.81		25.89
Salmonella in Hemp Oil CRM Extraction 2	Enriched	17.82		26.15

Salmonella in Hemp Oil CRM Extraction 3	Enriched	18.13		26.1
Salmonella in Hemp Oil CRM Extraction 3	Enriched	17.94		25.97
Positive Control		13.22		No Cq
NTC		No Cq		No Cq