PathoSEEK® Bile Tolerant Gram Negative (BTGN) Detection Assay with SenSATIVAx® Extraction Protocol for BTGN Detection in Cannabis Flower and MIP Matrices

Manufacturers Validation Document

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Abstract

Background:

Bile Tolerant Gram Negative (BTGN) bacteria can cause deterioration and decomposition of cannabis, and certain species of BTGN, such as *Shiga Toxin producing E. coli,* can cause infections in humans. The PathoSEEK® Bile Tolerant Gram Negative (BTGN) assay is a qPCR detection assay for the rapid detection and enumeration of BTGN in cannabis matrices.

Objective:

To evaluate the PathoSEEK® BTGN qPCR Detection Assay, using the SenSATIVAx® flower and extraction protocol for the detection and enumeration of BTGN in cannabis flower (delta 9-tetrahydrocannabinol >0.3%; 1g) and for presence/absence detection of BTGN in MIP (marijuana infused products).

Results:

Inclusivity and exclusivity results showed the PathoSEEK® BTGN v2 method is highly specific in discriminating target organisms found in cannabis flower and infused products from non-target organisms.

Conclusions:

The SenSATIVAx® flower and MIP extraction kits along with the PathoSEEK® BTGN v2 qPCR assay is a rapid, alternative method to traditional plating procedures for the detection of Bile Tolerant Gram Negative bacteria in cannabis flower and cannabis infused products.

The PathoSEEK® BTGNv2 method with SenSATIVAx® extraction produced comparable results to 3M Petrifilm EB and CC plates for the enumeration of Bile Tolerant Gram Negative bacteria in cannabis flower.

Materials

Test Kit Name: PathoSEEK® Bile Tolerant Gram Negative (BTGNv2) 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® Bile Tolerant Gram Negative Detection Assay v2 - P/N 420149
5. PathoSEEK® Bile Tolerant Gram Negative / Coliform / Entero Multiplex 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, 35 strains of bacteria were evaluated. Target strains were either cultured in Tryptic Soy Broth for 24 hours at 37℃ or purified DNA from ATCC was used. Thirteen exclusivity organisms were cultured under optimal conditions for growth of the organism or purified DNA from ATCC was used. Exclusivity cultures were analyzed undiluted. Inclusivity and exclusivity cultures were analyzed by the PathoSEEK® BTGNv2 method.

Results

Of the 35 Inclusivity strains tested, 35 were correctly detected by the PathoSEEK® BTGNv2 Method. Of the 13 exclusivity strains tested, all 13 were correctly excluded. Tables 1 and 2 present a summary of the results.

Table 1: Results for inclusivity of the PathoSEEK® BTGNv2

#	Species	ATCC#	Pathoseek BTGN v2 Result
1	*Aeromonas hydrophillia*	7965 DNA	Detected
2	*Aeromonas hydrophillia*	7966	Detected
3	*Aeromonas hydrophillia*	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 hermanii*	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	*Pseudomonas aeruginosa*	9027	Detected
25	*Rahnella aquatilis*	33991	Detected
26	*Salmonella bongori*	43975D-5	Detected
27	*Salmonella enterica sub species Newport*	6962	Detected
28	*Salmonella enterica subsp. arizonae*	BAA-731D-5	Detected
29	*Salmonella enterica subsp. diarizonae*	BAA-1579D-5	Detected
30	*Salmonella enterica subsp. houtene*	BAA-1580D-5	Detected
31	*Salmonella enterica subsp. indica*	BAA-15780D	Detected

		-5	
32	*Salmonelle enterica subsp. Salamae*	BAA-1582D-5	Detected
33	*Shigella flexneri*	29903D-5	Detected
34	*Vibrio cholerae*	39315D-5	Detected
35	*Yersinia enterocolitica*	9610	Detected

Table 2: Results for Exclusivity of the PathoSEEK® BTGNv2

#	Species	ATCC#	Pathoseek BTGN v2 Result
1	*Bacillus subtilis*	11774	Not Detected
2	*Clostridium sporogenes*	11437	Not Detected
3	*Lactobacillus acidophilos*	4357	Not Detected
4	*Listeria monocytogenes*	19115D-5	Not Detected
5	*Listeria seeligeri*	35967D-5	Not Detected
6	*Listeria welshimeri*	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

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 CC 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 by using the best fit line to these points. The resulting equation is y = -0.2855x+11.698.

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

$$Y = -0.2855X + 11.698$$

(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).

Cq to CFU Conversion Equation Table

Matrix	Microbial Test	Cq to CFU Conversion Equation
Flower	BTGN	$CFU/g = 10^{[(-0.2855* Cq\ Value)+11.698]}$
MIP	BTGN	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® BTGNv2 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 BTGNv2 Assays. The following organism was evaluated for LOD of the PathoSEEK® BTGNv2 Assay in the absence of cannabis matrix, *Escherichia coli* strain TY-2482 from ATCC.

Results

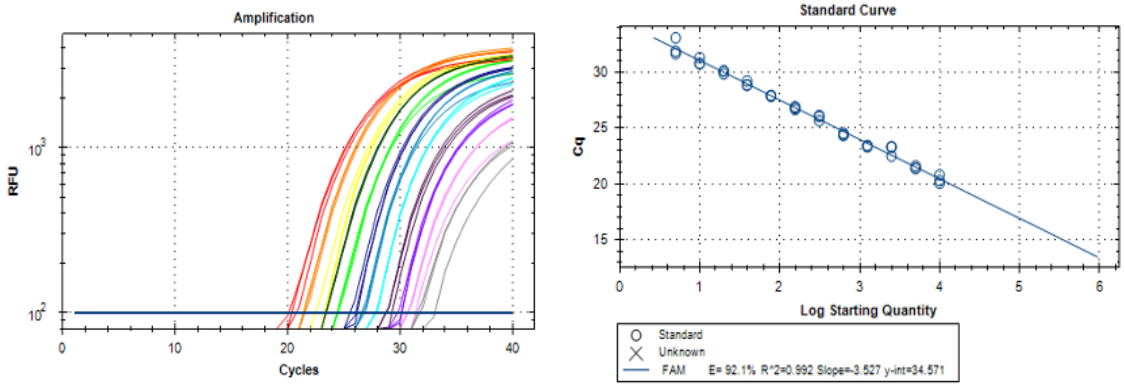
The organism demonstrated detection down to 5 genomic copies. Table 3 summarizes this data.

Table 3: Results for E.coli LOD

Assay	DNA Copies (*E. coli*)	Cq Value	%RSD
BTGNv2	10,000	20.07	1.90
BTGNv2	10,000	20.32	
BTGNv2	10,000	20.83	
BTGNv2	5,000	21.63	0.58
BTGNv2	5,000	21.40	
BTGNv2	5,000	21.42	
BTGNv2	2500	23.30	2.05
BTGNv2	2500	22.51	
BTGNv2	2500	23.35	
BTGNv2	1250	23.34	0.26

BTGNv2	1250	23.46	
BTGNv2	1250	23.40	
BTGNv2	625	24.55	0.42
BTGNv2	625	24.35	
BTGNv2	625	24.40	
BTGNv2	313	26.04	0.96
BTGNv2	313	25.66	
BTGNv2	313	26.13	
BTGNv2	156	26.80	0.46
BTGNv2	156	26.67	
BTGNv2	156	26.92	
BTGNv2	78	27.92	0.11
BTGNv2	78	27.86	
BTGNv2	78	27.90	
BTGNv2	39	28.86	0.73
BTGNv2	39	28.83	
BTGNv2	39	29.21	
BTGNv2	20	30.09	0.50
BTGNv2	20	29.84	
BTGNv2	20	30.12	
BTGNv2	10	31.31	1.01
BTGNv2	10	30.79	
BTGNv2	10	30.74	
BTGNv2	5	31.87	2.36
BTGNv2	5	31.65	
BTGNv2	5	33.06	
BTGNv2	Positive Control	13.52	
BTGNv2	NTC	ND	

Figure 1: BTGN qPCR Dilution Curves and qPCR Efficiency (E)



Action Limit Study

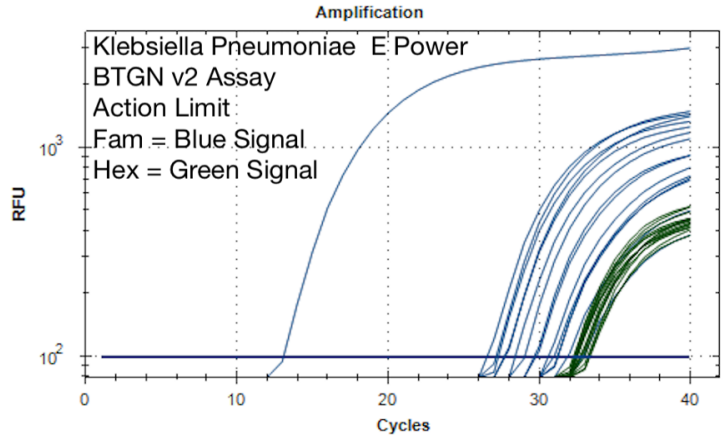
Flower Matrix

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 BTGNv2 User guide and qPCR was run on each extraction.

Table 4: Flower Matrix qPCR Results

Sample	Spike Level (CFU)	BTGN v2 Cq Fam	Average Cq Fam	Cannabis Cq Hex
Klebsiella pneumoniae	5K	27.1		32.84
Klebsiella pneumoniae	5K	27.22		33.28
Klebsiella pneumoniae	5K	26.63	26.98	32.99
Klebsiella pneumoniae	2.5K	28.34		33.23
Klebsiella pneumoniae	2.5K	27.8		33.14
Klebsiella pneumoniae	2.5K	27.76	27.97	32.71
Klebsiella pneumoniae	1K	29.87		32.49
Klebsiella pneumoniae	1K	29.76		32.3
Klebsiella pneumoniae	1K	29.02	29.55	32.36
Klebsiella pneumoniae	500	30.97		32.42
Klebsiella pneumoniae	500	31.15		32.47
Klebsiella pneumoniae	500	30.64	30.92	32.27
Klebsiella pneumoniae	250	31.17		32.2
Klebsiella pneumoniae	250	32.9		32.78
Klebsiella pneumoniae	250	31.9	31.99	32.34
Positive Control	NA	13.06	No Cq	No Cq
NTC	NA	No Cq	No Cq	No Cq

Figure 2: BTGN 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 BTGN v2 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 2 different lab technicians. Each technician ran 3 extractions from CRM vial and plated the sample on 3M EB and Coliform/E.coli Petrifilms.

Table 5: NSI Hemp CRM Results - Average of 2 Users

Sample	Extraction	Cq	Average CFU/g qPCR	Average CFU/g on 3M EB	Avg CFU/g on 3M CC
EB in Hemp	Extraction 1	25.77	437,264	**582,500**	**428,750**
EB in Hemp	Extraction 2	25.72	452,866		
EB in Hemp	Extraction 3	25.84	419,547		
	Average CFU/g or vial(qPCR) =		**436,559**		

MIP Matrix

Matrix Used - NSI CRM Salmonella in Hemp Oil. Part Number FM-611 (Lot numer 210921) Certified Reference Material testing was performed by 1 lab technician. Three extractions were performed from CRM vial after enrichment for 16 hours at 37℃. Each extraction was run in duplicate with PathoSEEK® BTGN v2 Detection Assay.

Table 6: NSI Salmonella in Oil CRM results

Sample	Spike Level (CFU)	BTGN v2 Cq Fam	Average Cq Fam	Cannabis Cq Hex
Salmonella in Hemp Oil CRM Extraction 1	Enriched	17.55	17.535	26.4
Salmonella in Hemp Oil CRM Extraction 1	Enriched	17.79		26.22
Salmonella in Hemp Oil CRM Extraction 2	Enriched	17.58		26.2

Salmonella in Hemp Oil CRM Extraction 2	Enriched	17.44		26.3
Salmonella in Hemp Oil CRM Extraction 3	Enriched	17.48		25.97
Salmonella in Hemp Oil CRM Extraction 3	Enriched	17.37		25.96
Positive Control		12.89		No Cq
NTC		No Cq		No Cq

Conclusions

The data derived from the use of commercially available Proficiency Tests/CRMs for bile tolerant gram negative bacteria indicate that the PathoSEEK® BTGN v2 Detection Assay with the SenSATIVAx® Extraction Protocol is equivalent for the enumeration of BTGN bacteria in cannabis flower and presence/absence detection in MIP. 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 BTGN v2 user guide. If BTGN is detected post enrichment, plating should be performed to achieve a total viable count for reporting of CFUs. If no BTGN is detected in a sample extracted after enrichment this sample can be reported as non-detect.

References

1. Emerald Scientific (2018) *Cannabis Testing Regulations by State: Increase Your Knowledge*. https://emeraldscientific.com/blog/cannabis-testing-regulations-by-state-increase-your-knowledge/ (Accessed February 2021)
2. Official Methods of Analysis (2019) 21st Ed., Appendix J: AOAC INTERNATIONAL, Rockville, MD, http://www.eoma.aoac.org/app_j.pdf (Accessed February 2021)